

Adult forms of glycogenosis type II

A defect in an early stage of acid α -glucosidase realization

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The activity of acid α -glucosidase in cultured fibroblasts from adult patients with the lysosomal storage disease glycogenosis type II is only 10% of normal. A normal activity per molecule is found for the mature as well as for the precursor form of acid α -glucosidase in adult mutant fibroblasts. Excessive lysosomal breakdown of mature enzyme purified from mutant fibroblasts and taken up by acceptor cells does not occur. However, the NH_4Cl -stimulated secretion of a precursor form of acid α -glucosidase by adult mutant fibroblasts is markedly reduced. The results are indicative of a defect during the production of acid α -glucosidase.

<i>Glycogenosis type II</i>	<i>Pompe disease</i>	<i>α-Glucosidase</i>	<i>Synthesis</i>	<i>Processing</i>
		<i>(Human skin fibroblast)</i>		

1. INTRODUCTION

Glycogenosis type II is an autosomal recessive glycogen storage disease, in which deficiency of lysosomal (acid) α -glucosidase (EC 3.2.1.20) is the basic defect [1]. The amount of acid α -glucosidase present in cultured fibroblasts [2] and urine [3] from patients with adult forms of the disease is reduced to 10% of normal. Since no kinetic, physical nor immunological parameters of the adult mutant α -glucosidase were found to be abnormal [2,3], it was hypothesized that the enzyme was either synthesized at reduced rate or degraded at increased rate [2].

Here, we show that not only the cellular but also the extracellular precursor form of acid α -glucosidase from adult mutant fibroblasts has a normal stability and normal specific catalytic activity. Moreover, the apparent biological half-lives of acid α -glucosidase from adult mutant and control fibroblasts are comparable. The only striking difference between control and mutant fibroblasts is, that the amount of precursor acid α -glucosidase, measured as NH_4Cl -stimulated enzyme secretion, is markedly reduced in mutant cells. We conclude that the defect in adult forms of glycogenosis type

II lies in an early step in the realization process of acid α -glucosidase.

2. MATERIALS AND METHODS

2.1. Secretion and uptake of enzyme in cell culture

Human skin fibroblasts were cultured in Ham's F10 medium supplemented with 10% fetal calf serum and antibiotics. For enzyme secretion studies, the cells were seeded in 24-well tissue culture plates and 48 h later, when the cultures had reached confluence, the medium was replaced by 500 μl fresh medium with added to it 5% inactivated (4 h, 37°C pH 10) fetal calf serum and NH_4Cl at 10 mM final conc. At given time points a 75 μl aliquot was taken and lysosomal enzyme activities were measured as follows: acid α -glucosidase; 50 μl medium + 100 μl 4-methylumbelliferyl- α -D-glucopyranoside, β -galactosidase; 10 μl medium + 20 μl 4-methylumbelliferyl- β -D-galactopyranoside, and β -N-acetylhexosaminidase; 5 μl + 20 μl 4-methylumbelliferyl- β -D-glucosaminide. Substrate concentrations were as in [4], and incubation times were 2 h, 1.5 h and 0.5 h, respectively, for the different enzymes. Reactions were stopped with 500 μl sodium carbonate

buffer (0.5 M) pH 10.7. Enzyme uptake and stability studies were also carried out in 24-well culture plates. 5–15 Units of acid α -glucosidase, purified from control cells or fibroblasts from patients with adult forms of glycogenosis type II (as in [5]) were added overnight to the medium (500 μ l) of acceptor cells. For this purpose we used fibroblasts from patients with the infantile form of glycogenosis type II, without any detectable acid α -glucosidase activity. 1 Unit hydrolyzes 1 nmol 4-methylumbelliferyl- α -glucopyranoside/h.

