RESIDUAL ALTERED α-MANNOSIDASE IN HUMAN MANNOSIDOSIS

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Summary: The apparent Km of residual acidic α-mannosidase detected in fibroblast extracts from four unrelated patients with mannosidosis was increased to >25mM for a fluorogenic substrate compared to 0.86-0.96mM for controls. The mutant enzyme was also more labile with heat treatment. These findings indicate a mutation in the structural gene for this enzyme. The altered kinetics of mutant enzyme can result in apparently normal enzyme specific activity at high concentrations of fluorogenic substrate creating potential for errors in the diagnosis of mannosidosis.

Mannosidosis was first described in 1967 (1) and is now recognized as a lysosomal storage disease characterized by visceral accumulation of mannose rich oligosaccharides, generalized deficiency of acid α-mannosidase (EC 3.2.1.24), autosomal recessive inheritance and clinical features resembling mucopolysaccharidosis (2). Two acidic α-mannosidase activities, designated A and B, and a neutral activity, designated C, have been described in human liver (3). Only a single acidic α-mannosidase has been observed in cultured fibroblasts although at least one neutral mannosidase is also present (4,5). The forms of α-mannosidase with acid pH optima are deficient in patients with mannosidosis. We have recently diagnosed a patient with mannosidosis who had been found previously to have normal α-mannosidase activity, suggesting a need to further examine diagnostic enzymic techniques for this condition. Using high concentrations of 4-methylumbelliferyl-α-D-mannoside substrate to study activity from cell extracts from mannosidosis patients, we have observed altered kinetic and stability properties which explain the potential for errors in the enzymic diagnosis of mannosidase deficiency and provide insight into the nature of the mutation present.

Materials and Methods: Primary cultured skin fibroblasts were obtained from S.A., a white male with typical findings of mannosidosis as described elsewhere (6). Cell line GM 654, from a case previously described (7), was

obtained from the Institute for Medical Research and lines TC 180 and TC 296 were obtained from Dr. George Thomas (5). Cells were cultured in Dulbecco's modified Eagles medium with 10% fetal calf serum with three times weekly medium changes. Cultured fibroblasts were harvested nine to eleven days after attaining confluence and leukocyte extracts were prepared using previously described methods (8). Mannosidase reactions were incubated 60 minutes at 37°C in a volume of 0.05 ml with the following final concentrations: 0.1 M sodium acetate (pH 4.0) except for 51°C heat treatment assays, 0.5 mg/ml bovine serum albumin (mannosidase free, Schwarz-Mann), 4-methylumbelliferyl-α-D-mannoside (Research Projects International) as indicated and 0-80 μg extract protein. The 4-methylumbelliferone released was quantitated as previously described (8) with the background value obtained by addition of enzyme after termination of the reaction subtracted. For heat stability studies at 56°C, enzyme was heated in 0.25 M sodium acetate, pH 4, in 0.02 ml with bovine albumin followed by chilling, addition of substrate and volume adjustment for mannosidase assay as described above. For heat stability studies at 51°C, enzyme was heated in 0.05 M sodium acetate, pH 4, in 0.02 ml with bovine albumin followed by chilling, addition of 0.01 ml of 1 M sodium acetate, pH 4, or 0.5 M-1.0 M citrate-phosphate, pH 6, and addition of substrate in a final assay volume of 0.05 ml as above.

Results: The α-mannosidase activities of fibroblast extracts from nine control individuals and four unrelated patients with mannosidosis are listed in Table 1. Enzyme deficiency was readily documented using 1 mM 4-methylumbelliferyl-α-D-mannoside, but at 15 mM substrate the values for extracts of patients all fell

Table 1. Fibroblast α-D-Mannosidase Specific Activity*

	4-Methylumbelliferyl-α-D-Mannoside	
Extract Source	1mM	15mM
Fibroblast		
S.A.	0.04	0.78
G.M. 654	0.07	1.14
T.C. 180	0.09	1.51
T.C. 296	0.09	0.87
Control Mean†	1.15	1.39
Control Range	0.63 - 2.36	0.77 - 2.53
Leukocyte		
S.A.	0.02	0.20
Control Mean†	4.32	4.72
Control Range	1.64 - 6.70	1.72 - 8.93

*n mol 4-methylumbelliferone released/minute/mg protein
†based on fibroblasts from nine and leukocytes from eight non-diseased individuals

within the normal range. Activity of patient extracts increased 10 to 20 fold at 15 mM substrate compared to 1 mM while control extracts increased only 21%. The mannosidase activity of the leukocyte extract from patient S.A. (Table 1) also increased 10 fold at higher substrate concentration, but the higher activity of the deficient extract was only 4% of the control mean.

Michaelis-Menten plots for fibroblast extracts for two controls and two mannosidosis patients are shown in Figure 1. There was a marked difference in the apparent Km for the artificial substrate with values of 0.86 mM and 0.96 mM for the two controls compared to >25 mM for the mutant extracts. The exact determination of the Km for the mutant extracts is limited by the solubility of the substrate. The activity of patient extracts was still increasing linearly at the highest concentration of substrate achieved so that the maximal specific activity was not clearly attained. The ordinate is plotted as the reciprocal of the specific activity since crude extracts were used for these studies. The specific activity of normal extracts is subject to some biological variation and these data do not allow accurate comparison of the Vmax of purified wild type and mutant enzyme. The α-mannosidase activity of control and mutant extracts was further distinguished by study of thermal lability at 56°C. Enzyme activity was measured with 15 mM substrate to detect

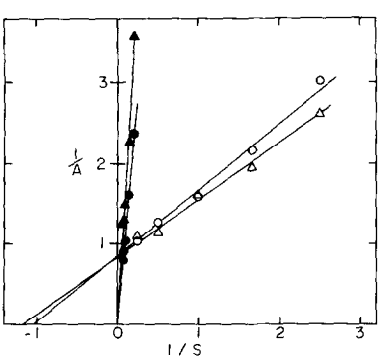

Figure 1. Michaelis-Menten analysis of acidic α-mannosidase activity of fibroblast extracts from two controls (○,△), S.A. (▲) and GM 654 (●). S is millimolar concentration of 4-methylumbelliferyl-α-D-mannoside and A is specific activity as in Table 1.

mutant and wild type activity. The α-mannosidase activity at pH 4 was inactivated in mutant extracts from all four patients while wild type enzyme was stable or significantly activated. The results for fibroblast extracts from our patient and TC 296, as well as results from the leukocyte extract of our patient, are shown in Figure 2.

