SYNTHESIS AND TURNOVER OF LYSOSOMAL GLYCOPROTEINS. RELATION TO THE MOLECULAR HETEROGENEITY OF THE LYSOSOMAL ENZYMES

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1. Introduction

Many, if not all, of the lysosomal acid hydrolases exist in several molecular forms. For example, using polyacrylamide gel electrophoresis [1] and isoelectric focusing [2,3] we found two to five distinct forms of seven different hydrolases in rat kidney lysosomes. That this molecular heterogeneity may have biological importance is strongly suggested by the recent discovery that the acidic (A) form of a lysosomal hydrolase is lacking in two inherited lipid storage diseases, namely aryl sulfatase A in metachromatic leukodystrophy [4,5] and hexosaminidase A in Tay-Sachs disease [6,7]. Apparently the corresponding basic (B) form of these hydrolases which is present in normal or elevated amounts in these diseases is incapable of hydrolyzing the accumulated sphingolipid, cerebroside sulfate in metachromatic leukodystrophy [4,5] and GM_2 ganglioside (and its asialo derivative) in Tay-Sachs disease [6,7]. These observations imply that the different molecular forms may display divergent substrate specificities and hydrolytic activities in vivo.

We have shown that the various acid hydrolases in purified lysosomal fractions from rat kidney and liver are glycoproteins (GPs) [1,8,9]. A study of the effects of bacterial neuraminidase on the electrophoretic mobility [8], and pIs [3] of a number of lysosomal hydrolases indicated that *N*-acetylneuraminic acid (NANA) residues are largely responsible for the solubility and electronegative charge of these enzymes. On the basis of the solubility and electrophoretic mobility of five acid hydrolases in rat kidney subcellular fractions [9,10], and radiochemical [11] and ultrastructural radioautographic [12] findings of isotope uptake into lysosomal GPs, we proposed that the various hydrolases are synthesized in a restricted portion of the rough endoplasmic reticulum as bound, basic GP enzymes. The soluble, acidic forms are generated from the latter by attachment of sugar sequences containing NANA as the nascent GP enzymes migrate through the Golgi apparatus en route to the lysosomes [10]. From this schema we inferred that the basic forms of these enzymes which occur in lysosomes may be produced from the acidic forms during intracellular digestion through autolytic cleavage of their NANA residues by lysosomal neuraminidase [10].

As part of a series of studies to determine the origin and physiological significance of this molecular heterogeneity we report here on the synthesis and turnover of rat kidney lysosomal GPs, labeled in the NANA and peptide moieties with *N*-acetyl-[^{3}H]mannosamine and [^{14}C]lysine. The results indicate that newly synthesized macromolecules are packaged in rat kidney lysosomes as very acidic, NANA-rich GPs. The labeled GPs become progressively more basic with the passage of time due to the fact that NANA residues turn over more rapidly than the peptide moieties. A preliminary account of this work has appeared [13].

2. Methods

Sprague–Dawley rats (150–200 g) received L-[UL-^{14}C]lysine (240 mCi/mmole, 10 μCi per rat) and/or *N*-acetyl-β-D-[UL-^{3}H]mannosamine (400 mCi/

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mmole, 40 μCi per rat), a precursor of NANA [14] (ICN Corporation). To study isotope incorporation, kidneys were removed 90 min after [^{14}C] lysine and/or 60 min after *N*-acetyl-[^{3}H] mannosamine injection. For turnover studies, kidneys were excised from animals 4.5, 8.5 and 12.5 days after [^{14}C] lysine, 4.5 and 8.5 days after *N*-acetyl-[^{3}H] mannosamine, and 1, 2, 4.5 and 30 days after both isotopes. Kidneys from five rats were pooled for each experiment and heavy lysosomal fractions isolated as described previously [9, 11]. Lysosomal fractions showed a 17- to 25-fold enrichment in acid hydrolase activities over the homogenate and contained a maximum of 5–10% contamination by mitochondria, peroxisomes and endoplasmic reticulum, as shown by assay of marker enzymes and electron microscopy [9, 11].