2.2. Immunotitration

Intracellular enzyme was purified from mass cultures of control or mutant fibroblasts. Extracellular (precursor) enzyme was obtained by stimulating fibroblasts for 2 days with 10 mM NH_4Cl to secrete newly synthesized lysosomal enzymes. In the latter case, 6×10^6 control cells or 60×10^6 mutant cells were maintained in medium with 1% inactivated fetal calf serum. The medium was 10–50-times concentrated and used as such. Cellular and medium enzyme was used in a concentration of 1–4 units/10 μ l. Samples of 10 μ l were incubated overnight at 4°C with 10 μ l serially diluted antiserum, raised in rabbits against highly purified placental acid α -glucosidase. Subsequently, 50 μ l substrate solution was added containing 50 mg glycogen/ml in 0.2 M sodium acetate buffer (pH 4.3). The samples were incubated for 1 h at 37°C and the reaction was terminated by heating for 1 min at 100°C. The amount of glucose formed in the reaction was measured by adding 200 μ l glucose reagent, as in [2].

3. RESULTS AND DISCUSSION

In the first series of experiments, acid α -glucosidase was purified from mass cultures of control fibroblasts and fibroblasts from adult patients, in order to compare the specific catalytic activities of the normal and mutant enzymes. The assay is based on the fact that acid α -glucosidase bound to antibody molecules loses its activity for the natural substrate glycogen. This feature results in an inverse linear relation between the activity of acid α -glucosidase and the amount of antiserum added to the reaction mixture (fig.1). As shown, parallel lines are found in all instances comparing the normal and mutant enzymes. Thus, equal

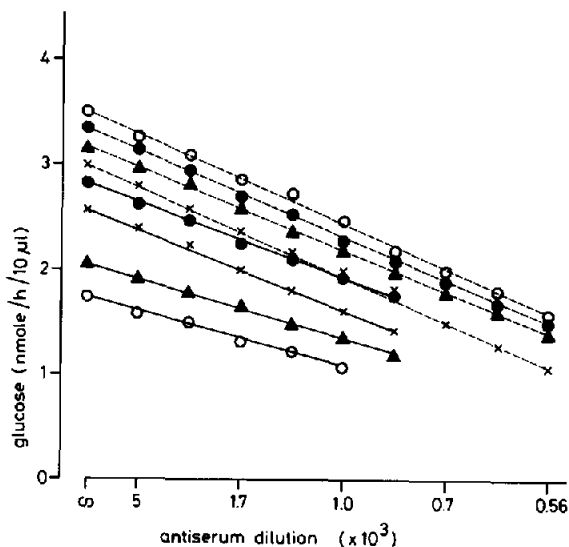


Fig.1. Immunotitration of normal and mutant α -glucosidase: (---) acid α -glucosidase purified from fibroblasts; (—) acid α -glucosidase in concentrated medium; (\blacktriangle — \blacktriangle) adult mutant (177LAD); (\circ — \circ) adult mutant (338 LAD); (\bullet — \bullet) control (261 LAD); (\times — \times) control (GRA).

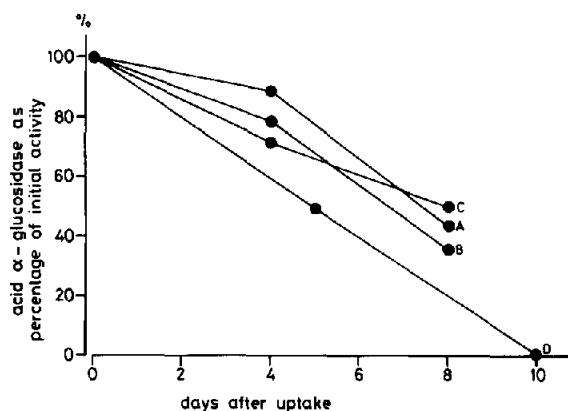


Fig.2. In vivo stability of endocytosed acid α -glucosidase. Addition of normal or adult mutant acid α -glucosidase over 16 h resulted in an intracellular activity of 2–10 units/mg cellular protein. The initial activity after 16 h uptake was set at 100%. Results are shown for acid α -glucosidase purified from: A, control cells (261LAD); B, adult patient cells (177LAD); C, control cells (GRA); D, adult patient cells (338LAD).

amounts of antibody are needed to inactivate a fixed amount of both normal enzyme and enzyme from mutant cells. The conclusion, therefore, is that the catalytic activity of the mutant enzyme per enzyme molecule is identical to that of normal acid α -glucosidase.

