The presence and orientation of ecto-5′-nucleotidase in rat liver lysosomes

Gerald A Maguire and J Paul Luzio

Department of Clinical Biochemistry, University of Cambridge, Adenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, England

Received 19 November 1984

Purified rat liver lysosomes contained 5′-nucleotidase activity which was 92 ± 2% [4] latent This latency was lost in response to a permeant sugar at a similar rate to that of the lysosomal marker enzyme β-*N*-acetylglucosaminidase indicating that the 5′-nucleotidase was genuinely located in the lysosome and not a plasma membrane contaminant Lysosomal 5′-nucleotidase exhibited the following properties characteristic of ecto-5′-nucleotidase inhibition by specific polyclonal antibodies binding to a monoclonal antibody, inhibition by 1 mmol/l αβ-methylene ADP, immunoreactive subunits of 70 and 38 kDa Lysosomes in addition contained immunoreactive species of intermediate molecular mass

Rat liver lysosome *5′-Nucleotidase* *Membrane cycling*

1. INTRODUCTION

5′-Nucleotidase (EC 3.1.3.5) has been used extensively as a plasma membrane marker but it is also present in varying amounts in many other subcellular structures [1]. It is possible that 5′-nucleotidase may be a plasma membrane component which is not segregated during endocytosis and may therefore prove to be a marker for plasma membrane elements during this process [2–4].

The lysosome is an important intracellular site for internalized plasma membrane, probably playing a role in the recycling of endocytosed membrane components back to the cell surface [6,7]. It is therefore important to establish that the 5′-nucleotidase associated with lysosomes is the same enzyme as that found in plasma membrane and that it is genuinely present in lysosomes and is not the result of contamination. We have addressed these questions in the present communication using lysosomes prepared from rat liver by a rapid and simple centrifugation procedure.

2. MATERIALS AND METHODS

2.1. *Materials*

All reagents used were of the highest grade available. Reagents and detergents for polyacrylamide gel electrophoresis were as in [8].

2.2. *Antisera, immunoadsorbents and immunoblotting*

A mouse monoclonal antibody to liver 5′-nucleotidase coded 5NE5 [8], and rabbit antiserum to immunoaffinity purified rat liver 5′-nucleotidase [9], were as previously described. Immunoadsorbents were prepared by coupling IgG factions to diazo-cellulose as in [10]. Lysosome or plasma membrane 5′-nucleotidase was solubilized with Sulphobetaine 14, adsorbed onto a 5NE5 monoclonal antibody immunoabsorbent, transferred to nitrocellulose paper and detected with rabbit antibodies as in [8].

2.3. *Preparation of purified rat liver lysosomes*

A rat liver was gently homogenised (4 up and

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/85/$3.30

down strokes on an aldridge-type homogeniser, driven at 2400 rpm) in 70 ml of 0.45 mol/l sucrose, 0.5 mmol/l EDTA (pH 7.0). The crude homogenate was centrifuged at 500 × g_{av} for 10 min to give a pellet (P1). The supernatant (S1) was centrifuged at 15000 × g_{av} for 10 min to give a supernatant (S2). The pellet was gently resuspended in 20 ml of 0.25 mol/l KCl, 1 mmol/l Tes–Tris (pH 7.4) and centrifuged at 500 × g_{av} for 10 min to give a pellet (P3). This treatment selectively sediments mitochondria [11]. The supernatant was diluted to 70 ml such that the final concentrations were 35% (v/v) Percoll, 0.25 mol/l sucorse, 10 mmol/l Tes–Tris (pH 7.4), 30 mmol/l KCl and centrifuged in 2 × 35 ml portions at 48000 × g_{av} for 10 min. The lower 4 ml of each self-formed Percoll gradient were pooled and diluted to 140 ml with 0.25 mol/l sucrose, 10 mmol/l Tes–Tris (pH 7.4) and centrifuged at 12000 × g_{av} for 20 min. The pellet constituted the lysosomal fraction (ly). The supernatant was combined with the remainder of the Percoll gradient and designated the S_4 fraction.