To ascertain the labeling of sugar residues by ^{3}H from *N*-acetyl-[^{3}H] mannosamine, one lysosomal preparation was isolated 90 min after isotope injection and hydrolyzed for 1 hr in 0.1 N H_2SO_4 at 80°C to split bound NANA. The residue was further hydrolyzed for 3 hr in 3 N HCl at 100°C to release neutral and amino sugars, and the sugars separated by column and paper chromatography [1]. Radioassay of the isolated sugars showed that 90% of the incorporated ^{3}H radioactivity occurred in NANA, and 10% in *N*-acetylglucosamine. Thus, *N*-acetyl-[^{3}H] mannosamine is an effective precursor of NANA in lysosomal GPs.

Rat kidney lysosomes contain an acid neuraminidase which cleaves the NANA of lysosomal glycoproteins [8, 15]. The following procedures, developed to minimize the autolytic degradation of lysosomal GPs [15], were used. Lysosomal pellets were cooled to −20°C immediately upon isolation and covered with 3 ml of 0.1 M sodium–glycinate buffer, pH 9.0, containing 0.2% Triton X-100 (v/v) and 0.1% *p*-nitrophenyloxamic acid (K and K Laboratories), an inhibitor of bacterial [16] and lysosomal [15] neuraminidase. After 30 min the pellet was dispersed and sonicated in this medium and the insoluble residue removed by centrifugation at 100 000 *g* for 1 hr at 4°C. The soluble lysosomal protein was immediately fractionated by isoelectric focusing in an ampholine gradient, pH 10–3, containing 0.1% *p*-nitrophenyloxamic acid in an LBK Model 7100 column at 4°C for about 16 hr [17]. 3 ml fractions were collected and aliquots dialyzed overnight at 4°C against several changes of 0.005 M sodium–glycinate buffer, pH 8.8, and 0.1% *p*-nitrophenyloxamic acid.

Aliquots of undialyzed fractions were used for measurement of pH and one or more of the following hydrolases, as described previously [9]: aryl sulfatase (aryl sulfate sulfohydrolase, EC 3.1.6.1; *p*-nitrocatechol sulfate substrate); β-glucuronidase (β-D-glucuronohydrolase, EC 3.2.1.31; phenolphthalein glucuronic acid); acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2; *p*-nitrophenylphosphate); β-*N*-acetyl-hexosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30; 4-methylumbelliferyl-β-D-glucosaminide); and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21; *p*-nitrophenyl-β-D-glucopyranoside). Dialyzed fractions were analyzed for protein [18] after precipitation with cold 7.5% trichloroacetic acid. For radioassay, 10 ml of Aquasol (New England Nuclear Corp.) was added to aliquots of fractions or total soluble lysosomal GP and ^{14}C and ^{3}H counted in a Beckman Model LS-250 liquid scintillation system.

3. Results and discussion

The radioactivity decay curves of ^{14}C and ^{3}H incorporated into the total soluble lysosomal GP after injection of [^{14}C] lysine and *N*-acetyl-[^{3}H] mannosamine are shown in fig. 1. The specific radioactivity (S.R.) of incorporated ^{14}C declined at a faster rate at the beginning [half-time ($t_{1/2}$) = 6.8 d] than toward the end of the experimental period ($t_{1/2}$ = 13.5 d). The S.R. of incorporated ^{3}H declined precipitiously between 1 hr and 4.5 days ($t_{1/2}$ = 3.2 d), but the rate of decline fell off subsequently ($t_{1/2}$ = 9.5 and 19.0 d) so that the S.R. of ^{3}H and ^{14}C were both down to about 10% of their original values by 30 days. Thus, the turnover rate of NANA was more than twice that of the polypeptide during the first few days.

