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Endocytosis of Lysosomal Alpha-Galactosidase A by Cultured Fibroblasts from Patients with Fabry Disease

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SUMMARY

The endocytosis of alpha-galactosidase A was studied in cultured fibroblasts from patients with Fabry disease. Alpha-galactosidase A was purified from human placenta by chromatography on concanavalin A-Sepharose, DEAE-cellulose, and *N*-epsilon-aminocaproyl-alpha-D-galactosylamine-Sepharose. Separation of the high-uptake form of the enzyme from the low-uptake form was accomplished by chromatography on ECTEOLA-cellulose. With the high-uptake form of the enzyme, the uptake was linear at low concentrations of enzyme and had a K_{uptake} of 0.01 U/ml of medium that corresponds to a K_m of 5.0×10^{-9} M. At high concentrations of enzyme, it became saturated. The high-uptake form could be converted to the low-uptake form by treatment with acid phosphatase. Mannose-6-P strongly inhibited the active uptake of the enzyme. Once taken up into the lysosomes of Fabry disease fibroblasts, alpha-galactosidase A activity was rapidly lost in the first 2 days of incubation at 37°C, but was fairly stable for the next 6 days. The half-life of internalized alpha-galactosidase A activity was calculated to be 4 days. Crosslinking of the enzyme with hexamethylene diisocyanate did not increase the intracellular stability of alpha-galactosidase A activity.

INTRODUCTION

Fabry disease is a X-linked inherited disorder of glycosphingolipid metabolism [1]. It is caused by a mutation that results in a deficiency of lysosomal alpha-

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galactosidase A activity [2, 3]. This metabolic block leads to the accumulation of ceramide trihexoside and other glycolipids [4]. The deposition of these glycolipids in the tissues causes a characteristic skin rash, pain and paresthesias of the extremities, and eventually renal and/or cardiac failure.

Fabry disease is one of the genetic disorders that might be amenable to enzyme replacement therapy [5]. Preliminary studies on infusion of purified alpha-galactosidase A into patients are very encouraging [6, 7]. However, more basic information is needed before the procedure can be perfected. Cultured fibroblasts are an excellent model system to study the process of enzyme replacement. Dawson et al. [8] showed correction in cultured Fabry fibroblasts by treatment with alpha-galactosidase from ficin. Here we describe the endocytosis of human alpha-galactosidase A by cultured fibroblasts from patients with Fabry disease.

MATERIALS AND METHODS

Reagents were purchased as follows: 4-methylumbelliferyl-alpha-galactoside from Research Products, Elk Grove Village, Ill.; tissue culture reagents from Gibco, Grand Island, N.Y.; mannose-6-P, glucose-6-P, galactose-1-P, *N*-acetyl glucosamine-1-P, and potato acid phosphatase (type III) from Sigma, St. Louis, Mo. The acid phosphatase was further purified by affinity chromatography on phosphocellulose [9].

Alpha-galactosidase was assayed fluorometrically as described by Beutler and Kuhl [10]. Alpha-galactosidase A was differentiated from alpha-galactosidase B by inhibiting the B enzyme with *N*-acetylgalactosamine [11]. A unit of activity is defined as that amount of enzyme that catalyzes the formation of 1 μmol of product/min at 37°C. Specific activity is expressed as U/mg of protein. Protein was determined by the method of Lowry et al. [12] with bovine serum albumin as standard.

Alpha-galactosidase A was purified as follows: All the purification steps were performed at 0°–4°C and at a pH range of 5.0–6.5, as the enzyme is very labile above pH 6.5 [13]. Human placenta that were refrigerated and not more than 24-hrs-old were obtained from the Department of Gynecology and Obstetrics, University of Oklahoma Health Sciences Center, stripped of the outer membrane, and extracted in a Waring blender with 12.5 mM succinate buffer, pH 6.0, containing 0.1% sodium azide and 25 mM sodium tartrate. Sodium tartrate was added to all solutions to inhibit phosphatases that cleave the phosphate from the recognition marker. After centrifuging the extract at 16,000 *g* for 30 min, the supernatant was saved for further purification. The first two steps of the purification procedure, concanavalin A-Sepharose and DEAE-cellulose chromatography, were the same as described in [13]. Fractions of alpha-galactosidase activity from the first peak of the DEAE-cellulose column were combined. This activity was determined to be alpha-galactosidase A by heat stability [10], precipitation with antibody against the A enzyme [13], and no inhibition with *N*-acetylgalactosamine [11]. The enzyme was further purified by affinity chromatography on *N*-epsilon-aminocaproyl-alpha-D-galactosylamine-Sepharose [14]. The purified product appeared to be homogeneous for alpha-galactosidase A as it showed one diffuse band of protein on polyacrylamide gel electrophoresis that had alpha-galactosidase activity with 4-methylumbelliferyl-alpha-galactoside. It had a final specific activity of 15.2. Separation of the high-uptake form of the enzyme from the low-uptake form was performed on ECTEOLA-cellulose. Purified alpha-galactosidase A (6.4 U) that had been dialyzed for 2 hrs against two changes of 1 liter each of 10 mM phosphate buffer, pH 6.5, with 2.5 mM sodium tartrate was added to a column (0.9 × 17 cm) of ECTEOLA-cellulose that had been equilibrated with the above buffer. The column was washed with buffer until the low-uptake form of the enzyme was eluted. The high-uptake form was then eluted with an NaCl gradient of 0–0.1 M.

