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AMOUNT AND PROPERTIES OF UPTAKE FORMS IN PREPARATIONS OF α-MANNOSIDASE FROM PIG KIDNEY

GÜNTHER MERSMANN, RAINER BECKER and ECKHART BUDDECKE

Institute of Physiological Chemistry, University of Münster, Waldeyerstr. 15, D-4400 Münster, (G.F.R.)

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Summary

α-Mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) from pig kidney has been shown to exist in multiple forms differing in their capability to be endocytosed by α-mannosidase deficient cultured cells. A method is presented to evaluate the amount of "uptake" forms in different preparations of the enzyme. Preparations with different rates of uptake were shown to contain different amount of "uptake" forms and "non-uptake" forms. The content of "uptake" forms in a preparation was identical with that of enzyme molecules bearing a phosphorylated carbohydrate group necessary for the recognition by cell surface receptors.

Introduction

Cultured cells of patients with enzyme deficiency disorders are a valuable model for studies of these diseases [1]. For several disorders it has been shown that the deranged metabolism of these cells can be corrected by addition of the deficient enzyme to the culture medium [1—13]. The corrective activities of different forms of the deficient enzyme depend on their endocytosis rate [3,5,10,13] and on the susceptibility of the intracellular storage material to degradation by the internalized enzyme [13]. Great differences in the endocytosis rates even of enzymes from the same source and preparation have been reported [14—16] and these different endocytosis properties have been described by the terms "high uptake" and "low uptake" forms [14]. We here give some indications that "high uptake" and "low uptake" forms of α-mannosidase from pig kidney just contain different amounts of "uptake" forms besides material not endocytosed or endocytosed very slowly ("non-uptake" forms).

Materials and Methods

DEAE-cellulose (DE 52) was purchased from Whatman (Maidstone, U.K.), neuraminidase from *E. coli* (20 U/mg) from Boehringer Mannheim (Mannheim, West Germany).

Enzyme preparation. α-Mannosidase was purified from pig kidney as described previously [17]. A preparation of specific activity 6.7 U/mg was used for the cell experiments and of 13.2 U/mg for the determination of the neuraminic acid content.

Cell experiments. The methods of cell culturing, harvesting, and homogenizing were exactly as given earlier [12], except that for short-time endocytosis experiments plates of 35 mm diameter with 1 ml medium and reduced volume of washing and trypsinisation solutions were used.

Isoelectric focusing. This was done in 110-ml columns for analytical purposes and in 440-ml equipment (both LKB, Bromma, Sweden) for the preparative runs, with 1% Ampholine pH 5—8 (LKB) as carrier ampholyte and a sucrose gradient exactly according to the manufacturer's instruction.

Neuraminic acid determination. The neuraminic acid content of α-mannosidase was determined by the thiobarbituric acid method in the modification of Aminoff [18]. 150 μg enzyme protein were hydrolysed in H_2SO_4 (0.05 mol/l) at 80°C for 1 h. Duplicate determinations of two different enzyme preparations gave identical results.

Results

Long duration endocytosis experiments with α-mannosidase

Purified α-mannosidase from pig kidney with an acidic pH optimum was added to the culture medium of cells from a mannosidosis patient and the intracellular α-mannosidase activity measured after 4—96 h incubation. It reached a maximum after about 24 h and then distinctly decreased (Fig. 1a). After 72 h, only about 1—2% of the α-mannosidase activity applied to the medium were endocytosed and more than 95% were still found in the medium.

The residual α-mannosidase of the medium after 72 h or more of incubation

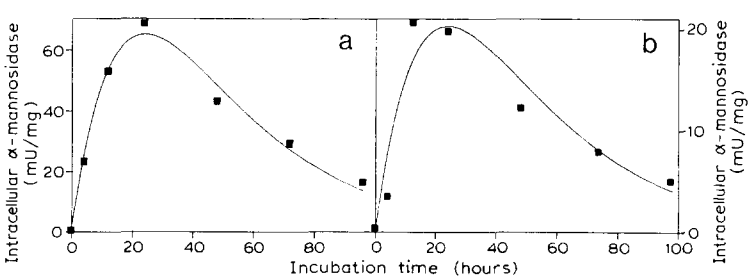

Fig. 1. Long-duration endocytosis of α-mannosidase by cultured mannosidosis fibroblasts. Confluent mannosidosis fibroblasts (25-cm² flasks) were incubated in 5 ml medium containing α-mannosidase. After 4—96 h the intracellular activity was determined, ■, experimental values; ———, time-dependence of intracellular activity calculated from Eqn. 1. (a) Medium contained 1800 mU unseparated α-mannosidase per 5 ml. (b) Medium contained 120 mU α-mannosidase B_2-form per 5 ml.