Fig. 2 shows the distribution of protein and several lysosomal enzymes in the ampholine gradient. The acidic form of all five hydrolases had pIs between 4.0 and 4.8 and occurred in GP fractions with pIs between 3.0 and 4.9. The basic form of these hydrolases exhibited pIs ranging from 6.1 (β-glucuronidase) to 8.1 (β-*N*-acetylhexosaminidase) and occurred in GP fractions with pIs between 5.8 and 10 or higher. A third

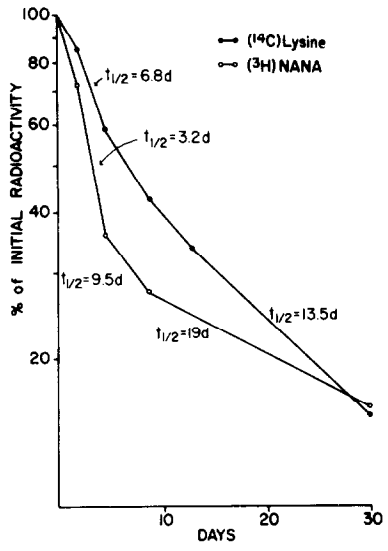

Fig. 1. Turnover of ^{14}C and ^{3}H from [^{14}C]lysine and *N*-acetyl-[^{3}H]mannosamine, a precursor of NANA, in the total soluble glycoprotein of rat kidney lysosomes. Rats were sacrificed 1.5 hr, 2, 4.5, 8.5, 12.5 and 30 days after receiving [^{14}C]lysine, and 1 hr, 2, 4.5, 8.5 days and 30 days after *N*-acetyl-[^{3}H]mannosamine by intraperitoneal injection. Kidneys from five rats for each time point were pooled, lysosomal fractions isolated and protein and specific radioactivities measured as described in the text. The specific radioactivities are plotted as the percent of the initial specific radioactivity (^{14}C = 330; ^{3}H = 840) on a logarithmic scale in the ordinate against a linear time scale in days in the abscissa. The half-times ($t_{1/2}$) are shown for several time intervals.

peak of acid phosphatase (pI 5.6) and small amounts of the basic forms of the other hydrolases were present in the intermediate GP fractions with pIs between 5.0 and 5.7. Acidic lipoglycoproteins without enzymatic activities, which comprise about 35% of the total soluble protein in rat kidney and liver lysosomes [19], were also present. These had a peak pI of 4.3 and occurred mainly in GP fractions with pIs between 3.8 and 5.3 [20].

When animals were given [^{14}C]lysine and *N*-acetyl-[^{3}H]mannosamine 90 and 60 min before death, the ^{14}C and ^{3}H radioactivity profiles of the fractionated lysosomal GPs were essentially congruent (fig. 3). 96 and 100% of the ^{14}C and ^{3}H, respectively, was recovered in GP fractions with pIs between 2.1 and 5.8, the bulk of both labels occurring in the very acidic GP fractions with pIs in the range of 2.0 to 4.9. The slight labeling of GPs with pIs between 5.0 and 6.6 probably represents uptake into GPs which had lower pIs in vivo but lost some NANA residues during extraction and processing due to the action of lysosomal neuraminidase [15].

With the passage of time the ^{14}C radioactivity profile of the GP fractions underwent a dramatic and progressive displacement from fractions of low pIs labeled initially into fractions of higher pIs that were originally unlabeled or sparsely labeled (fig. 4). By 4.5 days label from [^{14}C]lysine virtually disappeared from GPs with pIs of 2.7–4.2, whereas frac-