Cultures of fibroblasts were started from skin biopsies from a patient (DeHe) with Fabry disease and a normal subject (RiSi). The patient is a 31-year-old male. He has angiokeratomas over most of his body as the only clinical manifestation. His cells had no detectable alpha-galactosidase A activity [11]. A culture (GM 2775) of fibroblasts from a patient with Fabry disease was also obtained from the Human Genetic Mutant Cell Repository. Fibroblasts were grown on Eagle's minimum essential medium (MEM) with Earle's salts, 10% medium 199, 7.5% newborn calf serum, and 7.5% fetal calf serum.

For enzyme-uptake experiments, cells were grown to confluency in a T-25 flask and the monolayers rinsed twice with phosphate buffered saline (PBS) (Dulbecco's). Medium (MEM) with 5 mg/ml bovine serum albumin (essentially globulin-free), penicillin-streptomycin, purified alpha-galactosidase A, and other test substances were sterilized by filtration and added to the washed monolayers. The flasks were incubated at room temperature (26°C) for a given period of time. Monolayers were then rinsed twice with PBS and cells detached from the dish by trypsinization. After centrifuging the cells and washing with PBS, the cell pellet was suspended in 0.5 ml of PBS and the cells ruptured by freeze-thawing three times. Protein and alpha-galactosidase A activity were determined on the cell extract.

In the intracellular stability studies, confluent monolayers of Fabry disease fibroblasts were incubated for 20 hrs at 26°C in the presence of purified alpha-galactosidase A (7.7 m U/ml of medium) or alpha-galactosidase A (7.7 m U/ml of medium) crosslinked with hexamethylene diisocyanate [15]. The crosslinked enzyme had an increase in heat stability and a decrease in the rate of protease degradation similar to that described by Snyder et al. [15]. After removing the enzyme solution, the monolayers were washed twice with PBS. Complete medium was added and the flasks placed in an incubator at 37°C, pH 7.4. At various times, the cells were harvested and extracts prepared as described for the uptake experiments. Protein and alpha-galactosidase A activity were determined in these extracts.

For isolation of subcellular fractions, Fabry disease fibroblasts were loaded with enzyme (25 m U/ml of medium for 12 hrs) and harvested as described for the intracellular stability studies. The cells were suspended in 1.5 ml of 0.25 M sucrose solution buffered with 3 mM Tris-HCl, pH 7.4, and homogenized in a tight-fitting Dounce homogenizer. The homogenate was fractionated by differential centrifugation as follows: 700 *g* for 10 min to give the nuclear fraction in the precipitate, 24,000 *g* for 10 min to give the mitochondrial-lysosomal fraction in the precipitate, and 100,000 *g* for 1 hr to give the microsomal fraction in the precipitate and the soluble fraction in the supernatant. An aliquot from each subcellular fraction was assayed for alpha-galactosidase A, beta-hexosaminidase, and protein. Another aliquot from each fraction was applied to a cellulose nitrate-coated electron microscopy grid and negatively stained with 1% phosphotungstic acid, pH 7.0, for the identification of organelles using the electron microscope.

RESULTS

The stability of alpha-galactosidase A in tissue culture medium under various conditions is given in figure 1. Most of the activity was lost within 6 hrs when purified enzyme was added to tissue culture medium and incubated at 37°C, pH 7.4. However, the enzyme was very stable when incubated in tissue culture medium with bovine serum albumin (5 mg/ml) at 26°C, pH 6.8, with over 90% of the activity remaining after 6 hrs. The latter conditions were employed in all of the uptake studies.

