New form of acid phosphatase during lysosome biogenesis

G. Ramananda RAO,*† Harthattu N. AITHAL,* F. Gary TOBACK* and Godfrey S. GETZ†
*Department of Medicine, †Department of Biochemistry and †Department of Pathology, University of Chicago, Pritzker School of Medicine, Chicago, IL 60637, U.S.A.

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Lysosome formation was induced in cells of the renal medulla by feeding rats on a K⁺-deficient diet. The role of the endoplasmic reticulum in the production of acid phosphatase, a typical lysosomal enzyme, was examined. Lysosomal and microsomal fractions were prepared for study by differential centrifugation of homogenates of renal papilla and inner stripe of red medulla. Acid phosphatase activity in the microsomal fraction was distinguished from the activity in the lysosomal fraction in normal tissue by differences in pH optima, tartrate inhibition, distribution of multiple forms after polyacrylamide-gel electrophoresis and detergent-sensitivity. During progressive K⁺ depletion, acid phosphatase activity in both microsomal and lysosomal fractions of the tissue increased 3-fold. In the lysosomes, K⁺ depletion was associated with the appearance of a new band of acid phosphatase. The neuraminidase-sensitivity of this band on polyacrylamide-gel electrophoresis indicated that the enzyme protein had been modified by the addition of sialic acid residues. K⁺ depletion also altered the lysosomal enzyme so that thiol compounds were able to stimulate its activity.

Lysosome biogenesis and cell proliferation occur in the kidney medulla during K⁺ depletion (Oliver *et al.*, 1957; Spargo *et al.*, 1960; Morrison *et al.*, 1963; Toback *et al.*, 1976, 1979; Cleveland & Swank, 1978). We previously correlated the appearance of lysosomes during K⁺ depletion in rats with increased synthesis of a typical lysosomal enzyme, acid phosphatase (Aithal *et al.*, 1977). Although the mechanism by which renal cells produce new lysosomes is unknown, the presence of microsomal acid phosphatase suggests that this and other acid hydrolases are synthesized on the endoplasmic reticulum. In fibroblasts, lysosomal enzymes are synthesized as high-molecular-weight precursors and are subsequently processed to the shortened form found in the organelle (Hasilik & Neufeld, 1980a,b). The steps by which the enzyme molecule is packaged into the lysosome are unknown, although several attractive hypotheses have been proposed (Novikoff *et al.*, 1964; Hickman & Neufeld, 1972; Lloyd, 1977; von Figura & Weber, 1978). The aim of the present study was to determine the role of the microsomal fraction in lysosomal enzyme production. The results suggest that microsomal acid phosphatase activity is produced at an increased rate during K⁺ depletion, and that a new type of lysosomal isoform of the enzyme is produced by

post-translational modification of the enzyme protein. The term isoform is used to describe the multiple forms of acid phosphatase in this study. The term isoenzyme is used when the different forms of an enzyme are known to arise from genetically determined differences in primary structure (Dean, 1975).

Experimental

Animals

Male Sprague–Dawley rats were K⁺-depleted by feeding them *ad libitum* on a standard low-K⁺ but otherwise adequate diet (no. 170550; Teklad Test Diets, Madison, WI, U.S.A.). Control animals received the same diet supplemented with K⁺ (1.2 g of KCl/100 g of diet). All rats were allowed distilled water *ad libitum*.