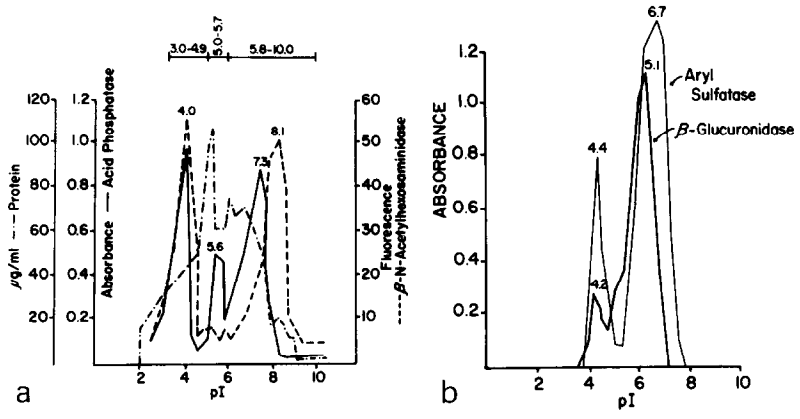

Fig. 2. Fractionation of soluble glycoproteins from rat kidney lysosomes by isoelectric focusing in a pH 10 to 3 gradient. The pH (pI) of the fraction at equilibrium is noted in the abscissa. a) Distribution of protein in (μg/ml), β-*N*-acetylhexosaminidase and acid phosphatase in fractions. b) Distribution of aryl sulfatase and β-glucuronidase in fractions. The acidic (pI 3–4.9), intermediate (pI 5–5.7) and basic (pI 5.8–10) GP fractions are indicated in the upper portion of fig. 2a.

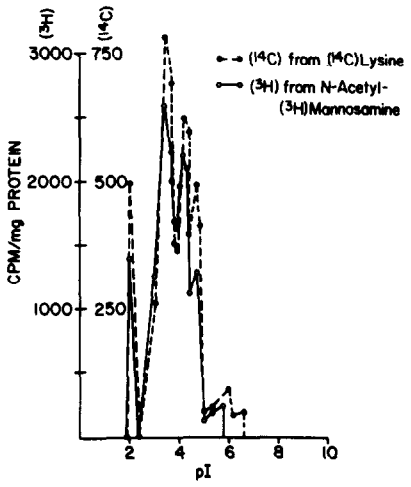

Fig. 3. Synthesis of peptide and NANA moieties of rat kidney lysosomal glycoprotein fractions. Rats received *N*-acetyl-[^{3}H]mannosamine and [^{14}C]lysine 1.0 and 1.5 hrs, respectively, before death. Lysosomal extracts were fractionated by isoelectric focusing in a pH 10 to 3 gradient. The specific radioactivity for ^{14}C and ^{3}H is shown in the ordinate.

tions with pIs of 5.0–6.9 showed a substantial increment in label over their original low levels. This shift progressed still further by 8.5 and 12.5 days when fractions with pIs in the range of 7.0–11.4 not originally labeled displayed appreciable radioactivity. The pI of the maximally labeled fraction rose from 3.3 at 1.5 hr to 4.7, 5.6 and 6.5 at 2, 4.5 and 8.5 days, and 7.1 at 12.5 and 30 days. ^{3}H label from *N*-acetyl-[^{3}H]mannosamine displayed a similar, though less conspicuous, shift with time due to the fact that the ^{3}H radioactivity decayed much more rapidly than the ^{14}C radioactivity at first (fig. 4). Furthermore, the basic GPs probably had a smaller NANA content than the acidic GPs.

Fig. 5 presents the mean specific radioactivity (S.R.) of ^{14}C of the acidic (pI 3–4.9) and basic (pI 5.8–10) GP fractions, which contain the acidic and basic forms of a number of acid hydrolases (fig. 2), and an intermediate fraction (pI 5–5.7) at the various time intervals. One and a half hours after isotope injection, the S.R. of the acidic GP fraction was 9- and 11-fold greater than that of the intermediate and