3. RESULTS AND DISCUSSION

3.1. *Purity of lysosomes*

The combination of low-speed centrifugation in 0.25 mol/l KCl and isopyanic centrifugation in Percoll allowed us to prepare lysosomes rapidly (<2 h) and led to a preparation exhibiting high latency of *β-N*-acetylglucosaminidase. The rapidly prepared lysosomes contained 17% of the marker enzyme *β-N*-acetylglucosaminidase with a specific activity 25-fold higher than the homogenate (table 1). For the present study it was important to use an intact lysosomal preparation. Tritosomes, although pure (57-fold purification), were previously shown to be less intact [12]. The main contaminants of the preparation used here were mitochondria, peroxisomes and microsomes; all <1.8% of their homogenate values. As these structures are not thought to contain 5′-nucleotidase their presence is not relevant to the present investigation. However, it was important to establish that the amount of 5′-nucleotidase associated with the lysosomal preparation was at least as low as in the most highly purified preparations. The recovery of 5′-nucleotidase compared with *β-N*-acetylglucosaminidase was 3%. This figure is similar to that found with 57-fold purified tritosomes [12] and about half that found by Pletsch and Coffey with 34-fold purified tritosomes [13] and by Wattiaux et al. with 70-fold purified lysosomes [14].

Table 1

Distribution of marker enzymes in the fractions obtained during lysosome preparation

	Percentage in fraction						Recovery (%)
	P_1	S_1	S_2	P_3	S_4	ly	
β-N-Acetylglucosaminidase (4)	50 8 ± 3.9	49.3 ± 3.9	8.3 ± 1.3	5.0 ± 0.4	10.3 ± 2.1	17.0 ± 2.1	91.3 ± 1.7
5′-Nucleotidase (3)	80.0 ± 4.1	20.0 ± 4 1	12.0 ± 2.0	3 7 ± 0.9	2.4 ± 0.95	0.5 ± 0.2	99.3 ± 0.33
Catalase (3)	36.3 ± 0.9	63.7 ± 0.9	35.7 ± 1.2	8.0 ± 2 1	17.8 ± 4.7	0.8 ± 0.2	98.7 ± 5.4
Glucose-6-phosphatase (3)	52.0 ± 2.7	48.0 ± 2.7	21.7 ± 3 4	8.5 ± 1.3	6.6 ± 2 3	0.90 ± 0.4	89 7 ± 0.7
Cytochrome oxidase (3)	46.0 ± 1.0	54.0 ± 1.0	2 2 ± 1.0	32.2 ± 2.7	1.9 ± 0.2	1.6 ± 0.4	83.7 ± 2.9
Galactosyl transferase (1)	49	52	29	nil	N.D.	0.1	80
Protein (4)	49.5 ± 2.5	50.5 ± 2 5	34.3 ± 2.1	8.2 ± 0.8	5.2 ± 1.3	0.68 ± 0.06	96.0 ± 2.1

The following markers were assayed as described: *β-N*-acetylglucosaminidase (EC 3.2.1.30) [12], 5′-nucleotidase (EC 3.1 3.5) [9], catalase (EC 1 11.1.6) [23], glucose-6-phosphatase (EC 3.1.3 9) [24], cytochrome oxidase (EC 1.9.3.1) [25], galactosyltransferase (EC 2.4.1.38) [26] and protein [27] Each tabulated value is the mean ± SE of the number of experiments in parentheses

3.2. *Latency studies*

Enzymes within intact lysosomes exhibit latency – they can only be detected when the lysosomal membrane has been disrupted. Both 5′-nucleotidase and β-*N*-acetylglucosaminidase were approx. 90% latent in purified rat liver lysosomes. Lysosomes remain intact for hours in isotonic solutions of impermeant substances such as sucrose, but are disrupted in low osmotic strength solutions [15]. When placed in isotonic (i.e., 250 mM) solutions of permeant substances such as glucose, a time-dependent rupture of lysosomes occurs. This is believed to be due to entry of the substance into the lysosome, followed by water being drawn into the lysosome with resultant swelling and eventual rupture leading to release of lysosomal enzyme activity [15]. The permeability of the lysosomal membrane towards the permeant substance determines the rate of release of lysosomal enzyme activity [16,17].

