Calcium Ion Activation of Rabbit Liver α1,2-Mannosidase*

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Rabbit liver α1,2-mannosidase is a calcium ion requiring enzyme involved in processing the asparagine-linked oligosaccharides of glycoproteins. Ca2+ activation occurs with an apparent $K_a$ of 1.1 μM. The major effect of the metal ion activator is on $K_m$ rather than $V_{max}$. The kinetic mechanism of the enzyme is that of an ordered equilibrium in which Ca2+ must bind before substrate and the metal ion cannot release once the substrate has added to the enzyme. Several other divalent cations including Co2+, Mn2+, and Zn2+ were competitive with Ca2+ and inhibited the enzyme. Significantly, Mg2+ had no effect on enzyme activity. 1-Deoxymannojirimycin and Tris, which inhibit glycoprotein processing in vivo, are inhibitors of the mannosidase competitive with substrate. The effect of Ca2+ on the affinity of the enzyme for substrate may be a determinant in regulation of enzyme activity in vivo.

α1,2-Mannosidases located in the rough endoplasmic reticulum and Golgi are involved in processing the asparagine-linked oligosaccharides of mammalian glycoproteins (1–9). These enzymes are required for the removal of four α1,2-mannosyl residues from a Man9GlcNAc2 intermediate structure. Although asparagine-linked oligosaccharides containing between 5 and 9 mannose units are all broadly classified as high-mannose structures, the Man9GlcNAc2 structure is physiologically quite distinct from oligosaccharides containing 6 or more mannose units, since only the Man9GlcNAc2 structure can serve as the immediate precursor of complex oligosaccharides initiated by the addition of an N-acetylgalactosaminyl residue catalyzed by N-acetylgalactosaminyltransferase I. All of the larger oligosaccharides contain at least 1 α1,2-linked mannose residue and are not substrates for the N-acetylgalactosaminyltransferase. Thus the activities of α1,2-mannosidases located in the rough endoplasmic reticulum and Golgi are critical in determining the final oligosaccharide structures of glycoproteins. At the present time, however, little is known about the kinetic properties of the α1,2-mannosidases, and few of the factors that regulate the activities of the enzymes have been identified. The activity of a second Golgi processing enzyme, N-acetylgalactosaminylphosphotransferase, has been shown to depend on specific protein domains in the glycoprotein substrates for the transferase which are all lysosomal hydrolases (10, 11). Since the oligosaccharide substrates for the α1,2-mannosidases reside on a wide variety of both soluble and membrane proteins, it is likely that alternative mechanisms may direct the specificities of the α1,2-mannosidases.

The high-mannose oligosaccharides on several glycoproteins have been shown to be sterically inaccessible to the action of α-mannosidases, and steric factors have been proposed to account for the presence of high-mannose chains on many glycoproteins, even though the full complement of other processing enzymes is present in the cell (12–16). This mechanism, however, does not account for the presence of high-mannose chains on glycoproteins such as thyroglobulin (17, 18), since the oligosaccharides on this glycoprotein are readily accessible to the action of the rabbit liver α1,2-mannosidase (12), suggesting that the activities of the α1,2-mannosidases may be subject to regulatory control. This latter possibility is consistent with results showing that the ratio of high-mannose to complex chains in thyroglobulin varies in different disease states (19), and that the ratio changes in tissue culture systems depending upon the culture conditions (20). Alternative control mechanisms for the processing enzymes are further indicated by results showing that the ratio of high-mannose to complex oligosaccharides in the human cancer cell line HT-29 is subject to change during cellular differentiation (21). In order to interpret these effects, it is necessary to have a better understanding of the properties of the α1,2-mannosidases.

Most investigations of the α1,2-mannosidases have assessed the properties of the enzymes utilizing assays carried out with isotopically labeled substrates at low concentrations. Although these assays were extremely sensitive, and allowed determination of the specificity of the enzymes toward various oligosaccharide structures, they were not well suited for determination of the kinetic properties of the enzymes. We have previously reported the purification of a calcium ion-activated α1,2-mannosidase from rabbit liver microsomes (12, 22). In order to obtain additional information, the kinetic properties have now been assessed under conditions that allowed determination of the kinetic constants and the kinetic mechanism of the enzyme.