TABLE I

UPTAKE OF PREINCUBATED AND NON-PREINCUBATED α-MANNOSIDASE INTO MANNOSIDOSIS FIBROBLASTS

The α-mannosidase-containing medium from flasks that had been incubated for 72 and 96 h of uptake (Fig. 1a) was collected and mixed with the same volume of fresh, α-mannosidase-free medium. For the control experiment, medium conditioned for 72 h and fresh medium containing non-incubated α-mannosidase were mixed 1 : 1 (v/v). Both samples were incubated with fresh mannosidosis cells and the intracellular α-mannosidase activity was measured after 12 h. Clearance is that volume of medium freed of α-mannosidase per h by 1 mg cell protein.

Preincubation of α-Mannosidase (h)	Extracellular α-Mannosidase (mU/ml medium)	Intracellular α-Mannosidase (mU/mg protein)	Clearance ($\mu l \cdot mg^{-1} \cdot h^{-1}$)
0	170	25.1	12.3
⩾72	170	4.5	2.2

was taken up in short-duration endocytosis experiments much less than fresh, non-preincubated enzyme (Table I).

Calculation of kinetic parameters

In uptake experiments with enzyme deficient cells and their deficient enzyme the intracellular enzyme activity a_i results from (a) the residual activity a_r, (b) the uptake rate of the extracellularly applied enzyme (rate constant k_{in}), and (c) the rate of inactivation and exocytosis of the intracellular enzyme (rate constant k_{ex}). The time course of a_i obeys an expression of the form [8]:

$$a_i(t) = a_{e_0} \cdot \frac{k_{in}}{k_{ex} - k_{in}} \cdot (e^{-k_{in} \cdot t} - e^{-k_{ex} \cdot t}) + a_r \qquad (1)$$

where a_{e_0} is the initial extracellular enzyme activity. k_{ex} can be determined separately by preincubating several plates of mannosidosis cells with α-mannosidase, then removing the extracellular enzyme and measuring the decrease of intracellular activity. k_{ex} was determined from a semilogarithmic plot as 0.038 h^{-1} (S.D. 0.005 h^{-1}). This corresponds to a intracellular half-life of 18.2 h. The residual acidic α-mannosidase activity of the cell line used was 0.38 ± 0.11 mU/mg of cell protein (mean ± standard deviation).

TABLE II

CALCULATION OF KINETIC PARAMETERS FROM LONG-DURATION ENDOCYTOSIS EXPERIMENTS

(Fig. 1)

Parameter	α-Mannosidase unseparated (Fig. 1a)	α-Mannosidase B_2-form (Fig. 1b)
Apparent initial extracellular activity $a_{e_0}^{app}$	6360 mU/mg (360 mU/ml)	348 mU/mg (24 mU/ml)
Rate constant for uptake k_{in}	0.046 h^{-1}	0.046 h^{-1}
Calculated initial extracellular activity a_{e_0}	161 mU/mg	49.2 mU/mg
$a_{e_0}/a_{e_0}^{app}$	0.025	0.141

With these values known a_{e_0} and k_{in} can be calculated by a computer program fitting the theoretical curve to the experimental values by a method minimizing the sum of squares of deviations (e.g. 19). The resulting values are given in Table II. The curve obtained with these parameters fits closely to the data points (Fig. 1a).

Partial purification and characterization of a "high uptake" form of α-mannosidase

α-Mannosidase was separated by DEAE-cellulose chromatography into three distinct fractions following an established procedure [17,20,21] (conditions see Fig. 2). The pooled forms A, B_1 and B_2 had isoelectric points of 6.05, 5.73, and 5.67, respectively. The endocytosis rate of these forms in short-duration experiments is distinctly different. The clearance (for definition see Table I and ref. 22) of these forms for a range of extracellular α-mannosidase activities of up to 270 mU/ml medium were 1.6, 9.5 and 18.0 $\mu l \cdot mg^{-1} \cdot h^{-1}$. In long-duration