When the lysosomal preparation was placed in 0.25 M glucose, β-*N*-acetylglucosaminidase activity was progressively released (fig.1) in agreement with earlier findings [16,17], indicating that progressive rupture of the lysosomal membrane occurred. As the lysosome ruptures, 5′-nucleotidase activity was also released (fig.1). The simplest explanation for the simultaneous release of lysosomal marker and 5′-nucleotidase activity is that they are present in the same organelle – the lysosome, and that the active site of 5′-nucleotidase is directed towards the interior of the lysosome. This result contradicts the earlier findings of Widnell and Little [1] who showed lead phosphate deposits on the cytoplasmic side of lysosomes in a cytochemical study of rat liver parenchymal cells.

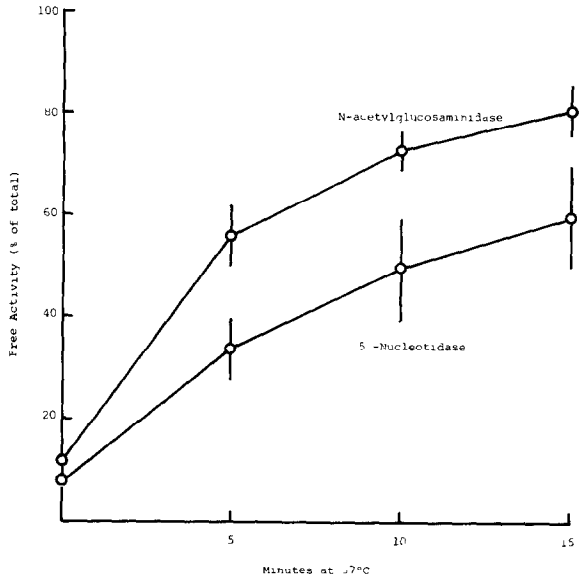

Fig.1 Release of enzyme activity in 0.25 mol/l glucose Purified rat liver lysosomes were suspended in ice-cold 0.25 mol/l glucose, 10 mmol/l, Tes–Tris (pH 7.4) At time zero the suspensions in thin-walled glass tubes were placed in a water bath at 37°C and the activities of β-*N*-acetylglucosaminidase [12] and 5′-nucleotidase [2] determined at intervals as indicated. The enzyme assays were in osmotically protecting buffer such that no further loss of latency occurred during the 20 min assay. Total activities (in 0.1%, w/v, Triton X-100) were determined at the end of the incubation. The error bars represent the SE of 4 separate experiments.

3.3. *Inhibition studies*

When lysosomes were incubated with rabbit polyclonal antirat 5′-nucleotidase antisera in the presence of 0.1% (w/v) Triton X-100, 85% inhibition of activity was observed (table 2). However, only 15% inhibition was observed if the lysosomes were incubated in the absence of detergent (table 2). Thus, in intact lysosomes, approx. 85% of the 5′-nucleotidase was not accessible to antisera. The figure is in good agreement with the latency (90%) of the 5′-nucleotidase of the preparation, and further supports the supposition that the active site of the enzyme is directed towards the interior of the organelle.

Lysosomal 5′-nucleotidase was 80% inhibited by 1 mmol/l αβ-methylene ADP (table 2). Thus, at least 80% of lysosomal 5′-nucleotidase is immunologically and catalytically similar to ecto 5′-nucleotidase and different from cytoplasmic 5′-nucleotidase [18]. The remaining lysosomal 5′-nucleotidase activity may not be due to ecto 5′-nucleotidase but to an acid phosphatase, since a similar proportion was inhibitable by 1 mmol/l tartrate, which does not inhibit ecto 5′-nucleotidase but does inhibit lysosomal acid phosphatase [19].

