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THE *SACCHAROMYCES CEREVISIAE* PROCESSING α1,2-MANNOSIDASE IS AN INVERTING GLYCOSIDASE

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The α1,2-mannosidase from *Saccharomyces cerevisiae*, which removes one specific α1,2-linked mannose residue from $Man_9GlcNAc_2$, is a member of the Class 1 α1,2-mannosidase family conserved from yeast to mammals. Although Class 1 α1,2-mannosidases are essential for the maturation of N-linked oligosaccharides in mammalian cells, nothing is known about their mechanism of action. The availability of sufficient quantities of recombinant yeast α1,2-mannosidase and its homology with the mammalian enzymes make it a good model to study the catalytic mechanism of this family of α1,2-mannosidases. The stereochemical course of hydrolysis of $Man_9GlcNAc$ by the yeast enzyme was followed by proton nuclear magnetic resonance spectroscopy. It was observed that β-D-mannose is released from the oligosaccharide substrate, thereby demonstrating that the enzyme is of the inverting type.

α1,2-Mannosidases are essential for the maturation of asparagine-linked oligosaccharides in mammalian cells. After trimming of $Glc_3Man_9GlcNAc_2$ to $Man_9GlcNAc_2$ by glucosidases, α1,2-mannosidases remove up to four mannose residues to form $Man_5GlcNAc_2$, which is then modified into complex oligosaccharide structures by the concerted action of α-mannosidase II and glycosyltransferases in the Golgi (1). Complex carbohydrates have been implicated in a variety of diseases (2), including cancer and viral infections. As a result, there has been a great deal of interest in the development of N-linked oligosaccharide processing enzyme inhibitors that may have therapeutic potential particularly as antitumor and antiviral agents (3). Several processing α1,2-mannosidases exist in the ER and Golgi of mammalian cells with slightly different properties and different sensitivities to inhibitors (for review, see 1). Inhibition of α1,2-mannosidase activity with 1-deoxymannojirimycin or with kifunensine completely prevents complex oligosaccharide formation with the accumulation of $Man_{8-9}GlcNAc_2$ (for review, see 1 and 4). Such inhibition affects important cellular recognition processes, such as angiogenesis (5), neutrophil adhesion to endothelial cells (6), and natural killer cell targeting (7). In addition,

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ABBREVIATIONS: GlcNAc, N-Acetylglucosamine; ER, endoplasmic reticulum.

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preventing mannose trimming can interfere with ER degradation of abnormal glycoproteins (8), suggesting that α1,2-mannosidases may be involved in quality control of newly synthesized glycoproteins. In view of the importance of these enzymes, a knowledge of the mechanism of action and active site topology of α1,2-mannosidases is essential for the development of more potent and specific inhibitors. Although there is a great deal of work on the mechanism of action of a wide variety of glycosidases (for review, see 9), there are no studies on the catalytic mechanism of any α1,2-mannosidases, because until now none of these enzymes were available in sufficient quantities.

Recently, amino acid sequence homology was observed between a series of α1,2-mannosidases cloned from yeast and mammals (1). These Class 1 α1,2-mannosidases are type II membrane proteins of 63-73 kDa with a large C-terminal catalytic region and an N-terminal transmembrane domain. All the enzymes in this class are inhibited by 1-deoxymannojirimycin, require Ca^{+2} for activity, and do not hydrolyze p-nitrophenyl-α-D-mannopyranoside. We have recently developed a system to produce and purify milligram quantities of the catalytic domain of the *Saccharomyces cerevisiae* Class 1 α1,2-mannosidase (10, 11) and showed that it has the same properties as the purified endogenous enzyme and removes one specific α1,2-linked mannose from $Man_9GlcNAc$ (Scheme 1) (12). The sequence homology between the yeast and mammalian catalytic domains makes the recombinant yeast enzyme a good model to study the mechanism of action of Class 1 α1,2-mannosidases. It is generally believed that glycosidases exhibit two distinct types of hydrolytic mechanisms. In one set, direct displacement at the anomeric center by a nucleophilic water molecule leads to inversion of the anomeric configuration ("inverting enzyme"). In the other set, there is net retention of the anomeric configuration as a glycosyl-enzyme intermediate is displaced by the nucleophilic water ("retaining enzyme") (13). The present work reports the stereochemical course of hydrolysis of $Man_9GlcNAc$ by the recombinant yeast α1,2-mannosidase using ^1H NMR spectroscopy (9, 14, 15).

