The Nature of the Residual α -Mannosidase in Plasma in Bovine Mannosidosis

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Acidic α -mannosidase (EC 3.2.1.24), optimum pH 4.25, is absent from the plasma of Angus calves with mannosidosis, and the residual α -mannosidase activity has an optimum pH of 5.5, intermediate between that of the acidic and neutral α -mannosidases. This 'intermediate' α -mannosidase differs from the acidic form in its kinetic properties, its lack of marked inhibition by EDTA and its thermolability at 55°C and physiological pH. Isoelectric focusing and ion-exchange chromatography show that it exists in at least two forms. The presence of a secondary peak at pH 5.5 in the pH/activity profile of normal plasma and the effect of heating at 55°C indicate that such a form is present in normal plasma. The residual activity in the plasma of a calf with mannosidosis is therefore probably not the product of the defective gene. A differential assay, based on their different stabilities at 55°C, has been developed for measuring the acidic and intermediate α -mannosidases in plasma. There was no correlation between the concentrations of the two enzymes in the plasma of Angus cows heterozygous for mannosidosis or in the plasma of normal animals. This precludes the use of the intermediate form as a reference enzyme for the acidic activity in a test for heterozygosity for mannosidosis based on the gene-dosage phenomenon. The concentrations of the intermediate activity were comparable in normal animals and animals homozygous or heterozygous for mannosidosis.

Mannosidosis is a disease of children and Angus cattle associated with the defective catabolism of glycoproteins resulting from an inherited deficiency of lysosomal α -mannosidase (EC 3.2.1.24) (Öckerman, 1967; Jolly, 1971; Hocking *et al.*, 1972; Carroll *et al.*, 1972; Jolly, 1975). In normal bovine tissues, α -mannosidase exists in at least three forms separable by ion-exchange chromatography on DEAE-cellulose (Phillips *et al.*, 1974a). Two components, A and B, have optimal activity at pH 4.25, but component C has a neutral pH optimum of 6.5. Components A and B with acidic activity are absent from the tissues of a calf with mannosidosis, and the neutral component, C, accounts for the residual activity. Acidic α -mannosidase is also absent from the plasma of a calf with mannosidosis, but the residual activity has a pH optimum of 5.5 and not of 6.5 as in other tissues. α -Mannosidase isoenzymes with different pH optima have been reported in several other mammalian species and in some detail in the rat and man (Marsh & Gourlay, 1971; Carroll *et al.*, 1972; Dewald & Touster, 1973; Masson *et al.*, 1974; Phillips *et al.*, 1974b). There is evidence that, in rat liver, the neutral activity is cytoplasmic in

origin and the acidic activity lysosomal (Marsh & Gourlay, 1971). A third type of α -mannosidase activity with a pH optimum of 5.3 and different properties from the acid and neutral activities has been described in the Golgi fraction of rat liver (Dewald & Touster, 1973).

In the present paper the nature of the residual α -mannosidase activity in the plasma of a calf with mannosidosis is examined and compared with the activity in normal plasma. The particular purpose of the study was to investigate whether α -mannosidase activity with an optimum pH of 5.5 also occurred in normal plasma, and, if so, whether it could be used as a convenient reference enzyme for the acidic activity in the detection of heterozygous individuals through application of the gene-dosage phenomenon.

Materials and Methods

Tissue

Bovine blood was collected in 10 ml heparinized Vacutainers (Becton, Dickinson and Co., Rutherford, NJ, U.S.A.) and plasma separated on the same

day by centrifugation at 1250g for 5 min. The kidneys from an Angus calf, which had been diagnosed clinically and by histopathology as suffering from mannosidosis, were stored frozen at -12°C until required.