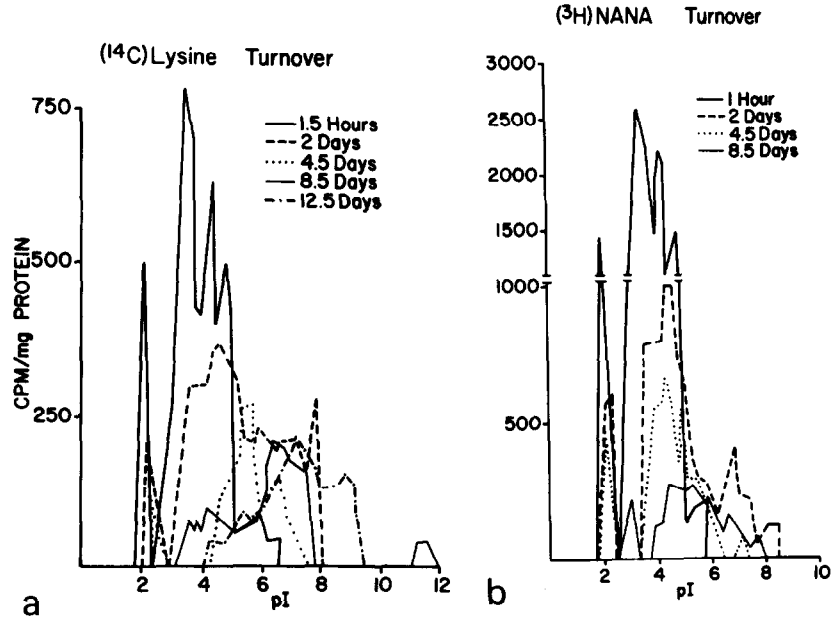

Fig. 4. Synthesis and turnover of the polypeptide and NANA moieties of rat kidney lysosomal glycoprotein fractions. a) Rats were given [^{14}C]lysine and killed 1.5 hr, 2, 4.5, 8.5, 12.5 and 30 (not shown) days later. The specific ^{14}C radioactivity of the glycoprotein fractions is shown in the ordinate. b) Rats were given *N*-acetyl-[^{3}H]mannosamine and killed 1 hr, 2, 4.5, 8.5 and 30 (not shown) days later. The specific ^{3}H radioactivity is given in the ordinate.

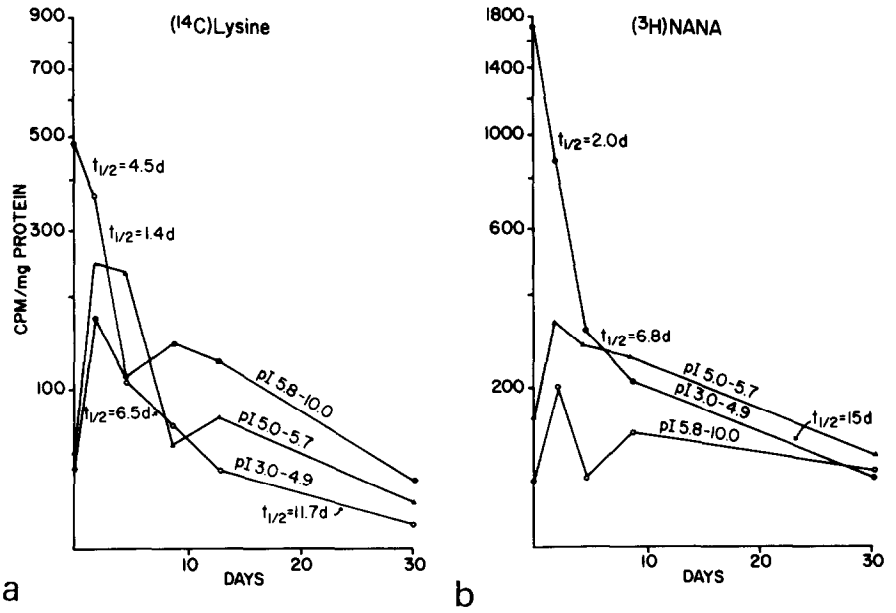

Fig. 5. Synthesis and turnover of the polypeptide and NANA moieties in the acidic (pI 3–4.9) intermediate (pI 5–5.7) and basic (pI 5.8–10) glycoprotein fractions in rat kidney lysosomes. Data are based on experiments summarized in fig. 4. The average specific radioactivity of each fraction is presented on a logarithmic scale in the ordinate against a linear time scale in days in the abscissa. a) Specific ^{14}C radioactivity from [^{14}C]lysine. b) Specific ^{3}H radioactivity from *N*-acetyl-[^{3}H]mannosamine.