Separation of the high-uptake form of alpha-galactosidase A from the low-uptake form on an ECTEOLA-cellulose column is shown in figure 2. In this procedure, the low-uptake form did not bind to the ECTEOLA-cellulose and ran straight through the column or was washed through with dilute phosphate buffer. The high-uptake form was eluted with an NaCl gradient. In this preparation, the

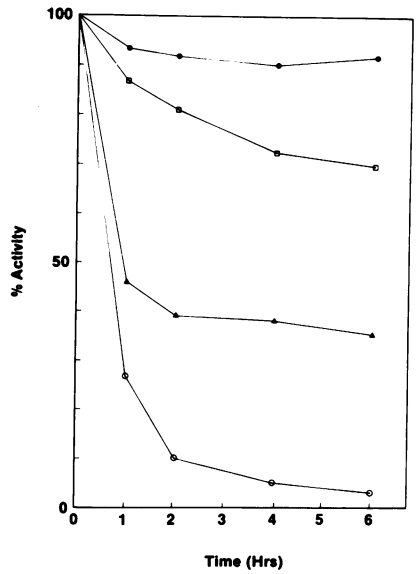

FIG. 1.—Stability of alpha-galactosidase A in tissue culture medium under various conditions. Values represent averages of duplicate assays. ● = MEM-BSA, 26°C, pH 6.8; □ = MEM, 26°C, pH 6.8; Δ = MEM-BSA, 37°C, pH 7.4; O = MEM, 37°C, pH 7.4.

high-uptake peak accounted for 31.2% of the total activity. In other preparations, it has ranged from 13% to 41%.

With the high-uptake form of the enzyme, the uptake was linear at low concentrations of enzyme (fig. 3), and a Lineweaver-Burk plot (insert, fig. 3) on these data gave an apparent K_{uptake} of 0.01 U/ml of medium that corresponds to 5×10^{-9} M for alpha-galactosidase A. At high concentrations of enzyme, the uptake became saturated. With high concentration of enzyme and long incubation, uptake became very significant, approaching half the activity of normal cells (table 1). Uptake was not limited to the DeHe cell strain, but the enzyme was taken up by another Fabry disease fibroblast strain as well as a control cell strain. Active uptake was abolished by mannose-6-P or treatment of the enzyme with acid phosphatase (table 2).

Isolation of subcellular fractions by differential centrifugation showed that alpha-galactosidase A was taken up into the mitochondrial-lysosomal fraction of the cell (fig. 4). The activity was in membrane-bound organelles as it was released with Triton X-100. Beta-hexosaminidase, a known lysosomal enzyme, was also assayed in these fractions, and it had a similar distribution and latency as the internalized alpha-galactosidase A. Fractionation of a normal fibroblast homogenate showed a similar pattern. All cell fractions were observed by electron microscopy, and the B fraction contained the majority of the lysosomes and mitochondria. These results would indicate that the enzyme was taken up into the lysosomes of the cell.