Subcellular fractionation

At various intervals of K⁺ depletion, rats were killed by decapitation, and their kidneys were removed and decapsulated. Slices from various renal zones were cut with a Stadie–Riggs microtome as described previously (Toback *et al.*, 1976). Tissue homogenates (10%, w/v) of different zones were prepared in 0.45 M-sucrose in 10 mM-Tris/HCl/1 mM-EDTA buffer, pH 7.5 (referred to below as sucrose medium) by using a Potter–Elvehjem homogenizer. Cell debris and nuclei were sedimented and discarded after centrifugation at 600 g for 10 min in a

† Present address: Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore, India.

refrigerated Sorvall RC-2B centrifuge. From the supernatant, mitochondria were sedimented and discarded after centrifugation at 5000g for 10 min. The lysosomal fraction was then sedimented by centrifugation of the resulting supernatant at 17000g for 15 min, washed once in sucrose medium, and finally suspended in fresh medium to yield a protein concentration of 5–6 mg/ml. The microsomal fraction of different renal zones was obtained by centrifuging the post-lysosomal fraction at 100000g for 1 h in a refrigerated Beckman ultracentrifuge. The microsomal sediment was suspended in sucrose medium at 5–7 mg of protein/ml. All homogenization and centrifugation procedures were conducted at 0–4°C.

Enzyme assay

Acid phosphatase activity in homogenates and cell fractions was determined with *p*-nitrophenyl phosphate or β -glycerophosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) as substrate (Michell *et al.*, 1970). A typical assay mixture (0.5 ml) with *p*-nitrophenyl phosphate contained 80 mM-cacodylate/80 mM-acetate buffer, pH 5.25 or 5.75, and about 25 μ g of protein. After incubation at 37°C for 10 min in order to inhibit maximally non-specific phosphatases, including glucose 6-phosphatase (Bartlett, 1972), the reaction was started by addition of 20 mM substrate. After further incubation for 10 min the reaction was stopped by the addition of 2.5 ml of 0.1 M-NaOH. The yellow colour due to *p*-nitrophenol liberated enzymically was measured spectrophotometrically at 410 nm. The results were expressed as nmol of *p*-nitrophenol formed/min per mg of protein by using the molar absorption coefficient of $1.84 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Michell *et al.*, 1970). The assay was linear with respect to concentration of protein (10–100 μ g) and of substrate (1.25–200 mM) and time of incubation (0–15 min). When β -glycerophosphate was used as substrate (50 mM), P_i released was measured by the method of Fiske & SubbaRow (1925), and the results were expressed as nmol of phosphate/min per mg of protein. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V; Sigma) as the standard.

Triton X-100 (0.5%, w/v) was usually included in the assay medium for enzyme activity. However, it was not included in measurements of microsomal activity, because this non-ionic detergent inhibited activity in this fraction. When enzyme activities in the microsomal and lysosomal fractions were compared, detergent was omitted. Various effectors were added to the assay mixture at a final concentration of 10 mM, and preincubated for 5 min. The effect of another non-ionic detergent, Nonidet P-40 (0.4%, w/v) (Gallard-Schlesinger Chemical Manufacturing Co., New York, NY, U.S.A.), was also studied. The

temperature-sensitivity of the enzyme activity was assessed by heating for 30 min at 50°C.

Polyacrylamide-gel electrophoresis

Disc gel electrophoresis (Buchler Instruments, Fort Lee, NJ, U.S.A.) of lysosomal and microsomal fractions (about 30 μ g of protein) was performed in 5% polyacrylamide gel (5 mm $\times$ 75 mm) consisting of 4.85% acrylamide (Sigma) and 0.15% *NN'*-methylenebisacrylamide (Sigma) with 35 mM- β -alanine/8 mM-acetate buffer, pH 4.0 (Lin & Fishman, 1972). The gels contained 0.5% Triton X-100 or 0.4% Nonidet P-40 as indicated. Electrophoresis was performed at a current of 4 mA/tube at 0–4°C maintained by circulating refrigerated water through the electrophoresis chamber. Electrophoresis was terminated after 90–120 min, and the activity of acid phosphatase was detected on the polyacrylamide gels by the coupling-dye method of Barka (1961). After electrophoresis, gels were incubated for 15–20 min at 37°C in a reaction mixture that contained 0.1% (w/v) sodium α -naphthyl acid phosphate (Sigma) and 0.1% (w/v) Diazo Fast Garnet GBC (Sigma) in 0.1 M-cacodylate/0.1 M-acetate buffer, pH 5.5. The reaction was stopped by immersing the stained gels in 7.5% (v/v) acetic acid. The stained gels were scanned at 600 nm with a Gilford spectrophotometer equipped with a gel-scanning attachment. The distribution of activity between the various bands was estimated by planimetry. Six control or K^+ -depleted animals were used to prepare the microsomal and lysosomal fractions for electrophoresis.