Assay of α -mannosidase activity

α -Mannosidase was assayed with the fluorogenic substrate, 4-methylumbelliferyl α -D-mannopyranoside (Koch-Light Laboratories, Colnbrook, Bucks., U.K.). Plasma (0.1 ml) suitably diluted in 0.85% (w/v) NaCl was incubated for 30 min at 37°C with 0.1 ml of a 10 mM solution of the substrate in 0.2 M-sodium phosphate adjusted to pH 4.0 or 5.5 with 0.1 M-citric acid. The reaction was stopped by the addition of 2 ml of 0.5 M-glycine, adjusted to pH 10.4 with NaOH. The fluorescence of the liberated 4-methylumbelliferone was measured in a Turner fluorometer (model III) by using a Turner 7-60 primary filter and a Turner 22 secondary filter. The phosphate/citrate buffer system was used to investigate the pH-dependence of the activity. Extracts of a kidney from a calf with mannosidosis were assayed in the same way. The effect of EDTA, Zn^{2+} and Co^{2+} on the activity was investigated by including 1 mM-EDTA, 1 mM- ZnSO_4 or 1 mM- CoSO_4 adjusted to the correct pH in the substrate solutions, giving a final concentration of 0.5 mM. All reagents were of A.R. grade. A unit of activity is that amount of enzyme which transforms $1\text{ }\mu\text{mol}$ of substrate/min under the specified conditions. K_m values were determined by the graphical analysis of Lineweaver & Burk (1934). A programmable calculator was used to obtain the line of best fit by a least-squares program for the regression line.

Separation procedures

Isoelectric focusing, ion-exchange chromatography on CM- and DEAE-cellulose (CM 32 and DE 32 respectively; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) and gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) were carried out as described previously (Phillips *et al.*, 1974a,b). Plasma was dialysed at 4°C overnight against 10 mM-sodium phosphate, pH 6.0, the equilibration buffer for the ion-exchange media, before analysis by ion-exchange chromatography. A longer incubation period, 1–3 h, was necessary to detect α -mannosidase activity in the fractions obtained by these separation methods because of the dilution of the activity.

Investigation of thermal stability

Plasma was heated at 55°C and, at suitable intervals of time, portions (0.1 ml) were removed and added to

2.4 ml of cold (4°C) 0.85% NaCl. The diluted plasma was assayed at pH 4.0 and 5.5 in the usual manner at 37°C .

Results

Characterization of residual plasma α -mannosidase activity

(1) *Effect of pH.* The pH/activity curve for α -mannosidase in normal bovine plasma has a pH optimum between 4.0 and 4.3 and a shoulder at pH 5.5 (Fig. 1a). By contrast, the curve obtained with the plasma of a calf with mannosidosis shows negligible activity at pH 4.0–4.3, but has an optimum at pH 5.5 and perhaps a further small shoulder about pH 6.5 (Fig. 1b). These data are consistent with a deficiency of acidic α -mannosidase in mannosidosis (Hocking *et al.*, 1972; Phillips *et al.*, 1974a) and also suggest that α -mannosidase with a pH optimum of 5.5 (intermediate α -mannosidase) is present in normal plasma.

(2) *Effect of substrate concentration.* As the acidic α -mannosidase is absent from the plasma of a calf with mannosidosis, the K_m at pH 5.5, the optimum pH for the activity in this plasma, is a property of the intermediate α -mannosidase (Table 1). The relatively high K_m value for this enzyme is consistent with the observation that the reaction velocity had not reached a maximum at the highest substrate concentration used (5 mM). There is negligible activity at pH 4.0–4.3, the pH optimum of the acidic α -mannosidase in normal plasma. It can therefore be assumed that if intermediate α -mannosidase is present in normal plasma, it does not contribute to the activity measured at pH 4.0 and that the K_m measured at this pH in normal plasma is characteristic of the acidic α -mannosidase. The difference in K_m values between the two types of α -mannosidase in normal plasma means that the shape of the pH/activity curve will depend on the substrate concentration. At relatively high substrate concentrations, i.e. above 3 mM, the apparent relative concentration of the intermediate enzyme will increase, because the acidic but not the intermediate enzyme will be saturated.

The residual α -mannosidase in plasma from a calf with mannosidosis also has a different K_m from neutral α -mannosidase, which occurs in tissues and has a pH optimum of 6.5.

(3) *Effect of Zn^{2+} , Co^{2+} and EDTA on activity.* Zn^{2+} (0.5 mM) increased the activity and slightly lowered the pH optimum of the residual activity in the plasma from a calf with mannosidosis (Fig. 1b). It had a similar effect on the acidic α -mannosidase in normal plasma (Fig. 1a). By contrast EDTA (0.5 mM) had very little effect on the α -mannosidase with a pH optimum of 5.5 but strongly inhibited the acidic α -mannosidase in normal plasma, especially below pH 5. Whereas Co^{2+} (0.5 mM) consistently inhibited

Fig. 1. pH-dependence of α -mannosidase in (a) normal plasma and (b) plasma from a calf with mannosidosis in the presence and absence of Zn^{2+} , Co^{2+} and EDTA