**EXPERIMENTAL PROCEDURES**

Materials—Materials were obtained from the following sources: GDP[14C]mannose (254 mCi/mmol), Du Pont-New England Nuclear; 1-deoxymannojirimycin, Genzyme; Chelex 100 (100–200 mesh), Bio-Rad; Mes* and Man-2-Man-CH2, Sigma. Nonionic detergents were enzyme grade and essentially peroxide free. All other supplies and chemicals were of the highest grade available from commercial sources. All solutions and buffers were prepared with Chelex-treated water or were passed through Chelex resin to remove contaminating calcium ions. A homogeneous preparation of rabbit liver α1,2-mannosidase was previously described (12). In order to have sufficient enzyme to carry out all of the assays required for the present investigation, the enzyme fraction purified through Cibacron blue-g agarose was utilized for most of these studies. This fraction had a specific activity of 13 μmol of mannose released per h/mg of protein utilizing Man-2-Man-CH2 as the substrate, and corresponded to a specific activity of 2546

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*The abbreviations used are: Mes, 2-(N-morpholinio)ethanesulfonic acid; Man-2-Man-CH2, methyl-2-O-α-D-mannopyranosyl-α-D-mannopyranoside.
activity of 5200 units/mg of protein in an assay with a radio-labeled Man\textsubscript{GlcNAc} \textsubscript{2} substrate (12) having the structure \(\text{[\text{C}]Manal-2Manal-2Manal-3(Manol-G)Man@GlcNAc@G1cNAc}\) (5). There were no significant differences in the kinetic parameters, including calcium ion activation, when this enzyme preparation was compared to a homogeneous enzyme preparation with a specific activity 952 \(\mu\)mol of mannose released per h/mg protein.

Buffers---The following buffers were used in these studies: A, 20 mM Mes/tetramethylammonium hydroxide, pH 6.0; B, 0.1 M Tris/HCl, pH 7.6, containing 1.3 mM mercaptoethanol and 6.4 mM MgCl\(_2\).

We had previously reported (12) that different lot numbers of Mes buffer had varying effects on the apparent \(K_a\) for the divalent cation of the \(\alpha,\beta\)-mannosidase. These effects were only noted, however, when the activity of the enzyme was assayed with the use of the \(\alpha\)-labeled substrate at low concentrations and at low ionic strength (\(>50\) mM). The effects of Mes buffer on Ca\(^{2+}\) activation were not detected in any of the studies reported in the present investigation.

Assays for \(\alpha,\beta\)-Mannosidase---\(\alpha,\beta\)-Mannosidase activity was assayed in standard reaction mixtures in a total volume of 0.1 ml of buffer A, Man-2-Man-CH\(_3\); at the indicated concentrations, 20 \(\mu\)M CaCl\(_2\), 0.1 M KCl, and 4.9 \(\mu\)g of protein. Assays at calcium ion concentrations lower than 10 \(\mu\)M were carried out in reaction mixtures prepared with CaCO\(_3\), pH 6.0, and buffered with the use of 5 mM EDTA, pH 6.0. After 30 min at 37°C the reactions were quenched by pipetting appropriate aliquots of the reaction mixtures (in duplicate) into sufficient buffer B to bring the volumes to 1.92 ml. The amount of mannose liberated by the \(\alpha,\beta\)-mannosidase was determined by the addition of 0.08 ml of fluorescamine (0.3 mg/ml in dioxane) with rapid mixing. The fluorescence was measured in cuvettes with a 1-cm light path on a Gilford Fluoro IV with an excitation wavelength of 390 nm (Table I). A plot of \(l/v\) versus calcium ion concentration as described under "Experimental Procedures." The effect of calcium ion concentrations on \(\alpha,\beta\)-mannosidase activity was assayed as described under "Experimental Procedures" at the indicated Ca\(^{2+}\) concentrations: 0.13 \(\mu\)M (\(\triangleright\)), 0.22 \(\mu\)M (\(\blacksquare\)), 0.49 \(\mu\)M (\(\triangle\)), and 2.7 \(\mu\)M (\(\bigcirc\)). Data obtained at 20 \(\mu\)M Ca\(^{2+}\) is almost identical to that at 2.7 \(\mu\)M and is not shown for clarity. The inset shows a plot of \(K_a/V_{\text{max}}\) versus 1/\(Ca^{2+}\). Ca\(^{2+}\) concentrations below 10 \(\mu\)M were prepared with CaCO\(_3\), pH 6.0, and buffered with 5 mM EDTA, pH 6.0.