In order to determine whether proteolysis *post mortem* plays a part in generating the multiple bands of enzyme activity on polyacrylamide-gel electrophoresis, kidney-medulla slices were incubated in sucrose medium for 90 min at 37°C in a metabolic shaker and then subjected to the above fractionation protocol.

To determine whether the isoforms of acid phosphatase on the gel arise as a result of variation in sialic acid content of this glycoprotein enzyme, samples were incubated with *Clostridium perfringens* neuraminidase type VI (Sigma) [50 units (μ mol/min)/mg of protein] for 2 h at 37°C (Neuberger & Ratcliffe, 1972) before gel electrophoresis.

Results

Fig. 1 shows that progressive K^+ depletion for 3 weeks was associated with increases of 2.5-fold in acid phosphatase specific activity in renal-medullary microsomal and lysosomal fractions when *p*-nitrophenyl phosphate was used as substrate. Similar increments in enzyme activity were observed in both fractions with β -glycerophosphate as substrate. The increment was 2-fold in both fractions when the activity was expressed per mg wet wt.

Fig. 1. Changes in acid phosphatase activity in renal-medullary homogenates (●) and in lysosomal (▲) and microsomal (○) fractions from rats fed on a K⁺-deficient diet for different periods of time

Enzyme activity was determined in the absence of detergents. Each value represents the mean for 9–12 animals assayed in groups of three; the S.E.M. was less than 5%. In three control animals total protein in the lysosomal fraction was 2.60 mg and in the microsomal fraction was 2.61 mg. After 21 days of K⁺ depletion, total protein in the lysosomal fraction from three rats was 3.45 mg and in the microsomal fraction was 4.50 mg.

of tissue, and 4-fold when expressed per 100 g body wt. Therefore total as well as specific enzyme activity increased during K⁺ depletion.

In control animals the pH optimum of acid phosphatase activity in the lysosomal fraction was 5.25, whereas that in the microsomal fraction was 5.75 (Fig. 2). K⁺ depletion did not change these pH optima, so that in subsequent experiments lysosomal and microsomal activities were assayed at pH 5.25 and 5.75 respectively.

To characterize acid phosphatase in each sub-cellular fraction enzyme activity was measured in the presence and absence of different effectors (Table 1). Tartrate inhibited the lysosomal enzyme by 53 and 65% in K⁺-depleted and control animals respectively, whereas the activity of the microsomal fraction was decreased by only 10–13%. Thus acid phosphatase activity in the microsomal and lysosomal fractions could be distinguished by differential inhibition by tartrate in control and K⁺-depleted animals. K⁺ depletion did not alter effector-sensitivity in the microsomal fraction, but induced

Fig. 2. pH optima of acid phosphatase activity in renal-medullary lysosomal (▲) and microsomal (○) fractions from control rats

Enzyme activity was assayed in 80 mM-cacodylate/80 mM-acetate buffer for the entire pH range in the absence of detergents. The pH of the buffer/substrate mixture was adjusted to the desired value before addition of enzyme. Each value represents the mean for six animals assayed in groups of three, with a variance of less than 6% of the mean.

several changes in the lysosomal fraction. The three thiol agents dithiothreitol, cysteine and glutathione stimulated lysosomal activity, as did alloxan. *N*-Ethylmaleimide, a thiol-blocking agent, inhibited acid phosphatase activity in lysosomes from control and K⁺-depleted rats by 30%. Prior incubation with a thiol-protective agent, dithiothreitol, prevented inhibition by *N*-ethylmaleimide.