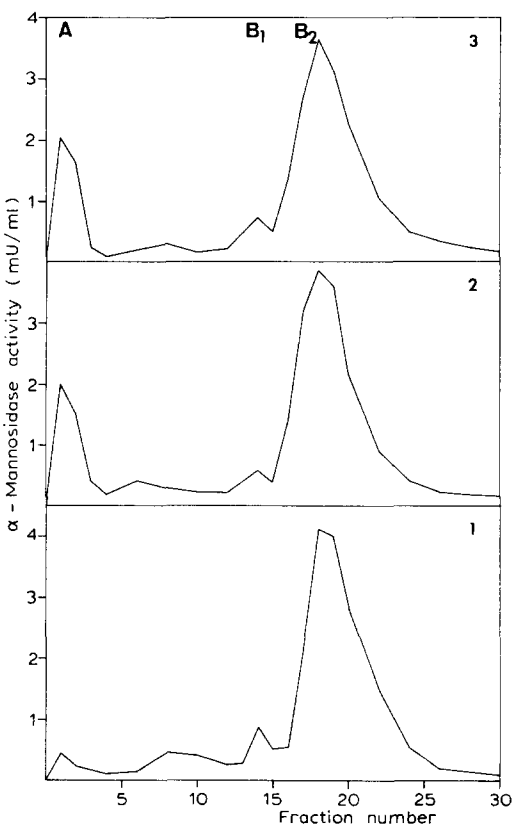

Fig. 2. Changes in the DEAE-cellulose elution pattern of α-mannosidase B_2-form after neuraminidase treatment. α-Mannosidase B_2-form was treated with neuraminidase or with buffer, as described in Table III. 10 μl of untreated enzyme (curve 1), of α-mannosidase incubated with neuraminidase (curve 2) and of α-mannosidase incubated only with citrate/phosphate buffer (curve 3) were, after dialysis against 0.01 M sodium phosphate, pH 6.0, applied to a column (0.8 × 4.2 cm) of DEAE-cellulose equilibrated with the same buffer. The column was eluted with 12 fractions (1.65 ml each) of the starting buffer and then with a linear gradient of 75 ml starting buffer and 75 ml 0.2 M NaCl in the starting buffer.

TABLE III

UPTAKE OF NEURAMINIDASE-TREATED α-MANNOSIDASE B_2-FORM INTO CULTURED MANNOSIDASIS FIBROBLASTS

Preincubation conditions: Solutions: α-Mannosidase B_2-form (4.8 U/ml) in 0.01 M sodium phosphate buffer, pH 6.0, 0.14 M NaCl (PBS); Neuraminidase (1 mg/ml) in 0.06 M sodium phosphate/0.03 M citric acid, pH 5.0. Incubation at 37°C for 24 h, second additions of neuraminidase or with buffer after 12 h. Sample (a): 50 μl α-mannosidase + 2 × 10 μl neuraminidase; sample (b): 100 μl α-mannosidase + 2 × 20 μl phosphate/citrate buffer; sample (c): 50 μl PBS + 2 × 10 μl neuraminidase. Endocytosis conditions: To confluent plates (10 cm^2) of mannosidosis fibroblasts in 1 ml medium were added: Sample I: 50 μl sample (a) + 50 μl PBS; sample II: 50 μl sample (b) + 50 μl sample (c); control: 50 μl sample (b) and 50 μl PBS. Intracellular activity was determined after 12 h of uptake.

Sample	Endocytosis (% of control)
I B_2-Form incubated with neuraminidase	145
II Mixture of separately incuated B_2-form and neuraminidase	135

endocytosis studies with the B_2-form a similar time course as for the unseparated α-mannosidase was obtained (Fig. 1b). The kinetic parameters k_{in} and a_{e_0} were calculated as described above (Table II, right column).

α-Mannosidase B_2-form was then subejcted to preparative isoelectric focusing and the material after the run separated into four fractions of almost identical activities. The pI and clearance values of these fractions were (a) pI 5.0—5.40: 27.4 $\mu l \cdot mg^{-1} \cdot h^{-1}$; (b) pI 5.42—5.65: 15.2 $\mu l \cdot mg^{-1} \cdot h^{-1}$; (c) pI 5.67—5.85: 7.3 $\mu l \cdot mg^{-1} \cdot h^{-1}$; (d) pI 5.88—6.50: 8.4 $\mu l \cdot mg^{-1} \cdot h^{-1}$.