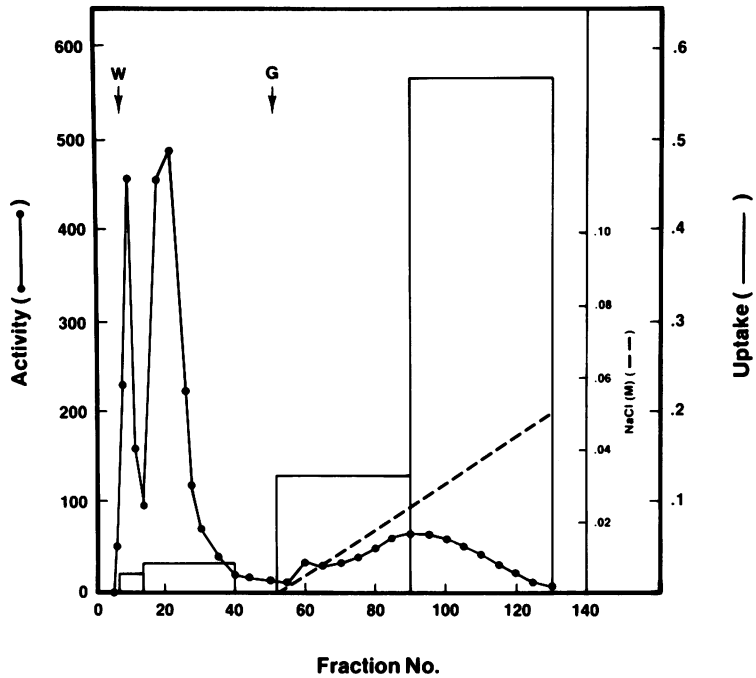

FIG. 2.—ECTEOLA-cellulose chromatography of purified alpha-galactosidase A. Conditions for chromatography are described in text. Activity is given as fluorescent readings. Uptake (10 m U of alpha-galactosidase A per ml of medium for 17 hrs) is given as m U of alpha-galactosidase A per mg of cell protein.

Intracellular stability of the endocytosed enzyme is shown in figure 5. The half-life of alpha-galactosidase A activity at 37°C was 4 days. Enzyme that was crosslinked with hexamethylene diisocyanate had the same intracellular stability as the native enzyme.

DISCUSSION

It is well established that lysosomal enzymes exist as low-uptake forms and high-uptake forms for endocytosis by cultured fibroblasts [16]. Since the two forms differ in that the high-uptake form contains phosphate [17], it would seem plausible to separate the two forms by anion exchange chromatography. After trying several materials, we were able to separate the high-uptake form and low-uptake form of alpha-galactosidase A by chromatography on an ECTEOLA-cellulose column. The high-uptake peak accounted for about 30% of the total activity. It is important that the enzyme be isolated in the cold and in the presence of a phosphatase inhibitor such as sodium fluoride or sodium tartrate, or the amount of high-uptake form will decrease. In early experiments, the concanavalin A-Sepharose chromatography was run at room temperature and without phosphatase inhibitor, and no high-uptake form was observed.

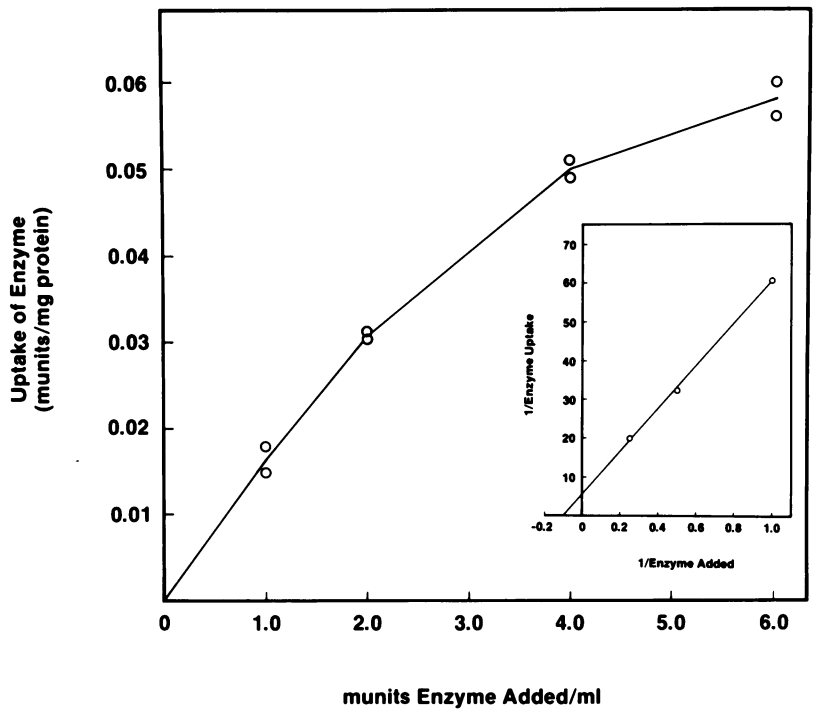

FIG. 3.—Uptake of alpha-galactosidase A by Fabry disease fibroblasts as a function of enzyme concentration. Results are averages of duplicate flasks as shown. *Insert* is a Lineweaver-Burk plot of the data at low enzyme concentration.