### RESULTS

#### Effect of Calcium Ions on \(\alpha,\beta\)-Mannosidase Activity---We have previously demonstrated that rabbit liver \(\alpha,\beta\)-mannosidase has an absolute requirement for calcium ions for activity (19, 22), but the activation constant and the mechanism of the activation were not established. With the exception of an enzyme from calf liver (9), the effects of Ca\(^{2+}\) on the activities of other mammalian \(\alpha,\beta\)-mannosidases have not been reported. \(\alpha,\beta\)-Mannosidases isolated from yeast (26) and from higher plants, however, have also been shown to be activated by Ca\(^{2+}\) (27, 28).

The activity of the rabbit liver \(\alpha,\beta\)-mannosidase was determined as a function of substrate concentration at varying calcium ion concentrations (Fig. 1). As previously shown, the \(\alpha,\beta\)-mannosidase has relatively low affinity for the saccharide substrate with a \(K_m\) of about 0.6 mM for Man-2-Man-CH\(_3\) at Ca\(^{2+}\) concentrations of 20 \(\mu\)M and higher. The concentration of divalent cation was found to have a significant effect on \(K_m\), but little or no effect on \(V_{\text{max}}\) (Table I). A homogeneous enzyme preparation gave essentially identical results indicating that other Ca\(^{2+}\)-binding proteins were not affecting the affinity of the enzyme for the divalent cation (Table I). A plot of \(K_m/V_{\text{max}}\) versus 1/Ca\(^{2+}\) (Fig. 1, inset) gave an apparent \(K_m\) for the divalent cation of 1.1 \(\mu\)M.

As shown in Fig. 1, the lines cross on the vertical axis which is a diagnostic pattern for equilibrium ordered reactions (29) in which the activator binds first, followed by substrate in an obligatory order according to Equation 1:

\[
1/v = K_{\text{cat}}/V_{\text{max}}(1 + K_{d}Ca^{2+}/1/S) + 1/V_{\text{max}}.
\]

where \(K_{d}\) is the dissociation constant for Ca\(^{2+}\). When the same data was plotted as a function of Ca\(^{2+}\) concentration (Fig. 2), the pattern intersected to the left of the vertical axis, whereas, a replot of the slopes as a function of 1/S (Fig. 2, Table I).
inset) goes through the origin. This pattern is also a characteristic of equilibrium ordered reactions showing that $K_a$ was immeasurably small compared to $K_m$ and indicating that the rate of dissociation of Ca$^{2+}$ is much faster than the rate of the reaction (29). This mechanism results from the addition of an activator, followed by substrate in obligatory order, with the step for addition of the activator being at equilibrium. The activator need not dissociate during each catalytic cycle, but it is essential that it cannot be free to dissociate once the substrate has been added to the enzyme.

**Effect of pH on Enzyme Activity**—The pH optimum of the rabbit liver $\alpha_1,2$-mannosidase was previously reported to be between 5.0 and 6.0 (12), although the assays employed in those studies utilized a radiolabeled substrate at low concentrations and therefore did not measure $V_{\text{max}}$. In the present investigation, both $K_m$ and $V_{\text{max}}$ were assessed as a function of pH (Fig. 3). $V_{\text{max}}$ was found to be optimal in the range pH 5.0–6.0, and $K_m$ values were at a minimum. At values above or below the pH optima, $V_{\text{max}}$ decreased and an increase in $K_m$ was noted.

**Inhibition by Divalent Cations**—A number of divalent cations were found to be potent inhibitors of the mannosidase including Co$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ (Fig. 4). When plotted as a function of substrate concentration, the divalent cations appeared to be competitive inhibitors with respect to substrate having apparent $K_i$ values of 0.21, 0.63, and 0.25 mM, respectively. Based on the proposed equilibrium ordered enzyme mechanism, however, displacement of Ca$^{2+}$ at the divalent cation-binding site would also have an effect on the apparent $K_m$ for substrate with no significant effect on $V_{\text{max}}$. Competition between Ca$^{2+}$ and Zn$^{2+}$ at the cation-binding site was demonstrated by experiments showing that the inhibition by Zn$^{2+}$ could be reversed by increasing calcium ion concentrations. By comparison, 1 mM Mg$^{2+}$ had no effect on enzyme activity in regards to either $K_m$ or $V_{\text{max}}$ (Fig. 4).