Plasma diluted 20-fold in 0.85% (w/v) NaCl (100 μ l) was incubated with 2.0 mM-4-methylumbelliferyl α -D-mannopyranoside (100 μ l) and buffer (200 μ l) at a series of pH values in the presence and absence of 0.5 mM- Zn^{2+} , Co^{2+} or EDTA. $\bullet$, Control; $\circ$, ZnSO_4 ; $\blacksquare$, CoSO_4 ; $\square$, EDTA.

acidic α -mannosidase, its effect on the residual activity in the plasma of calves with mannosidosis was found to be variable. In two cases it increased the activity (as shown in Fig. 1b) and in another two it caused a 20% decrease in the activity at pH 5.5. In each sample, and irrespective of its effect on the total activity, the presence of Co^{2+} resulted in two peaks in the pH/activity curve at approx. pH 5.25 and 5.75.

Table 1. Values of K_m for the hydrolysis of 4-methylumbelliferyl α -D-mannopyranoside by bovine α -mannosidases

Numbers of animals are given in parentheses.

	pH	K_m (mM)	
		Mean	Range
Acidic activity in normal plasma	4.0	0.64 (4)	0.60–0.69
Residual activity in plasma from calves with mannosidosis	5.5	3.8 (3)	2.73–5.45
Neutral activity in kidney from calves with mannosidosis	6.5	0.66 (2)	0.65–0.68

The effect of the added Co^{2+} could depend on the endogenous concentration of Co^{2+} in the plasma. If the latter was high, the added Co^{2+} would have little effect, but if it was low and insufficient to saturate the enzymic activity, the added Co^{2+} would stimulate the activity. Courtois & Mangeot (1972) reported that Co^{2+} has little effect on bovine serum α -mannosidase at pH 5.4, the optimum pH. The pH-dependence of the effects of Zn^{2+} , Co^{2+} and EDTA on the activity in normal plasma is consistent with the presence of the two types of α -mannosidase.

(4) Evidence for multiple forms of the α -mannosidase activity with a pH optimum of 5.5. The isoelectric-focusing profile (Fig. 2) for the α -mannosidase activity in the plasma from a calf with mannosidosis shows that the activity exists in more than one form. The major components have pI values of 5.8 and 6.5. The residual neutral activity in the liver of a calf with mannosidosis has pI 5.40 (N. C. Phillips & B. G. Winchester, unpublished work). The heterogeneity of the activity was confirmed by ion-exchange chromatography on DEAE- and CM-cellulose. At pH 6.0, which is intermediate between the pI values of the two major forms, 50–60% of the activity was not absorbed on the ion exchangers and the rest was eluted with a NaCl gradient. The residual neutral activity in tissues is completely absorbed on DEAE-cellulose at pH 6.0 (Phillips *et al.*, 1974a).

A single peak of activity only slightly retarded by the column was obtained when the activity was analysed by gel filtration on Sephadex G-200. It cannot be separated from the acidic and neutral α -mannosidase activities by this technique, as they have comparable elution volumes (Phillips *et al.*, 1974a).

(5) Thermal stability of α -mannosidase activity in plasma. The thermal stability of the α -mannosidase activity in normal plasma and in plasma from a calf with mannosidosis was investigated at 55°C (Fig. 3). The residual activity in the mannosidosis plasma was rapidly inactivated within 20 min, whereas the acidic

Fig. 2. Isoelectric focusing of residual α -mannosidase in plasma from a calf with mannosidosis

Plasma (1 ml) was focused for 60 h at 400V and 0.5mA over the range of pH3–10. The fraction size was 1.5 ml. ●, α -Mannosidase assayed at pH5.5; ----, pH.

Fig. 3. Thermal stability at 55°C of the α -mannosidase in normal plasma and in plasma from a calf with mannosidosis

Plasma was heated at 55°C and samples were removed at suitable time-intervals and assayed for α -mannosidase activity at pH4.0 and 5.5. ●, Normal plasma assayed at pH4.0; ○, normal plasma assayed at pH5.5; ■, plasma from a calf with mannosidosis assayed at pH5.5.