With the high-uptake form of the enzyme, the uptake at low enzyme levels was linear with concentration and had a K_{uptake} of 0.01 U/ml of medium. With a specific activity of 15.4 and a mol. wt. of 132,000 (our unpublished observations, 1980) for purified alpha-galactosidase A, this corresponds to a K_m of 5×10^{-9} M. This is similar to that reported for alpha-L-iduronidase [18]. The high-uptake form of alpha-galactosidase A could be converted to the low-uptake form by treatment

TABLE 1

UPTAKE OF ALPHA-GALACTOSIDASE A BY VARIOUS FIBROBLAST STRAINS

STRAIN	GENOTYPE	ALPHA-GALACTOSIDASE A ACTIVITY*		
		Control	Enzyme treated†	Uptake
DeHe	Fabry disease	0	0.305	0.305
GM 2775	Fabry disease	0	0.264	0.264
RiSi	Normal	0.666	1.000	0.334

* m U/mg cell protein.
† 40 m U/ml of medium for 16 hrs.

TABLE 2

INHIBITORS OF UPTAKE OF ALPHA-GALACTOSIDASE A

Treatment	Uptake (m U/mg protein)	Inhibition (%)
None	0.258	...
Mannose-6-P (2 mM)	0.005	98
Glucose-6-P (2 mM)	0.233	10
N-acetyl glucosamine-1-P (2 mM)	0.299	0
Galactose-1-P (2 mM)	0.314	0
Acid phosphatase	0.011	96

with acid phosphatase, and mannose-6-phosphate strongly inhibited the active uptake of the enzyme. This would indicate that the high-uptake form has a mannosyl-phosphate recognition marker that has been reported for other lysosomal enzymes [17–20].

Once taken up into the lysosomes of Fabry disease fibroblasts, alpha-galactosidase A activity was rapidly lost in the first 2 days of incubation at 37°C, but was fairly stable for the next 6 days. Crosslinking of the enzyme with hexamethylene diisocyanate has been reported to increase the heat stability and decrease the rate of protease degradation of the enzyme [15], and we were able to repeat these observations (our unpublished observations, 1981). However, this did not increase the intracellular stability of alpha-galactosidase A activity.

Beutler [21] has recently questioned the feasibility of enzyme replacement therapy in Fabry disease on the basis that chimeric female carriers manifest the signs of the disease. Presumably, there is no cross correction between the normal and mutant cells. There are several possible reasons why cross correction does not occur. First, the secreted enzyme may not have the correct recognition marker to be taken up by the mutant cells. These studies demonstrate that the recognition marker for alpha-galactosidase A is mannosyl-phosphate for fibroblasts, but recognition markers necessary for uptake of this enzyme by other cell types are not

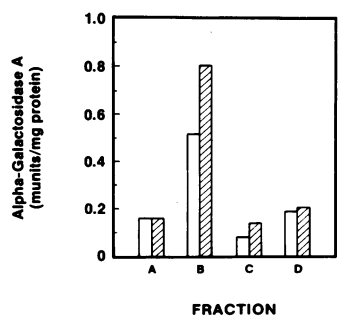

FIG. 4.—Alpha-galactosidase A activity in subcellular fractions after internalization of the enzyme by Fabry disease fibroblasts. *A* = nuclear fraction; *B* = mitochondrial-lysosomal fraction; *C* = microsomal fraction; *D* = soluble fraction. *Hatched bars* represent fractions treated with Triton X-100 (0.1%).

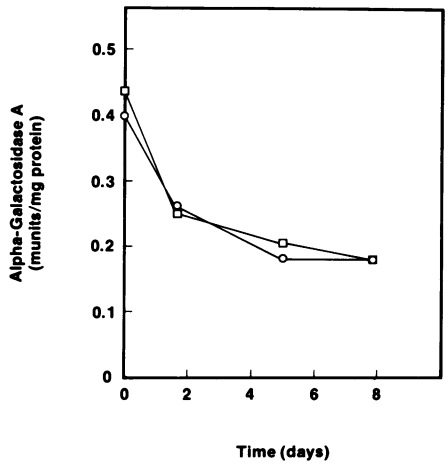

FIG. 5.—Intracellular stability of internalized native alpha-galactosidase A (□) and crosslinked alpha-galactosidase A (○). *Each point* represents the average of duplicate flasks.

known. Second, the enzyme is very labile. When added to plasma at 37° C, pH 7.4, the enzyme activity has a half-life of 9–17 min [5, 13]. Therefore, when the enzyme is secreted from a normal cell, the activity may be lost before it is taken up by a mutant cell. In contrast, these studies show that the enzyme activity is fairly stable once the enzyme has been internalized by Fabry disease fibroblasts. If alpha-galactosidase A can be targeted to the tissue and quickly removed from the bloodstream, it should retain enough of its activity to be effective in enzyme replacement therapy in Fabry disease.

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