In the second set of experiments, the purified normal and adult mutant enzymes were added to the culture medium above fibroblasts from a patient with infantile glycogenosis type II, which are fully deficient in acid α -glucosidase. The aim was to test the intralysosomal stability of the normal and the adult mutant enzymes after endocytosis by the acceptor cells. Results of these experiments are illustrated in fig.2. Over 16 h, 1–5% of the added enzyme, purified from control fibroblasts or cells from adult patients, is internalized and recovered as active enzyme in the acceptor cells. The uptake efficiency is the same for normal and mutant enzymes (not shown). Fig.2 illustrates that there is a gradual decline of intracellular activity over a period of 10 days following uptake. The estimated half-life is 5–7 days for the normal enzyme as well as for the adult mutant acid α -glucosidase.

From this observation it can be concluded that the 90% reduction of acid α -glucosidase activity in fibroblasts from patients with the adult form of glycogenosis type II is not caused by an increased susceptibility of a mutant enzyme to intralysosomal degradation. Moreover, when purified normal acid α -glucosidase was administered to fibroblasts from adult patients, a normal uptake and intracellular stability was observed. This excludes that in the adult form of glycogenosis type II, degradation of what appears to be a normal enzyme is promoted by lack of protection against lysosomal proteases. Such a genetically determined lack of protection has been described for β -galactosidase in certain patients with a combined deficiency of neuraminidase and β -galactosidase [6,7].

Since lysosomal enzymes undergo, during their synthesis, a series of post-translational modifications involving glycosylation and size reductions [8–10], in vivo measurement of enzyme synthesis will give the net result of some or all of these steps in enzyme realization. It has been shown, however, that transport of newly formed lysosomal enzymes to the lysosomes can be blocked by adding NH_4Cl to the culture medium of fibroblast, whereby 85–90% of newly synthesized high M_r precursor en-

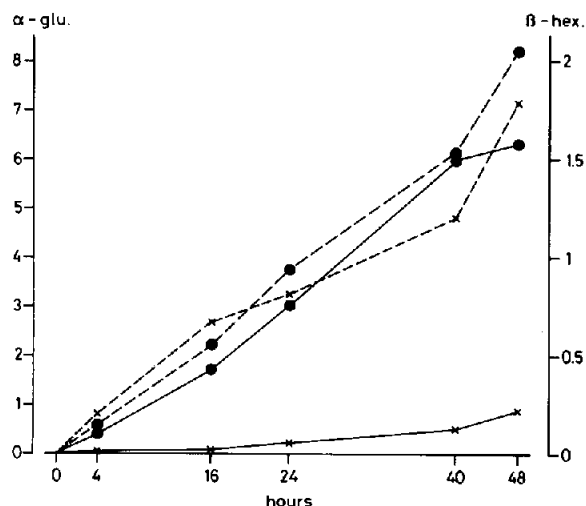


Fig.3. NH_4Cl -stimulated secretion of α -glucosidase and β -hexosaminidase. At intervals of 4, 16, 24, 40 and 48 h after addition of NH_4Cl the activities of α -glucosidase and β -hexosaminidase in the medium were measured. The activity of α -glucosidase is expressed in $\text{nmol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$, that of β -hexosaminidase in $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg protein}^{-1}$: (—•—) acid α -glucosidase; (---•---) β -hexosaminidase; (—•—•—) control cells (GRA); (—x—x—) cells from an adult patient (338LAD).

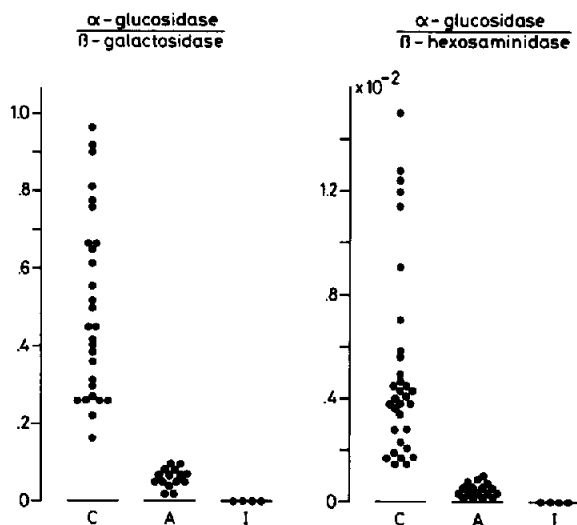


Fig.4. Secretion of acid α -glucosidase. At intervals of 16, 24, 40 and 48 h after addition of NH_4Cl , medium samples were taken and the lysosomal enzyme activity ratios for each individual cell line were plotted: C, 6 different control cell lines; A, 3 different cell lines from adult patients (196LAD, 338LAD, 177LAD); I, 2 cell lines from patients with the infantile form of glycogenosis II (266LAD, 217LAD).