Electrophoresis of microsomal and lysosomal fractions from control tissue on polyacrylamide gel containing Triton X-100 resolved acid phosphatase activity into two discrete bands (Fig. 3). In the microsomal fraction 75% of the activity was found in a slow-moving positively charged band located at 2–7 mm on the gel (band 1). A second relatively faster-moving negatively charged band at 15–20 mm contained 20% of the activity (band 2). In the lysosomal fraction two bands were also observed, but the distribution of activity differed from that for the microsomal fraction in that 50% of the activity was in band 1 and 45% in band 2.

During K⁺ depletion, increased activity in the microsomal fraction was confined to band 1. In the lysosomal fraction there was an increase in the relative amount of activity in band 1 to 75% of the total, and a decrease in band 2 to 15%. A new,

Table 1. *Influence of effectors on acid phosphatase activity in microsomal and lysosomal fractions from control and K⁺-depleted rats*

Microsomal and lysosomal fractions from renal medulla were prepared from control and 5-week-K⁺-depleted rats. Enzyme activity was assayed in the absence of detergents. Each value represents the mean for at least 12 animals assayed in groups of six, with a variance of less than 5% of the mean. Acid phosphatase specific activity (nmol/min per mg of protein) in the absence of effectors in control animals was 95 in microsomal fraction and 112 in lysosomes, and in K⁺-depleted animals 212 in microsomal fraction and 308 in lysosomes. Negative values indicate enzyme inhibition and positive values denote activation.

Effector	Change (%)			
	Control rats		K ⁺ -depleted rats	
	Microsomal	Lysosomal	Microsomal	Lysosomal
Tartrate	-13	-65	-10	-53
Dithiothreitol	0	+8	0	+35
Cysteine	-13	-5	0	+25
Glutathione	0	0	0	+20
Alloxan	0	-10	+4	+30
Oxalate	-6	-15	0	+13
Fluoride	-92	-96	-92	-93
Molybdate	-90	-91	-88	-88

faster-moving, band appeared at 35–45 mm on the gel and contained 10% of the total enzyme activity (band 3). This third band was not detected on the gel when lysosomes were obtained from slices of renal medulla from control animals incubated for 90 min before isolation of the lysosomal fraction. Therefore it is unlikely that band 3 was an autolytic product of acid phosphatase protein. This isoform of acid phosphatase was detected only in lysosomes obtained from the inner stripe of the red medulla of K⁺-depleted rats, and not from cortex or papilla of control or K⁺-depleted animals. The new isoform was first detected after 3 weeks of K⁺ depletion. The intensity of this band on the gel did not increase as the duration of K⁺ depletion was prolonged up to 7 weeks. In contrast, the intensity of the positively charged isoform (band 1) increased progressively from 2 to 7 weeks.

Incubation of microsomal and lysosomal fractions from control and K⁺-depleted animals with neuraminidase before electrophoresis resulted in a relative decrease in the amount of the negatively charged isoform in band 2, and a relative increase in band 1 (Fig. 4). In addition, neuraminidase treatment caused the disappearance of the new isoform, band 3, in the lysosomal fraction from K⁺-depleted rats. These results suggest that bands 2 and 3 contain sialic acid residues that were removed by neuraminidase.

Heating of microsomal and lysosomal fractions to 50°C for 30 min inhibited enzyme activity in bands 1 and 2 of control animals and in band 1 of K⁺-depleted rats by about 30% (Fig. 5). Incubation with dithiothreitol before heating offered only slight

protection against heat-induced inhibition. In band 2 of the microsomal fraction from K⁺-depleted animals, heat decreased enzyme activity by 70%, and dithiothreitol provided only marginal protection. The increased heat-sensitivity of band-2 enzyme suggests that this microsomal isoform has been altered during K⁺ depletion. In band 2 of the lysosomal fraction heat induced a 50% inhibition, which was largely overcome by pretreatment with dithiothreitol. Inhibition by heat treatment in band 3 was completely prevented by dithiothreitol.