Neuraminidase treatment and neuraminic acid determination of α-mannosidase

α-Mannosidase forms A and B_2 were reported to differ in their neuraminic acid content [21]. The B_2-form was subjected to neuraminidase degradation. The treatment did not markedly reduce the total α-mannosidase activity and led to the formation of about 20% of a material that in DEAE-cellulose chromatography behaved like the A-form of the enzyme. A similar amount of conversion was, however, achieved in a control experiment, when α-mannosidase B_2-form was incubated in buffer in absence of neuraminidase (Fig. 2). The endocytosis rate of α-mannosidase B_2-form treated with neuraminidase is shown in Table III. Mild acid hydrolysis of highly purified α-mannosidase and determination of neuraminic acid in the hydrolysate gave a neuraminic acid content of less than 0.15%, i.e. less than 0.2 neuraminic acid residues per α-mannosidase subunit (mol. wt. 44 000 [17]).

Effect of alkaline phosphatase treatment of α-mannosidase B_2-form

We have shown elsewhere in detail [23] that treatment of α-mannosidase B_2-form with alkaline phosphatase from *Escherichia coli* leads to (a) the almost entire reduction of endocytosis (residual rate 1% of control) and (b) to the transformation of 13% of the B_2-form into material which in DEAE-cellulose chromatography behaves like the A-form.

Discussion

The valitidy of Eqn. 1 for the reflection of long-duration uptake of extracellularly applied enzyme into cells deficient of this enzyme has been shown

by Von Figura and Kresse [8] for α-*N*-acetylglucosaminidase and cultured Sanfilippo B fibroblasts. In contrast to their system, the characteristic feature of which was that a constant level of intracellular activity was reached after an initial phase of increase (obtained from Eqn. 1 for $k_{ex} >> k_{in}$), the time course of intracellular α-mannosidase activity in mannosidosis fibroblasts incubated in presence of pig kidney acidic pH-optimum α-mannosidase shows a maximum after about 24 h with a distinct subsequent decrease of intracellular activity (Fig. 1a, b). Such a time course is obtained if $k_{in} \geqslant k_{ex}$. This leads to a rapid decrease of extracellular activity and hence of the uptake rate which soon becomes lower than the rate of inactivation and exocytosis, leading to the early overall decrease of intracellular activity.

The values for k_{ex} calculated from a separate chase experiment and for k_{in} determined by a curve fitting procedure are in agreement with this model. Surprising was the value obtained for the initial extracellular activity a_{e_0} which was only 1/40 of the α-mannosidase activity added to the medium. The suggested explanation for this result, that only about 2—5% of the enzyme exists in an "uptake" form, was confirmed by the following further findings:

The uptake balance showed that at the time where the endocytosis rate obviously had markedly decreased still more than 95% of the extracellular α-mannosidase activity were left in the medium and only about 1—2% had been internalized into the cells. The decrease of uptake could hence not be due to the decrease of total α-mannosidase in the medium but only to the shortage of the "uptake" forms. The possibility that the decrease of uptake rate after long-duration endocytosis was caused by change or damage to the recognition and uptake mechanisms of the cells was excluded by uptake experiments with the conditioned enzyme and fresh cells (Table I) which showed that the endocytosis of the preincubated enzyme was much less than that of the fresh one.

The calculation of the kinetic parameters k_{in} and a_{e_0} for a distinct α-mannosidase form with high endocytosis rate (B_2-form) isolated by DEAE-cellulose chromatography had two interesting results: the rate constant k_{in} for that part of the enzyme preparation that can be taken up is identical with that found for the "uptake" forms of the original preparation and the content of this forms had increased from 2.5% originally to 14% in the partially purified material. This supports the model that different enzyme molecules do not gradually differ in their uptake properties (as suggested by the terms "high uptake" and "low uptake" forms [14] but rather that enzyme preparations differing in their endocytosis rate consist of different proportions of enzyme molecules that can be taken up ("uptake" forms) and of others that cannot be ("non-uptake" forms). The "uptake" and "non-uptake" molecules are not necessarily homogeneous other than in their uptake properties (hence the name in the plural).