3.4. *Immunoblotting*

Rat liver lysosomal 5′-nucleotidase could be absorbed onto monoclonal antibody immunoabsor-

Table 2

Inhibition of lysosomal 5′-nucleotidase

Inhibition	Activity % of control
Non-immune rabbit serum	101 ± 5
Rabbit anti-5′-nucleotidase serum	15 ± 3
Tartrate (1 mmol/l)	85 ± 7
α-β-Methylene ADP (1 mmol/l)	20 ± 4

Antiserum inhibition experiments were carried out by incubating lysosome samples with 1:1000 dilutions of rabbit antiserum or non-immune serum for 1 h at 37°C in the presence of 0.1% (w/v) Triton X-100, prior to addition of radioassay cocktail. Incubation with antiserum in the absence of Triton X-100 resulted in the retention of 85 ± 2% of control activity. Other inhibitors were added to the enzyme at the start of the radioassay. The final concentrations in the 5′-nucleotidase assay were 0.3 IU/l of 5′-nucleotidase, 0.2 mM [1-^{3}H]AMP, 1.5 mM adenosine, 2 mM $MgSO_4$, 20 mM β-glycerophosphate, 0.25 M sucrose, 0.1% (w/v) Triton X-100 and 50 mM Tris–HCl (pH 8.0). Assay incubation was for 20 min at 37°C. Each tabulated value is the mean ± SD of the quadruplicate observations from a single representative experiment

bent after solubilization with sulphobetaine 14. Subsequent electrophoresis, blotting and localization with polyclonal anti-rat liver ecto 5′-nucleotidase antibodies revealed a major band at 70 kDa and a minor band at 38 kDa. These bands were in identical positions to bands produced by immunoaffinity purified rat liver 5′-nucleotidase (fig.2). In addition, some broadening of both bands was shown by the lysosomal enzyme with antibody staining proteins between the two major bands. The 38-kDa subunit of the ectoenzyme is formed by a proteolytic cleavage of a 70-kDa subunit which loses a cell surface glycosylated domain [8]. It is tempting to speculate that the 38-kDa fragment is generated in the lysosomes by proteolytic digestion with the other minor bands observed being intermediates in this breakdown process.

In contrast with previous studies where different 5′-nucleotidase activities have been observed in the lysosome [1,13,20] the present results show that the majority of the rat liver lysosomal enzyme is identical to the ectoenzyme with its active site directed towards the intra-lysosomal space. Internalisation and phago-lysosomal inactivation of the

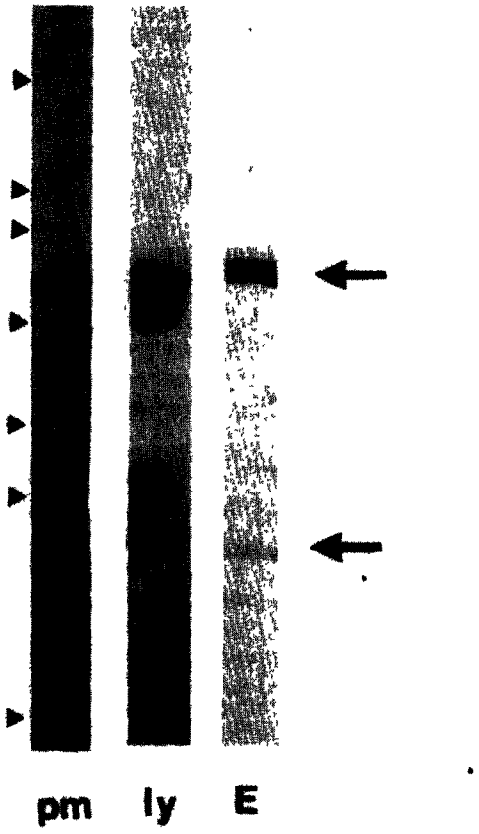

Fig.2. Immunoblotting of rat liver lysosomal 5′-nucleotidase. Aliquots of lysosomes (ly), plasma membrane (pm) [22] and immunoaffinity purified 5′-nucleotidase (E) [8] each approx. 100 mU enzyme were solubilised with 2% (w/v) sulphobetaine 14 and absorbed onto 0.5 mg monoclonal antibody (5NE5) immunoadsorbent, 60 h, at 4°C. Binding of enzyme to immunoadsorbent was 90% in each case. After SDS-polyacrylamide gel electrophoresis the protein was electrophoretically transferred to nitrocellulose. This was treated with polyclonal rabbit anti-(5′-nucleotidase) IgG (20 μg/ml) and labelled bands visualised with immunoperoxidase labelled second antibody. The markers on the left show the positions of molecular mass standards 200; 116; 92.5; 66; 50, 45, 25 and the arrows on the right indicate the 70 and 38-kDa bands