Enzyme activities in microsomal and lysosomal fractions could also be distinguished from each other by the effect of treatment with non-ionic detergents. Triton X-100, which was necessary for electrophoresis on polyacrylamide gel, inhibited activity only in the microsomal fraction (by 50%) in both control and K⁺-depleted animals. Nonidet P-40, another non-ionic detergent, inhibited activity in the microsomal fraction by 50% and that in the lysosomal fraction by 15% in control and K⁺-depleted rats. Electrophoresis revealed that the loss of activity in the presence of Nonidet P-40 was confined to band 1, with no modification of the activities of the faster-migrating bands 2 and 3.

Discussion

During the onset of K⁺-depletion nephropathy in man and laboratory animals, lysosome biogenesis has been demonstrated in the renal papilla (Spargo, 1954; Spargo *et al.*, 1960; France, 1962; Morrison *et al.*, 1963; Aithal *et al.*, 1977; Cleveland & Swank, 1978; France *et al.*, 1978). New lysosomes first

Fig. 3. Mobility of (a) microsomal and (b) lysosomal acid phosphatase from control (—) and 3-week- K^+ -depleted (---) rats on polyacrylamide-gel electrophoresis

Acid phosphatase activity on the gel was detected histochemically. The intensity of the stain was measured spectrophotometrically at 600nm and expressed in arbitrary units on the ordinate. The fastest-moving, most negatively charged, species, band 3, was detected only in the lysosomal fraction from K^+ -depleted animals.

appear in cells of the papillary tip after 32h in rats fed on a K^+ -deficient diet (Toback *et al.*, 1976). Later, new lysosomes fill cells of the remainder of the papilla, and then appear in cells of the inner stripe of red medulla (Spargo *et al.*, 1960; Wilson *et al.*, 1973). Increased synthesis of typical lysosomal-enzyme proteins, acid phosphatase and β -glucuronidase, has been demonstrated in K^+ -depleted animals (Aithal *et al.*, 1977; Cleveland & Swank, 1978). Rapid renal growth also takes place during K^+ depletion and is most striking in cells of the collecting tubules, which exhibit adenomatous hyperplasia in the inner stripe of red medulla (Oliver *et al.*, 1957; Toback *et al.*, 1976, 1979). The return of K^+ to the diet is associated with cessation of renal

Fig. 4. Effect of neuraminidase on the isoforms of lysosomal acid phosphatase

Lysosomes were prepared from renal-medullary tissue of control and 5-week- K^+ -depleted rats, and preincubated with neuraminidase where indicated below. Samples were then applied to polyacrylamide gels, subjected to electrophoresis, and stained to detect acid phosphatase activity. From left to right gels contain lysosomes from: control animals, controls after neuraminidase treatment, K^+ -depleted animals and K^+ -depleted animals after neuraminidase. Band 3, the strongly negatively charged isoform of acid phosphatase, was detected only in lysosomes of K^+ -depleted animals (arrow), and disappeared after neuraminidase treatment.

growth, regression of the hyperplastic cells and condensation and disappearance of lysosomes in medulla and papilla (Ordóñez *et al.*, 1977).

In the present study, we have shown that progressive K^+ depletion was associated with increased specific and total activity of acid phosphatase in microsomal and lysosomal fractions of renal papilla and inner stripe of red medulla (Fig. 1). Acid phosphatase in the microsomal fraction was distinguished from that in the lysosomal fraction in normal tissue by differences in pH optima, tartrate inhibition, distribution of isoforms after polyacrylamide-gel electrophoresis and detergent-sensitivity. During K^+ depletion modifications were detected in enzyme activity in both fractions. The isoform of the enzyme in band 2 in the microsomal fraction acquired increased sensitivity to heat that was not ameliorated by the presence of dithiothreitol (Fig. 5). A new highly negatively charged anionic isoform (band 3) appeared only in lysosomes from K^+ -depleted animals, and was restricted to cells of the inner stripe of red medulla (Figs. 3 and 4). In addition, the enzyme in the lysosomal fraction exhibited a new sensitivity to stimulation by thiol reagents (Table 1). Only the negatively charged