The dependence of the endocytosis properties of lysosomal enzymes from their pI had earlier been shown [14—16] and was confirmed here for α-mannosidase. A possible explanation for these findings could be the participation of an acidic group on the enzyme molecule in the recognition process. As the α-mannosidase forms A and B_2 had been reported to differ in their content of sialic acid groups [21], we tried to modify the endocytosis rate of the B_2-form by treatment with neuraminidase. This procedure did not, however, decrease the uptake but slightly increased it (Table III). DEAE-cellulose chromatog-

raphy revealed that only about 20% of the B_2-form was converted into material chromatographically similar to the A-form and that the same effect was achieved in a control experiment without neuraminidase (Fig. 2). This and the very low sialic acid content found for α-mannosidase weaken the evidence for the model that α-mannosidase forms A and B_2 differ only in the absence or presence of neuraminic acid groups. No explanation could be given until now for the increase of endocytosis in the presence of neuraminidase. Whilst this work was in progress it was shown that the acidic group involved in the recognition and uptake of lysosomal enzymes into fibroblasts is hexose phosphate, most probably mannose 6-phosphate [23—25]. The finding that the treatment of α-mannosidase B_2-form with alkaline phosphatase leads to the formation of about 13% of a α-mannosidase form not bound to DEAE-cellulose and to an almost total loss of endocytosis [23] is further evidence for the existence of about 10—15% "uptake" forms in the B_2-form of α-mannosidase mixed with "non-uptake" forms. The "uptake" forms seem to differ from the latter in the presence of a phosphoric ester group necessary for the recognition of the enzyme by the cell surface receptors. The nature of the acidic groups in the "non-uptake" forms not involved in the recognition process remains unknown.

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References

1 Neufeld, E.F. and Cantz, M. (1973) in Lysosomes and Storage Diseases (Hers, H.G. and van Hoof, F., eds.), pp. 262—275, Academic Press, New York
2 Barton, R.W. and Neufeld, E.F. (1971) J. Biol. Chem. 246, 7773—7779
3 Shapiro, L.J., Hall, C.W., Leder, I.G. and Neufeld, E.F. (1976) Arch. Biochem. Biophys. 172, 156—161
4 Hall, C.W., Cantz, M. and Neufeld, E.F. (1973) Arch. Biochem. Biophys. 155, 32—38
5 Brot, F.E., Glaser, J.H., Roozen, K.J., Sly, W.S. and Stahl, P.D. (1974) Biochem. Biophys. Res. Commun. 57, 1—8
6 Hickman, S. and Neufeld, E.F. (1972) Biochem. Biophys. Res. Commun. 49, 992—999
7 Von Figura, K. and Kresse, H. (1972) Biochem. Biophys. Res. Commun. 48, 262—269
8 Von Figura, K. and Kresse, H. (1974) J. Clin. Invest. 53, 85—90
9 O'Brien, J.S., Miller, A.L., Loverde, A.W. and Veath, M.L. (1973) Science 181, 753—755
10 Mersmann, G., Von Figura, K. and Buddecke, E. (1974) Biochim. Biophys. Acta 364, 88—96
11 Kresse, H. and Neufeld, E.F. (1972) J. Biol. Chem. 247, 2164—2170
12 Mersmann, G., Von Figura, K. and Buddecke. E. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 641—648
13 Mersmann, G., Wilde, F.-J. and Buddecke, E. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 421—425
14 Nicol, D.M., Lagunoff, D. and Pritzl, P. (1974) Biochem. Biophys. Res. Commun. 59, 941—946
15 Glaser, J.H., Roozen, K.J., Brot, F.E. and Sly, W.S. (1975) Arch. Biochem. Biophys. 166, 536—542
16 Von Figura, K. (1977) in The Cultured Cell and Inherited Metabolic Diseases (Harkness, R.A. and Cockburn, F., eds.), pp. 105—119, MTP press, Lancaster
17 Mersmann, G. and Buddecke, E. (1977) Int. J. Biochem. 8, 763—768
18 Aminoff, D. (1961) Biochem. J. 81, 384—392
19 Braess, D. (1974) Nichtlineare Anpassung von Messdaten nach der Methode der kleinsten Quadrate, Schriftenreihe des Rechenzentrums der Universität Münster, Vol. 6

20 Carroll, M., Dance, N., Masson, P.K., Robinson, D. and Winchester, B.G. (1972) Biochem. Biophys. Res. Commun. 49, 579—583
21 Chester, M.A., Lundblad, A. and Masson, P.K. (1975) Biochim. Biophys. Acta 391, 341—348
22 Lagunoff, D., Nicol, D.M. and Pritzl, P. (1973) Lab. Invest. 29, 449—453
23 Ullrich, K., Mersmann, G., Weber, E. and Von Figura, K. (1978) Biochem. J. 170, 643—650
24 Kaplan, A., Achord, D.T. and Sly, W.S. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2026—2030
25 Sando, G.N. and Neufeld, E.F. (1977) Cell 12, 619—628