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ALTERED SERUM α-D-MANNOSIDASE ACTIVITY IN MUCOLIPIDOSIS II AND MUCOLIPIDOSIS III

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Summary

Normal human serum contains at least three forms of α-D-mannosidase: an acidic form which has a pH optimum of 4.25, is inhibited by Co^{2+} and is thermostable; an intermediate form, which has a pH optimum of 5.6-5.7, is stimulated by Co^{2+} and is heat labile at 50°C; and a neutral form with a pH optimum of 6.0-6.5. In Mucolipidosis II and III sera, the acidic α-mannosidase activity persists while the intermediate activity is absent or altered. Heating the serum does not affect the pH activity curve, the electrofocusing profile or the response to Co^{2+} of α-mannosidase. During heat inactivation at 55°, 90-100% of the pH 5.6 α-mannosidase activity is lost in normal sera while less than 40% is lost from ML sera. The effect on sera from ML obligate heterozygotes is intermediate. The absent or altered intermediate mannosidase may be responsible for aberrant biochemical properties reported for other glycosidases in Mucolipidosis II and Mucolipidosis III.

Unlike other well-characterized inherited neurometabolic disorders in which a single lysosomal hydrolase is affected (1), Mucolipidosis (ML) II (I-cell disease) and ML III demonstrate multiple lysosomal enzyme deficiencies in cultured human fibroblasts with a concomitant increase of several lysosomal enzyme activities in the culture medium (2,3). The most consistent finding in autopsied tissues from ML II patients is a marked decrease in the activities of acid β-D-galactosidase (2,4,5), while serum and urine from ML II and ML III patients demonstrate elevated levels of several acid hydrolase activities (6-8). The molecular defect responsible for ML II and ML III is unknown. Experiments in our laboratory have revealed that lysosomal hydrolases from ML patients do not bind as well to Concanavalin A-Sepharose 4B

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as do normal enzymes (5,8). Other studies indicate ML enzymes are not pinocytosed as readily as normal enzymes by cultured fibroblasts with specific hydrolase defects (9). Uptake of normal enzyme in these cells can be inhibited by mannose and mannose-phosphate (10-12). Thus the post-translational modification of mannose-containing oligosaccharide chains of lysosomal hydrolases may be affected in ML. In this report we present evidence for the absence or alteration of the activity of the Golgi-associated, intermediate pH form of serum α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) in ML II and ML III.

EXPERIMENTAL

All procedures were carried out at 0-4°C unless otherwise stated. Mutant and pathological control serum samples were obtained by the attending physicians and shipped frozen to our laboratory. Venous blood was collected from healthy laboratory personnel and the serum separated on the same day by centrifugation at 3,000 rpm for 20 min. All samples were stored at -20°C until used.

The α-D-mannosidase activity was determined using a 10 µl aliquot of serum incubated for 15 min with 50 µl of 2 mM 4-methylumbelliferyl (4MU) α-D-mannopyranoside (Koch-Light Ltd.), prepared in 0.2 M sodium phosphate adjusted to pH 4.25 or pH 5.65 with 1.0 M citric acid. A unit of activity is defined as the amount of enzyme that will hydrolyze one nanomole of substrate per minute at 37°C. The buffers for the pH activity profile experiments were prepared by titrating 0.2 M sodium phosphate with 1.0 M citric acid to various pH values ranging from 2.8 to 6.8. Enzyme assays were then performed as described above. The effect of Co^{2+} on the α-D-mannosidase activity was investigated by the addition of $CoCl_2$ to the assay mixture at 10 mM final concentration. Assays of other forms of acid hydrolase activities were performed as previously described (2).

Isoelectric focusing was performed by the method of Haglund (13) using a 30 ml electrofocusing column designed from the LKB 8101 column. Electrofocusing was routinely performed for 16-18 hr, with a 2.5% (v/v) (pH 5-8) concentration of carrier ampholytes in a gradient of 0-67% (w/v) sucrose. The voltage was maintained at 600 V establishing a current of 3-5 mA. After 14 hr, the voltage was increased to 800 V for 2-4 hr (the final current was 0.5 mA). When electrofocusing had been completed, 100 column fractions were collected containing 5 drops per fraction. Assays for α-D-mannosidase activity present in these fractions were performed by incubating 10 µl to 30 µl from every other fraction for 1-3 hr with 50 µl of substrate buffered at pH 4.25 or 5.65. Samples from every other tube were then combined with the subsequent tube and the pH determined on a Beckman digital pH meter at 0-2°C.

Electrofocusing and pH activity studies were performed following heat inactivation. Aliquots of 400 µl of mutant serum samples were heated in parafilmed tubes at 55 - 60°C for 30 min followed by centrifugation at 40,000 xg for 30 min. For normal serum samples, 400 µl of serum was heat inactivated as just described prior to the pH activity studies and 1.5 ml heat inactivated prior to electrofocusing.