ectoenzyme has previously been shown in macrophages phagocytosing latex beads [2], though in this system plasma membrane components become trapped in the phago-lysosomes. In liver only a small proportion of the total 5′-nucleotidase (3%) is found in lysosomes and we

do not yet know the extent to which internalised 5′-nucleotidase needs to pass through the lysosome on recycling routes, whether the lysosome is an important functional site for the enzyme or whether it is simply a site for its ultimate degradation.

ACKNOWLEDGEMENTS

We thank the MRC for financial support and Professor C.N. Hales for his encouragement.

REFERENCES

[1] Widnell, C C and Little, J S (1977) Membranous Elements and Movements of Molecules (Reid, E ed) pp.149–162, Ellis Horwood, Chichester
[2] Stanley, K.K , Edwards, M.R and Luzio, J P (1980) Biochem J 186, 59–69
[3] Widnell, C C , Schneider, Y J., Pierre, B , Baudhuin, P. and Trouet, A. (1982) Cell (Cambridge, MA) 28, 61–70
[4] Luzio, J.P and Stanley, K K. (1983) Biochem J 216, 27–36.
[5] Widnell, C.C. (1984) Methods Enzymol. 98, 395–404
[6] Schneider, Y.J., Tulkens, De Duve, C and Trouet, A. (1979) J Cell Biol. 82, 466–474
[7] Ottosen, P.D , Courtoy, P.J. and Farquhar, M.G (1980) J. Exp. Med. 152, 1–19.
[8] Bailyes, E.M , Soos, M , Jackson, P., Newby, A C , Siddle, K and Luzio, J P. (1984) Biochem J 369–377
[9] Stanley, K.K , Burke, B., Pitt, T , Siddle, K and Luzio, J.P (1983) Exp Cell Res. 144, 39–46
[10] Siddle, K , Bailyes, E M. and Luzio, J P (1981) FEBS Lett 128, 103–107
[11] Reeves, J P (1979) J Biol Chem 254, 8914–8921
[12] Maguire, G A , Docherty, K and Hales, C N (1983) Biochem J 212, 211–218
[13] Pletsch, Q A and Coffey, J W (1972) Biochim Biophys Acta 276, 192–205
[14] Wattiaux, R , Wattiaux-de Coninck, S , Ronveaux-Dupal, M.F and Dubois, F (1978) J Cell Biol 78, 349–368
[15] Berthet, J , Berthet, L , Appelmans, F and De Dure, C. (1951) Biochem J. 50, 182–189
[16] Lloyd, J.B. (1969) Biochem. J 115, 703–707
[17] Docherty, K , Brenchly, G V and Hales, C N (1979) Biochem J 178, 361–366.
[18] Worku, Y and Newby, A C (1983) Biochem J 214, 325–330
[19] Hollander, V.P (1971) The Enzymes, 3rd edn (Boyer, P D ed) pp 449–498
[20] Arsenis, C and Touster, O (1978) Methods Enzymol 51, 271–275
[21] Werb, Z and Cohn, Z A. (1972) J Biol Chem 247, 2439–2446.
[22] Pilkis, S J , Exton, J H., Johnson, R A and Park, C R. (1974) Biochim Biophys Acta 343, 250–267
[23] Beers, R. and Sizer, J W (1952) J. Biol Chem 195, 133–140
[24] Kitcher, S.A , Siddle, K and Luzio, J.P (1978) Anal. Biochem. 88, 29–36.
[25] Cooperstein, S.J. and Lazarow, A L (1951) J Biol Chem 189, 665–670
[26] Bretz, R , Bretz, H and Pallade, G E (1980) J Cell. Biol. 84, 87–101.
[27] Udenfriend, S., Stein, S., Bohlen, P , Dairman, W , Leinguter, W and Weigele, M (1972) Science 178, 871