zymes are secreted [11,12]. Such intervention in the normal processing pathway of lysosomal enzymes allows us to discriminate between defects in early or late steps in the realization process.

As early as 4 h after the addition of NH_4Cl , the secretion of catalytically active acid α -glucosidase can be measured in the medium. The accumulation of enzyme proceeds almost linearly for up to 2 days (fig.3). This figure also shows that the secretion of acid α -glucosidase by adult mutant fibroblasts is much reduced, whereas the mutant cells secrete similar amounts of β -hexosaminidase compared to control cells.

Although lesser in amount, the specific catalytic activity of acid α -glucosidase secreted by adult mutant fibroblasts was the same as that of the enzyme secreted by normal cells (fig.1). Moreover, these precursor enzymes from control and mutant cells were equally stable and they lost $< 10\%$ of activity over 52 h in 37°C culture medium. The data from several series of experiments, with different cell lines, are summarized in fig.4. The secretion of acid α -glucosidase is presented as the ratio of acid α -glucosidase to β -galactosidase and to β -hexosaminidase, to reduce the effect of biological variation. The mean ratio of α -glucosidase to β -hexosaminidase is 5×10^{-3} for control cell lines and 0.4×10^{-3} for cell lines from adult patients. The ratio of α -glucosidase to β -galactosidase is 0.5 for control vs 0.06 for mutant cells. On the average, fibroblasts from adult glycogenosis II patients secrete only 8–12% of the normal amount of precursor acid α -glucosidase. Fibroblasts from patients with the infantile form of this disease do not secrete any active acid α -glucosidase.

On the basis of the combined observations we conclude that the mutation in adult forms of glycogenosis type II results in a diminished net production of a precursor form of acid α -glucosidase.

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REFERENCES

- [1] Hers, H.G. (1963) *Biochem. J.* 86, 1–6.
- [2] Reuser, A.J.J., Koster, J.F., Hoogeveen, A. and Galjaard, H. (1978) *Am. J. Hum. Genet.* 30, 132–143.
- [3] Schram, A.W., Brouwer-Kelder, B., Donker-Koopman, W., Loonen, C., Hamers, M.N. and Tager, J.M. (1979) *Biochim. Biophys. Acta* 482, 89–97.
- [4] Galjaard, H. (1980) *Genetic Metabolic Diseases*, Elsevier Biomedical, Amsterdam, New York.
- [5] Beratis, N.G., LaBadie, G.U. and Hirschhorn, K. (1978) *J. Clin. Invest.* 63, 1262–1274.
- [6] Van Diggelen, O.P., Hoogeveen, A.T., Reuser, A.J.J. and Galjaard, H. (1982) *Biochim. Biophys. Acta* 703, 69–76.
- [7] d'Azzo, A., Hoogeveen, A., Reuser, A.J.J., Robinson, D. and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. USA* 79, in press.
- [8] Erickson, A.H., Conner, G.E. and Blobel, G. (1981) *Proc. Natl. Acad. Sci. USA* 78, 11224–11231.
- [9] Neufeld, E.F. (1981) in: *Lysosomes and Lysosomal Storage Diseases*, pp. 115–130, Raven, New York.
- [10] Sly, W.S., Natowicz, M.R., Gonzalez-Noriega, A., Grubbe, J.B. and Fischer, H.D. (1981) in: *Lysosomes and Lysosomal Storage Diseases*, pp. 131–146, Raven, New York.
- [11] Hasilik, A. and Neufeld, E.F. (1980) *J. Biol. Chem.* 255, 4946–4950.
- [12] Waheed, A., Hasilik, A. and von Figura, K. (1982) *Eur. J. Biochem.* 123, 317–321.