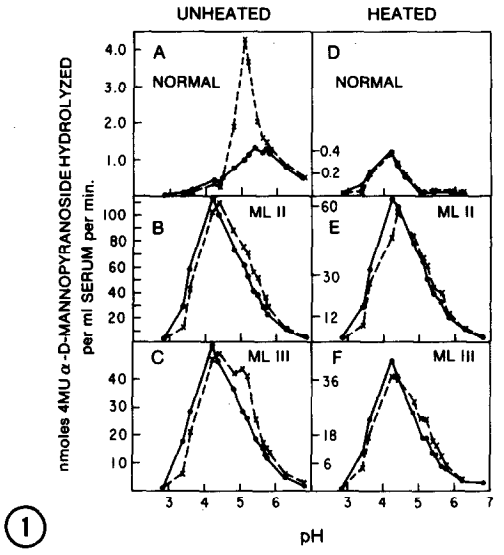
Time course heat inactivation studies were performed separately by incubating 30 µl of mutant and normal sera at 50°C in a shaking water bath in tubes sealed with parafilm. Tubes were removed at various timed intervals, placed into an ice-water bath and assayed for α-D-mannosidase activity as described earlier.

RESULTS

Figure 1 shows the effect of pH on α-D-mannosidase activity in normal control, ML II and ML III serum samples. The enzyme in normal sera displayed a major pH optimum between 5.5 and 5.7 with a minor optimum at pH 4.25 (panel A), while in ML II and ML III serum samples a single pH optimum at pH 4.25 was observed (panels B and C). Serum α-D-mannosidase activity from ML II heterozygotes Tay-Sachs disease (TSD), cystic fibrosis and Mucopolysaccharidosis (MPS) I patients had pH profiles comparable to that of normal controls. Sera from two patients with mannosidosis showed a marked decrease in α-D-mannosidase activity at pH 4.25 and a normal pH activity profile above pH 5.0. ML II and ML III serum samples demonstrated normal pH activity profiles for β-glucuronidase, α-galactosidase, and hexosaminidase.

Incubating control sera at 55 - 60°C for 30 min resulted in the loss of α-D-mannosidase activity above pH 5.0 (Fig. 1, panel A). This pattern was similar to those obtained for unheated and heated ML II and ML III samples (Fig. 1, panels B,C,E, and F). Heat inactivation of two mannosidosis serum samples resulted in a complete loss of α-D-mannosidase activity.

Co^{2+} inhibited acid α-D-mannosidase while maximally stimulating the enzyme activity at pH 5.1-5.2 in normal, ML II and ML III sera (Fig. 1, panels A-C). Similar results were obtained



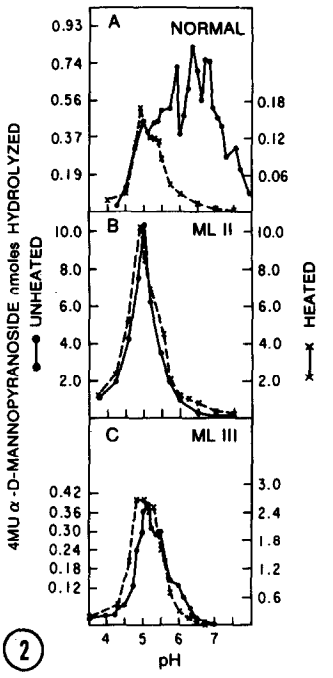


Figure 1 pH dependence of α-mannosidase activity in heated and untreated normal, ML II and ML III sera in the presence and absence of Co^{2+}

(●-●) control; (x-x) + 10 mM Co^{2+}

Figure 2 Isoelectric focusing (pH 5-8) of serum mannosidase

normal: unheated (1.0 ml, 1.9 units); heated (1.0 ml, 0.2 units);

ML II: unheated (0.072 ml, 11 units); heated (0.30 ml, 20 units);

ML III: unheated (0.083 ml, 4 units); heated (0.33 ml, 13 units).

(●-●) unheated; (x-x) heated

with the two mannosidosis serum samples. After heat treatment, the Co^{2+} effect in the ML II and ML III serum samples remained unchanged (Fig. 1, panels E,F) while in normal sera, Co^{2+} inhibited the residual activity (panel A).

Electrofocusing of control serum α-mannosidase revealed two major regions of enzymatic activity with pH values of 5.0-6.0 and

6.3-7.0 with a minor component at 7.6 (Fig. 2, panel A). However, the serum α-mannosidase pattern of ML II and ML III demonstrated only one region of activity at pH 4.8-6.0 (Fig. 2, panels B,C). Isoelectric focusing patterns in pathological control sera (TSD and MPS I) were similar to controls. Electrofocusing of mannosidosis sera showed the major α-D-mannosidase activity between pH 6.3 and 7.0 although some activity focused in the pH 5.0-6.0 range. Heat inactivation of α-D-mannosidase from normal sera resulted in isoelectric focusing profiles which resembled ML II and ML III samples (Fig. 2, compare panel A to panels B and C), while the profiles of the heat inactivated ML II and ML III sera did not change (Fig. 2, panels B,C).