**Inhibition by 1-Deoxymannojirimycin**—1-Deoxymannojirimycin is an analogue of mannose and is routinely used as an inhibitor of the $\alpha_1,2$-mannosidase in vivo in studies designed to investigate the role of glycoprotein processing on a number of different metabolic processes (30). 1-Deoxymannojirimycin has previously been reported to be a noncompetitive inhibitor of Golgi $\alpha_1,2$-mannosidase I from rat liver, with an apparent $K_i$ of 2.7 mM (Fig. 5). Inhibition of deoxymannojirimycin could not be reversed with increasing Ca$^{2+}$ concentrations demonstrating that 1-deoxymannojirimycin was not competitive with the divalent cation.

**Inhibition by Tris**—Tris has been reported to inhibit the activity of a rat liver $\alpha_1,2$-mannosidase in vitro (4), but the mechanism of the inhibition was not determined. It was recently reported that 20 mM Tris also inhibited glycoprotein processing reactions in vivo, including the activity of the $\alpha_1,2$-mannosidase, (32). The results suggested that Tris might also be a useful inhibitor for studies of the metabolic role of glycoprotein processing. In this study, Tris was found to be an inhibitor of the rabbit liver $\alpha_1,2$-mannosidase, competitive with Man-2-Man-CH₂ having a $K_i$ of 7 µM (Fig. 5). Inhibition of deoxymannojirimycin could not be reversed with increasing Ca$^{2+}$ concentrations demonstrating that 1-deoxymannojirimycin was not competitive with the divalent cation.

**DISCUSSION**

The rabbit liver $\alpha_1,2$-mannosidase is capable of removing $\alpha_1,2$-linked mannosyl units found in high-mannose oligosaccharide structures, but little information has been available about the kinetic properties of the enzyme since suitable assays have not been utilized. Previous studies on the rabbit...
liver and other α1,2-mannosidases have, for the most part, utilized radiolabeled high-mannose oligosaccharides at suboptimal substrate concentrations. In addition, the substrates also contained multiple terminal nonreducing α1,2-mannosyl residues that could be removed by the enzyme. It was therefore difficult to assess kinetics for the rates of removal of each of the individual mannose units.

In this investigation we have demonstrated that calcium ion activation of the rabbit liver α1,2-mannosidase is an ordered equilibrium mechanism in which the activator must be added prior to the substrate and cannot be released during the reaction. Since the apparent $K_a$ of 1.1 μM for Ca$^{2+}$ approximates the physiological concentration range for this cation, it is conceivable that calcium ion concentrations could modulate the activity of the α1,2-mannosidase in vivo. The mannosidase was inhibited by several divalent cations including Co$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$. Zinc ions were competitive with Ca$^{2+}$ and it is likely that the other two divalent ions were also competitive with Ca$^{2+}$. Magnesium ions, however, had no effect on enzyme activity in the concentration ranges tested. The enzyme was also inhibited by 1-deoxymannojirimycin and by Tris. Both inhibitors were competitive with the substrate having $K_a$ values of 7 μM, and 2.7 μM, respectively. A previous report (31) that 1-deoxymannojirimycin was a non-competitive inhibitor for a rat liver Golgi α1,2-mannosidase employed a different substrate, possibly accounting for the difference in the mechanism.

The rabbit liver α1,2-mannosidase has a relatively low affinity for the nonphysiological substrate methyl-2-O-α-D-mannopyranosyl-α-D-mannopyranoside with a $K_m$ of 0.6 mM (16). Although one might expect that the α-mannosidase would have higher affinity for oligosaccharide structures found in the normal processing pathway, we have previously shown that the $K_m$ for glycopeptide GP-IV, isolated from ovalbumin, was 0.55 mM (12). In the same study, native thyroglobulin was found to have an apparent $K_m$ of 20 μM, but this glycoprotein contains 5 or 6 high-mannose oligosaccharides. Since each high-mannose chain has 3 terminal nonreducing α1,2-linked mannose units, the concentration of potential substrates for the α-mannosidase at the apparent $K_m$ for thyroglobulin approximated 0.36 mM. These constants were all obtained, however, with a solubilized and purified preparation of the mannosidase. Since the α1,2-mannosidase is a membrane enzyme, it is possible that in vivo the enzyme has higher apparent affinity for the glycoprotein substrates, many of which are also membrane bound. The activity of the mannosidase could then also be regulated through differential physical interactions with substrates adjacent to the enzyme on the two-dimensional surface of the membrane.

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