basic GP fractions, respectively. However, between 1.5 hr and 4.5 days the S.R. of the acidic GP fraction declined sharply (average $t_{1/2}$ = 2.3 d), and then decreased more gradually, whereas the S.R. of the intermediate and basic GP fractions increased 4.6- and 4-fold, respectively, between 1.5 hr and 2 days and surpassed that of the acidic GP fraction shortly thereafter. The S.R. of the intermediate GP fraction declined abruptly after 4.5 days, but the S.R. of the basic GP fraction was maintained at a relatively high level through 12.5 days, exceeding that of the intermediate GP fraction after about 6 days.

The pattern of ^{3}H radiodecay generally resembled that of ^{14}C (fig. 5). Thus, the S.R. of the acidic GP fraction was 11- and 25-fold greater than that of the intermediate and basic GP fractions 1 hr after isotope injection. The S.R. of the acidic GP fraction dropped sharply between 1 hr and 4.5 days ($t_{1/2}$ = 2 d) and then more slowly, whereas the S.R. of the intermediate and basic GP fractions rose 2- and 3-fold between 1 hr and 2 days and then declined at a slow rate.

The present experiments demonstrate that newly synthesized macromolecules, labeled in the NANA and peptide moieties, are packaged in rat kidney lysosomes primarily, if not exclusively, in the form of highly acidic, NANA-rich GPs with pIs in the range of 2.1 to 4.9. At first the NANA residues turn over much more rapidly than the peptide moieties and the labeled GPs become progressively more basic with the passage of time. A similar rise in the pIs of lysosomal GPs and several acid hydrolases has been observed after the removal of NANA residues in vitro with bacterial or lysosomal neuraminidase [3, 15].

Our results provide the first direct evidence for the schema proposed earlier [10] that each newly completed hydrolase is packaged in lysosomes in a highly acidic form. The finding that the basic GP fraction, which contains the basic forms of a number of lysosomal hydrolases, is essentially inlabeled shortly after isotope administration and becomes significantly labeled at a later time indicates that it is unlikely that the basic forms of these enzymes are synthesized and packaged as separate enzymes. However, it provides strong support for the earlier suggestion [10] that the basic lysosomal isoenzymes are generated

from the corresponding acidic isoenzymes during biodegradation. This view is supported by the finding that hexosaminidase A and B [21,22] and β-glucuronidase I (A) and III (B) [23] are immunologically identical and thus presumably possess a common polypeptide structure.

Lysosomal extracts can digest specific GPs of non-lysosomal origin during incubation in vitro through the concerted action of neuraminidase, various glycosidases and proteases present in the lysosomal enzyme complement [4,5]. We recently found that lysosomal GPs underwent autolytic cleavage of NANA, neutral and amino sugars and peptides during incubation at pH 5 [15]. Coincidentally, the pIs of the partially degraded GPs and of several hydrolases increased [3,15]. The present investigation suggests that peptides, NANA, and probably sugar residues as well, are lost from lysosomal GPs during physiological turnover. A partial removal of NANA, sugar and (glyco?)peptide residues would be expected to modify the molecular conformation of acidic lysosomal isoenzymes and alter their physicochemical and catalytic properties. Therefore, it seems unlikely that the basic isoenzymes of lysosomal hydrolases are merely asialo derivatives of the corresponding acidic isoenzymes, as suggested earlier [10]. Moreover, in recent studies involving the treatment of purified aryl sulfatase A [26] and α-galactosidase A [27] with bacterial neuraminidase, the desialylated enzymes showed the expected change in electrical charge, but otherwise retained the properties of the native enzymes. These considerations prompt us to propose that the various basic isoenzymes present in lysosomes are generated during physiological hetero- and autophagy through a partial autolytic digestion of the corresponding acidic isoenzymes. We are currently extending this investigation to one or two purified lysosomal enzymes in order to gather more precise data on this important subject.

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