Time course of thermal inactivation at 50°C of ML II and ML III sera revealed that α-D-mannosidase activity measured at pH 5.65 was more stable than corresponding normal controls (Fig. 3). The kinetics of inactivation of the ML II and ML III enzyme yielded a single slope, suggesting the presence of a single form of α-D-mannosidase. This would be in agreement with the electrofocusing results shown in Fig. 2, panels B and C. Normal control serum α-D-mannosidase activity was more thermal labile at pH 5.65 than the enzyme from ML II and ML III patients and demonstrated an inflection between 10-15 min, suggesting the presence of more than one form of α-D-mannosidase. This observation was also supported by electrofocusing (Fig. 2, panel A). The heat inactivation profile of the serum enzyme from a patient with mannosidosis was similar to that of normal controls. Serum α-mannosidase from ML II heterozygotes demonstrated intermediate levels of enzyme activity after heating at 50°C for 30 min.

DISCUSSION

The reports of altered properties of ML II and ML III lyso-

somal hydrolases that can be attributed to changes in the structural composition, orientation, and/or accessibility of mannose residues on the lysosomal hydrolases (5,8,10-12) stimulated us to investigate the possible involvement of α-D-mannosidase in these diseases. Normal human serum contains three forms of α-D-mannosidase: an acidic (lysosomal), intermediate (Golgi), and neutral (cytosolic) form, all thought to be under separate genetic control (14-16). The ML II and ML III serum α-D-mannosidase pH activity and electrofocusing profiles suggest the preferential secretion of the lysosomal form of α-D-mannosidase into the serum. The inability of heat treatment of ML II and ML III serum α-D-mannosidase to change the pH activity curves, the Co^{2+} effect, or the electrofocusing profiles suggests that the heat labile (intermediate) form of serum α-D-mannosidase is absent or altered. Heat treatment inactivates the intermediate pH form of normal serum α-D-mannosidase and results in pH activity and electrofocusing profiles that mimick the ML II and ML III serum α-D-mannosidase.

Alternatively, the intermediate form of serum α-D-mannosidase may not be absent or altered but due to the dramatic increase of acidic α-mannosidase activity, the subtle changes in the pH and electrofocusing profiles after heat inactivation of the intermediate enzyme may not be recognized.

After heat inactivation the α-D-mannosidase assayed at pH 5.65 is relatively stable in ML II and ML III serum samples but is thermolabile in normal serum (Fig. 3). When normal, ML II and ML III serum samples were heat treated in a similar manner and assayed for α-mannosidase activity at pH 4.25, the heat inactivation curves were indistinguishable from the ML II and ML III curves assayed at pH 5.65 (data not shown), again suggesting the

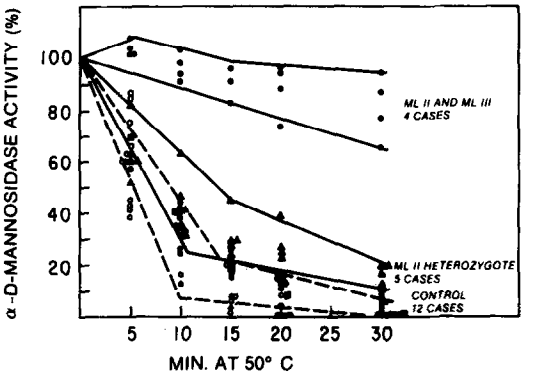


Figure 3 Time course heat inactivation of serum α-mannosidase assayed at pH 5.65. (●-●) ML II and ML III; (▲-▲) ML II heterozygotes; (○-○) normal control; (■-■) mannosidosis.

presence of a single heat stable α-D-mannosidase in ML II and ML III. Finally, the intermediate levels of residual α-mannosidase activity in the five ML II heterozygote serum samples assayed at pH 5.65 after heating at 50°C for 30 min may establish a criteria for detecting carriers of ML II.

The significance of the absence or alteration of the intermediate (Golgi-associated) α-D-mannosidase in the serum ML II and ML III patients is unknown. It is possible that this enzyme is an endomannosidase which participates in the normal processing of the lysosomal hydrolases by cleaving mannose chains on the enzyme prior to their secretion or incorporation into lysosomes. The absence or alteration of this enzyme activity could explain previous results from our laboratory and others demonstrating alterations in both the binding properties of the lysosomal hydrolases to Concanavalin A-Sepharose 4B and the specific pinocytosis into cultured fibroblasts.

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