EXPERIMENTAL PROCEDURES

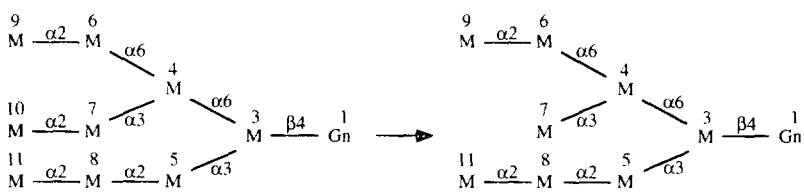
Recombinant yeast α1,2-mannosidase was produced as a secreted soluble form without its transmembrane domain using a *Saccharomyces cerevisiae* expression system and then initially purified by chromatography on S-Sepharose as previously described (12). In order to further purify the recombinant enzyme, it was dialyzed in 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM sodium azide, loaded on a Q-Sepharose column, and eluted with a 0 to 0.2 M sodium chloride gradient. The enzyme eluting at about 0.1 M sodium chloride was dialyzed against 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM sodium azide, and then concentrated to approximately 1.4 mg/ml using polysulfone centrifugal ultrafilters with a molecular weight cut-off of 30 000 (Millipore). Enzyme concentration was determined spectrophotometrically (16). A volume of 0.2 ml of enzyme solution was lyophilized and stored in a vacuum dessicator containing P_2O_5. The substrate, $Man_9GlcNAc$, was prepared from soybean agglutinin as described previously (17). Two mg of the oligosaccharide were lyophilized five times from D_2O and then stored in a vacuum dessicator containing P_2O_5. Sodium phosphate buffer (10 mM), pH 6.8, containing 1 mM sodium azide was also lyophilized twice from D_2O.

Just prior to the NMR experiment the dried buffer was redissolved in D_2O, and 0.6 ml of 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM sodium azide in D_2O was added to 2 mg of dried $Man_9GlcNAc$ (2mM, final concentration). Proton NMR spectra were recorded on a 500 MHz ^1H NMR spectrometer (Varian) using 5 mm sample tubes at 25°C. An initial spectrum was recorded, and 5 μL (7.5 μg) of α1,2-mannosidase, redissolved in D_2O, were added to start the reaction. The stereochemical course of hydrolysis was monitored by collecting spectra every two minutes for 96 minutes with a final spectrum at 134 min.

RESULTS AND DISCUSSION

^{1}H NMR spectroscopy is a convenient method to follow the stereochemical course of glycosidase reactions provided that sufficient substrate and enzyme are available (15). The time course for the hydrolysis of $Man_9GlcNAc$ to $Man_8GlcNAc$ (Scheme 1) by the recombinant yeast α1,2-mannosidase is shown in Figures 1 and 2. The signals corresponding to the anomeric protons of the mannose residues and GlcNAc residue in the substrate and product are shown in the spectra of Figure 1. These signals have been characterized previously (18, 19). Figure 1A depicts the spectrum of the substrate before addition of the α1,2-mannosidase. Six minutes after addition of the enzyme, the appearance of the hemiacetal anomeric proton of β-D-mannose at δ 4.89 is observed (Figure 1B). At this early time there is no signal at δ 5.17 representing the anomeric proton of α-D-mannose, clearly demonstrating that a β-D-mannose is released from the substrate. Concomitant appearance of a doublet representing the anomeric proton of GlcNAc in $Man_8GlcNAc$ is observed at δ 5.25, J = 3.4 Hz. After 16 minutes (Figure 1C), the reaction has proceeded to approximately 40 % completion as judged by the size of the GlcNAc doublets of $Man_9GlcNAc$ and $Man_8GlcNAc$ at δ 5.23 and δ 5.25, respectively. A new resonance at δ 5.17 from α-D-mannose formed by mutarotation of the β-anomer begins to appear. Fifty-six minutes after the addition of enzyme (Figure 1E), all the starting oligosaccharide has been converted to product. After 134 min (Figure 1F) the equilibrium anomeric ratio of D-mannose has been established. Figure 2A illustrates the time course of disappearance of the doublet representing the anomeric proton of GlcNAc in $Man_9GlcNAc$ and concomitant appearance of the GlcNAc doublet of $Man_8GlcNAc$. In Figure 2B one first sees the appearance of the signal corresponding to the anomeric proton of β-D-mannose which augments in intensity until the substrate has disappeared completely. This signal then begins to decrease in intensity until the equilibrium anomeric ratio is established through mutarotation. Finally, Figure 2C shows the progressive appearance of the anomeric proton of α-D-mannose through mutarotation of the β-isomer which augments in intensity until equilibrium is reached. These results demonstrate that the yeast Class 1 α1,2-mannosidase catalyzes hydrolysis with net inversion of the α-anomeric configuration.