Heat inactivation studies at 51°C were helpful in evaluating the possibility that the apparent acid α-mannosidase activity of mutant extracts might be due to activity of residual neutral mannosidase activity (mannosidase C). When fibroblast extracts were heated under these milder conditions, acid mannosidase activity was relatively stable for control and mutant extracts. However, mannosidase activity assayed at pH 6 was rapidly inactivated in control and patient extracts with some residual activity that appeared consistent with acid mannosidase activity being detected at the higher pH. In the case of patient extracts, the persistence of activity at pH 4 when activity at pH 6 was rapidly inactivated suggested that the acid mannosidase activity observed was not related to activity of the neutral mannosidase component.

Discussion: Electrophoretic studies by others, as well as our own unpub-

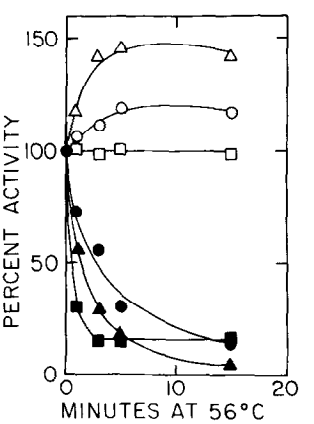

Figure 2. Heat inactivation of acid mannosidase at 56°C: Δ and O, control fibroblast (0.96 and 0.53); □, control leukocyte (5.66); ● TC-296 fibroblast (0.59); ▲ SA fibroblast (1.55) and ■ SA leukocyte (0.13). The values in parentheses are the n mol product per assay for the unheated samples.

lished studies, indicate only a single form of acid α-mannosidase in cultured skin fibroblasts although two forms have been reported in liver. Our studies suggest that the deficiency of acid mannosidase in fibroblast extracts in mannosidosis is caused by a mutation in the structural gene which alters the Km for the artificial substrate used and alters the heat stability of the acidic enzyme. Differences in Km for substrates have been reported for human mutant enzymes (9), but the difference between control and mutant mannosidase of at least 25 fold in our studies was particularly marked. The residual acidic mannosidase activity of mannosidosis extracts was clearly more heat labile than that from controls and clearly more heat stable than neutral mannosidase of control or mannosidosis extracts. Four unrelated patients demonstrated similar alteration in Km and increased heat lability. This could reflect an unusual susceptibility of this gene product to retain residual altered activity with mutation or to a chance distribution of such an allele. The ability to detect mutant enzyme by assay at high substrate concentration should facilitate purification of mutant enzyme for structural analysis.

Although explanations other than structural gene mutation could explain our data, these seem unlikely. Based on heat inactivation studies, the appar-

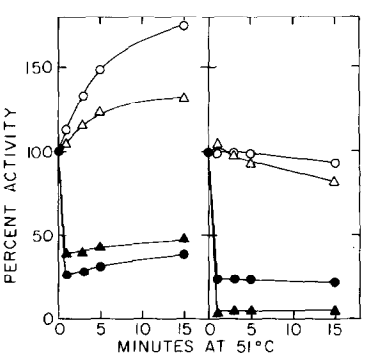

Figure 3. Heat inactivation at 51°C assayed at pH 6 (solid symbols) and pH 4 (open symbols). Unheated values in n mol product per assay at pH 4 and 6, respectively, were: left panel, control (triangles), 0.38 and 0.075; and second control (circles), 0.45 and 0.17. For the right panel the values were: SA (triangles), 0.63 and 0.57; and GM 654 (circles) 0.97 and 0.52.

ent acid mannosidase activity of patient extracts appeared to be independent of neutral mannosidase. The activity of mutant fibroblast extracts appeared not to be derived from fetal calf serum since the acid mannosidase in the serum was at least as heat stable as wild type human enzyme and had a Km for the fluorogenic substrate of approximately 0.06 mM. In addition, although the mutant leukocyte extract activity was only 4% of the control activity, the response to increasing substrate concentration and the heat lability of the residual activity were similar to the results with mutant fibroblasts. The possibilities exist that artificial substrate might be hydrolyzed by some other lysosomal glycosidase at high concentrations, or that the substrate itself might have a significant contamination with another pyranoside. Either of these explanations should have resulted in increasing activity with control extracts at high concentrations, but this was not observed.

The observation of residual mannosidase activity with altered kinetic properties in fibroblast extracts from four unrelated patients with mannosidosis suggests a general hazard in the clinical enzymatic diagnosis of this condition. Our studies demonstrate that the diagnosis, even once suspected, can be easily discarded based on misleading enzyme data obtained with commonly used substrate concentrations (10). The in vitro level of α-mannosidase activity in extracts is subject to multiple variables such as ionic strength, choice of buffer, pH, and extract preparation methodology. These results emphasize the general need, 1) for analysis at limiting as well as saturating substrate concentrations if more defective enzymes are to be detected, and 2) for the inclusion of known deficient extracts as well as normal control extracts in routine diagnostic enzymology.

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