Fig. 5. Inhibition of lysosomal and microsomal isoforms of acid phosphatase by heat and protection from inhibition by dithiothreitol

Microsomal fractions (a) and lysosomes (b) were prepared from renal tissue of control and 4-week-K⁺-depleted rats. Where indicated, samples were first preincubated with 10 mM-dithiothreitol for 15 min at 30°C. All samples were then heated for 30 min at 50°C before gel electrophoresis. Acid phosphatase activity in each gel was detected histochemically and scanned at 600 nm. The intensity of each band was estimated by planimetry and expressed as a percentage of that of the unheated samples. Inhibition by heat was most marked in band 2 of the microsomal fraction of K⁺-depleted animals and was not prevented by pretreatment with dithiothreitol. □, Heat treatment; ▨, dithiothreitol + heat treatment.

isoforms of the enzyme in the lysosomal fraction could be protected from heat-inhibition by dithiothreitol (Fig. 5).

Extralyosomal acid phosphatase activity has been demonstrated in many tissues (Neil & Horner, 1964, 1965; Maggi *et al.*, 1966; Lin & Fishman, 1972; Sasaki & Fishman, 1973; Miyayama *et al.*, 1975; Dean, 1975). An attractive hypothesis that relates the microsomal and lysosomal forms of the enzyme is that the lysosomal enzyme protein is first synthesized on the rough endoplasmic reticulum and then undergoes metabolic transformation into the lysosomal form (de Duve & Wattiaux, 1966; Cohn & Fedorko, 1969; Lin & Fishman, 1972; Goldstone & Koenig, 1973, 1974; Goldstone *et al.*, 1973). Novikoff *et al.* (1971) have suggested that the formation of primary lysosomes occurs in an analogous manner to that of protein-secretion granules. Thus acid hydrolases are synthesized on the endoplasmic reticulum and these membranes are transferred to the Golgi apparatus by a process of blebbing. The enzyme is further modified and finally released in the primary lysosomes (Cook, 1973). The results of the present study do not provide evidence for or against this model of lysosome biogenesis. However, if there is a precursor-product relationship between microsomal and lysosomal acid phosphatase, the differences in properties between the enzyme activities of the two fractions observed in the present study suggest that some modification of the enzyme molecule takes place in transit between

the endoplasmic reticulum and the lysosomes. On the other hand, the role of the micro-environment in which the enzyme molecules are located may contribute to the apparent differences in their properties, as suggested by the differential detergent-sensitivity.

That acid hydrolases are subjected to post-translational modification by the addition of sialic acid (Goldstone *et al.*, 1971, 1973; Barrett, 1972; Goldstone & Koenig, 1973) is also supported by the results of the present study. The addition of sialic acid residues increases the anionic character of the enzyme protein. In control animals, band 2 is relatively more prominent in the lysosomes than in the microsomal fraction. When anabolic processes of renal-medullary cells are accelerated during K⁺ depletion, there is a notable redistribution of enzyme activity in favour of the presumed least-modified isoform (band 1). This slow-migrating isoform of the enzyme is prominent in both lysosomes and microsomal fraction from K⁺-depleted rats.

The most noteworthy observation made in this study is the demonstration of a new, rapidly migrating, isoform of acid phosphatase (band 3) in lysosomes from K⁺-depleted rats. This new isoform was found only in cells of the inner stripe of red medulla after 3 weeks of K⁺ depletion. The isoform disappeared when treated with neuraminidase, thereby supporting the proposal that post-translational modification by the addition of sialic acid residues imparted more negative charge to the enzyme

molecules. Band 3 could represent a highly modified (post-translational) form of the enzyme, although the reason for the appearance of this isoform at the time that the least-modified form (band 1) is so prominent remains to be explained.

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