α -mannosidase in normal plasma was relatively stable for 1 h. The partial loss of activity measured at pH5.5 in the normal plasma would be due to inactivation of

Fig. 4. Effect of storage at -12°C on the thermal stability at 55°C of the acidic α -mannosidase in normal plasma

Plasma, which had been stored at -12°C for various lengths of time, was assayed for acidic α -mannosidase activity at 37°C before and after heating at 55°C for 1 h. ●, Percentage of activity in unheated sample remaining after heating.

the intermediate enzyme whose pH/activity profile overlaps with that of the acidic form. Subsequent investigation of the heat stability of acidic α -mannosidase of numerous individual samples of normal plasma showed variability in heat stability but confirmed its stability relative to the intermediate-pH form. In particular there was a loss of heat stability relative to storage time at -12°C (Fig. 4).

Differential assay for the intermediate α -mannosidase activity

Although the intermediate activity with a pH optimum of 5.5 has negligible activity at pH4.0, the acidic activity has considerable activity at pH5.5. Therefore it is not possible to assay the intermediate activity directly in the presence of the acidic activity. However, the difference in thermal stabilities between the two types of activity forms a basis for the assay of the intermediate activity in the presence of the acidic activity. It can be assumed that when normal plasma is heated at 55°C for 20 min, all the intermediate activity is destroyed, and that the residual activity at pH5.5 is due exclusively to the acidic α -mannosidase. Therefore the difference in activity at pH5.5 between an unheated and a heated sample of normal plasma is a measure of the intermediate activity present. The acidic α -mannosidase is the activity measured at pH4.0 in the heated or unheated sample.

Because of the small individual variations in heat stability of the acidic activity, an assay method that takes this variation into account was used to calculate the intermediate activity. Plasma samples were assayed at pH4.0 and pH5.5 both before and after heating at 55°C for 20 min. Residual enzyme activity at pH5.5 after heating would be due to the acidic enzyme, which is also active at that pH. A measure of the intermediate form 'I' is therefore obtained by

subtracting this heat-stable enzyme activity from the initial activity measured at pH 5.5. As there may have been some partial loss (or increase) of the acidic activity on heating, a correction calculated from any change in activity measured at pH 4.0 is applied, i.e.

$$I = A_{5.5}^U - \left(A_{5.5}^H \times \frac{A_{4.0}^U}{A_{4.0}^H} \right)$$

where $A_{4.0}$ and $A_{5.5}$ are the activities measured at pH 4.0 and 5.5 respectively, and U and H denote the unheated and heated sample respectively.

By using the differential assay, the intermediate and acidic activities were measured in the plasma from a herd of 48 Angus cows that were considered normal with regard to the mannosidosis genotype. No significant correlation was found between the two activities ($n = 48$; $r = +0.078$). In another experiment there was no significant difference ($P > 0.05$; Student's $t = 0.48$; degrees of freedom = 32) between the intermediate α -mannosidase activities in a group of normal and in a group of heterozygous animals of same age, sex and from the same herd. The mean concentration of intermediate activity in the 19 normal animals was 3.39 munits/ml of plasma (range 1.90–4.32) and in the heterozygous animals 3.64 munits/ml of plasma (range 2.67–4.40). The heterozygotes were diagnosed as such on the basis of their plasma acidic α -mannosidase activity (Jolly *et al.*, 1973, 1974a,b). There is thus no evidence of a compensatory increase in this form of α -mannosidase in mannosidosis heterozygotes. The concentrations of the intermediate α -mannosidase recorded for normal and heterozygous animals are comparable with those found in five calves with mannosidosis (mean activity, 3.56 munits/ml of plasma; range 2.99–4.35).

Discussion

There is no evidence to suggest that the residual α -mannosidase activity with a pH optimum of 5.5 in the plasma from a calf with mannosidosis is the product of the defective gene. The presence of a secondary peak at pH 5.5 in the pH/activity curve for normal plasma and the thermal stability experiments indicate that the intermediate activity is present in normal plasma. It has different kinetic and physico-chemical properties from the predominant acidic α -mannosidase in normal plasma, and its presence in mannosidosis suggests that it is under different genetic control from the acidic enzyme. Isoelectric focusing and ion-exchange chromatography suggest that the intermediate activity exists in more than one form.

Acidic and intermediate α -mannosidase also occur in human plasma, but the concentration of the intermediate activity in man is greater than that of the acidic activity (Öckerman, 1969; Masson *et al.*, 1974). Antiserum raised against human liver acidic

α -mannosidase precipitated the acidic activity but not the intermediate activity from human plasma, providing further evidence that the mammalian acidic and intermediate α -mannosidases are structurally and genetically distinct (Phillips *et al.*, 1975).