Accumulated data on various inverting glycosidases, such as soybean β-amylase (20), glucoamylase from *Aspergillus awamori* (21), and endocellulase E2 from *Thermomonospora fusca* (22), whose crystal structures have been determined, suggest that they contain two carboxylic acid functional groups which catalyze the hydrolysis reaction. One of these acts as a



$Man_9GlcNAc$ $Man_8GlcNAc$

Scheme 1

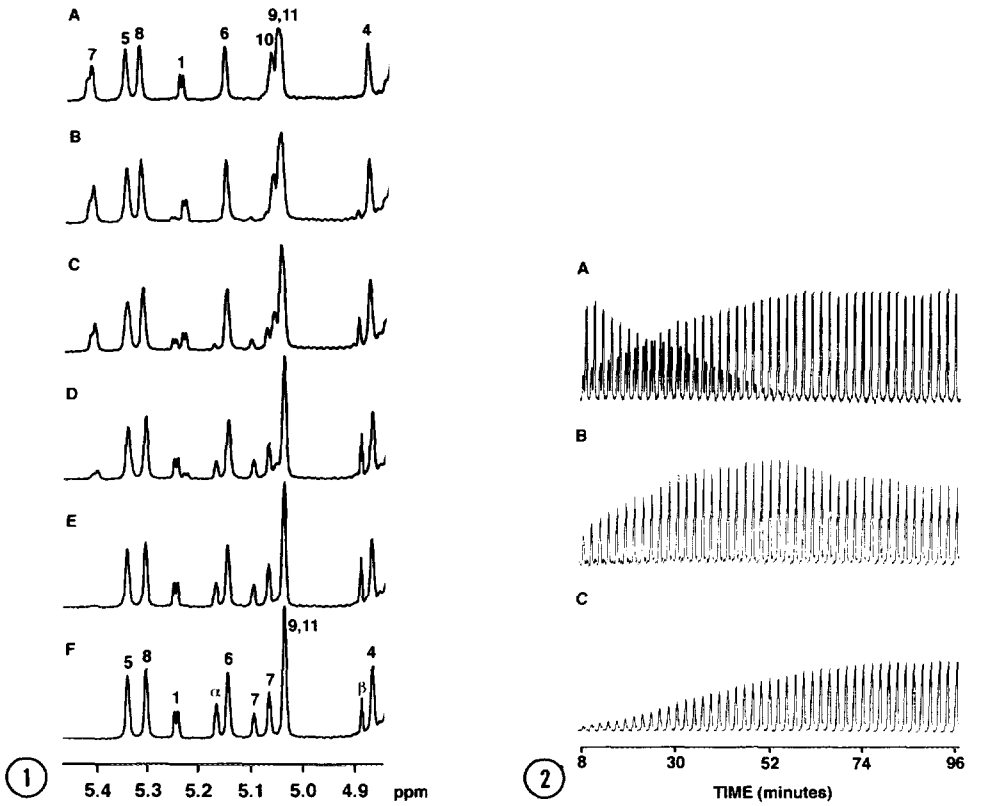


Figure 1. Partial ^{1}H NMR spectra of the anomeric region taken at different times of incubation of $Man_9GlcNAc$ with the yeast α1,2-mannosidase. ^{1}H NMR spectra were taken according to Experimental Procedures and correspond to: (A) $Man_9GlcNAc$ (2 mM) before the additon of α1,2-mannosidase and at different times after mixing; (B) 6 min, (C) 16 min, (D) 42 min, (E) 56 min,(F) 134 min (equilibrium). The numbers above each signal in spectra A and F correspond to the anomeric proton of the sugar residues indicated in Scheme 1. The α and β in spectrum F indicate the signal for the anomeric proton of α and β-D-mannose, respectively.

Figure 2. Time course of α1,2-mannosidase action on $Man_9GlcNAc$. Individual signals from the spectra of Figure 1 were selected and plotted with time to show: (A) disappearance of the doublet at δ 5.23 representing $Man_9GlcNAc$ with concomitant appearance of the doublet at δ 5.25 representing $Man_8GlcNAc$, (B) appearance of the signal at δ 4.89 representing β-D-mannose, and (C) appearance of the signal at δ 5.17 representing α-D-mannose.

general base catalyst to abstract the proton from an incoming water molecule, while the other acts as a general acid catalyst and donates a proton to the leaving group (13). There are several aspartic and glutamic acid residues highly conserved between the yeast and mammalian α1,2-mannosidases which could participate in this type of catalysis, but additional work with active site directed irreversible inhibitors will be required to identify the specific residues.

By comparing the stereoselectivity of β-1,4-glucanases and β-1,4-xylanases with similar amino acid sequences, Gebler *et al.* (15) found that members of the same enzyme family have the same stereoselectivity most likely due to similar folding patterns, active site topologies, and catalytic mechanisms. It is highly probable that the mammalian Class 1 α1,2-mannosidases which share significant amino acid similarity with the yeast enzyme are also inverting glycosidases.

This is the first report on the catalytic mechanism of any α-mannosidase. Most importantly, this information will aid in the design of more potent and specific inhibitors of Class 1 α1,2-mannosidases.

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