It is interesting to compare the effects of EDTA, Zn^{2+} and Co^{2+} on the intermediate α -mannosidase, which accounts for the residual activity in the plasma of a calf with mannosidosis, with their effects on the neutral α -mannosidase, which accounts for the residual activity in other tissues of a calf with mannosidosis. It has been shown previously (Phillips *et al.*, 1974a) that the neutral activity is only very slightly inhibited by EDTA but is activated by Co^{2+} at pH 6.5. Zn^{2+} also activates the neutral enzyme, but only at pH 5.0–5.5. Further experiments have shown that the activation by Co^{2+} is also greatest at pH 5.0–5.5 (B. Winchester, unpublished work). This suggests that the intermediate type of activity found in plasma also occurs in tissues. Its presence would normally be obscured by the large amounts of acidic and neutral α -mannosidase present. A similar suggestion has been made to explain the effect of various metal ions on the neutral α -mannosidase activity in normal human liver (Phillips *et al.*, 1974b). Dewald & Touster (1973) have shown that a Golgi fraction of rat liver contains α -mannosidase activity with an optimum pH of 5.3, which can be distinguished from the acidic lysosomal and neutral cytoplasmic forms described previously (Marsh & Gourlay, 1971). Thus three types of α -mannosidase possibly also exist in normal bovine tissues, acidic (optimum pH 4.0–4.5), neutral (optimum pH 6.3–6.5) and activity with an intermediate optimum pH of 5.5. In mannosidosis the acidic enzyme is absent, but the other two types of activity are present in tissues. However, the neutral enzyme does not appear to be present in plasma to any great extent, possibly because of its very low stability. An alternative explanation for its apparent absence could be conversion into the intermediate activity in an extracellular environment.

Measurements of enzyme activity are recorded relative to some parameter such as volume, mg of protein, weight of tissue or some variable factor such as the activity of another enzyme. Use of such a reference parameter is only valid if there is a positive correlation between it and the enzyme in question. Enzyme activities in plasma or serum are usually expressed as activity per ml. However, as demonstrated for hexosaminidase A in the screening programme for Tay-Sachs heterozygotes (Kaback *et al.*, 1974), this may not necessarily be the best parameter. In bovine plasma, acidic α -mannosidase varies with age, season and even within different groups of similar animals on the one property (Jolly *et al.*, 1973, 1974a). Measurement of bovine plasma acidic α -mannosidase activity is the basis of a heterozygote testing scheme

currently operative to control mannosidosis in the Angus-bull breeding herds (Jolly *et al.*, 1974*a,b,c*). The variability in enzyme concentration can be avoided by comparing enzyme concentrations in groups of animals of the same age, sex, nutritional status and from the same environment, but this is not always practical. An alternative way of avoiding these varying factors might be to relate the acidic α -mannosidase activity to a reference enzyme activity. Reference enzymes have been used for detection of heterozygotes in several similar lysosomal storage diseases (Hirshorn *et al.*, 1969; Harzer, 1973; Matsuda *et al.*, 1973; Masson *et al.*, 1974), although correlations between the test and reference enzymes have not been recorded. In the present study the use of the intermediate α -mannosidase with a pH optimum of 5.5 as a reference enzyme for the acidic-enzyme activity in plasma was investigated. The differential assay for the acidic and intermediate α -mannosidase activities in plasma was based on their different thermal stabilities and as such resembled the method of O'Brien *et al.* (1970) for measuring hexosaminidase A in human serum. Although a small positive correlation between the acidic and intermediate activities was previously reported (Jolly *et al.*, 1975) the present experiment failed to show a significant correlation. It must be concluded therefore that there is little or no correlation between the two activities. As such the intermediate α -mannosidase is not a suitable reference enzyme under the assay conditions used. Its use for the detection of heterozygotes may give an illusion of separating these animals from the normal, but this is not likely to be significantly more efficient than if another unrelated parameter was used.

In a small preliminary experiment with known human mannosidosis heterozygotes, Masson *et al.* (1974) related α -mannosidase activity at pH 4.0 to α -mannosidase activity at pH 6.0. The ratio failed to distinguish between heterozygotes and controls, but perhaps further investigations along the lines outlined above are warranted.

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