Calcium Binding to the Class I α-1,2-Mannosidase from *Saccharomyces cerevisiae* Occurs Outside the EF Hand Motif†

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ABSTRACT: Class I α-1,2-mannosidases are a family of Ca^{2+}-dependent enzymes that have been conserved through eukaryotic evolution. These enzymes contain a conserved putative EF hand Ca^{2+}-binding motif and nine invariant acidic residues. The catalytic domain of the α-1,2-mannosidase from *Saccharomyces cerevisiae* was expressed in *Pichia pastoris* and was shown by atomic absorption and equilibrium dialysis to bind one Ca^{2+} ion with high affinity ($K_D = 4 \times 10^{-7}$ M). Ca^{2+} protected the enzyme from thermal denaturation. Mutation of the 1st and 12th residues of the putative EF hand Ca^{2+} binding loop (D121N, D121A, E132Q, E132V, and D121A/E132V) had no effect on Ca^{2+} binding, demonstrating that the EF hand motif is not the site of Ca^{2+} binding. In contrast, three invariant acidic residue mutants (D275N, E279Q, and E438Q) lost the ability to bind $^{45}Ca^{2+}$ following nondenaturing polyacrylamide gel electrophoresis whereas D86N, E132Q, E503Q, and E526Q mutants exhibited binding of $^{45}Ca^{2+}$ similar to the wild-type enzyme. The wild-type enzyme had a K_m and k_{cat} of 0.5 mM and 12 s^{-1}, respectively. The K_m of E526Q was greatly increased to 4 mM with a small reduction in k_{cat} to 5 s^{-1} whereas the k_{cat} values of D86N and E132Q(V) were greatly reduced (0.005−0.007 s^{-1}) with a decrease in K_m (0.07−0.3 mM). The E503Q mutant is completely inactive. Asp^{275}, Glu^{279}, and Glu^{438} are therefore required for Ca^{2+} binding whereas Asp^{86}, Glu^{132}, and Glu^{503} are required for catalysis.

The *Saccharomyces cerevisiae* Ca^{2+}-dependent α-1,2-mannosidase which removes one mannose residue from $Man_9GlcNAc_2$ to form one isomer of $Man_8GlcNAc_2$ (*1*−*3*) is a member of Class I α-1,2-mannosidases involved in the processing of asparagine-linked oligosaccharides in the ER[1] and Golgi apparatus (*4*). The Class I α-1,2-mannosidases have been conserved through eukaryotic evolution and have been classified as Family 47 glycosidases (*5*). In addition to the yeast processing enzyme (*6*), this family includes fungal (*7*, *8*), insect (*9*, *10*), and mammalian (*11*−*15*) α-1,2-mannosidases. Besides their sequence similarity, the Class I α-1,2-mannosidases have similar biochemical properties. They specifically cleave α-1,2-linked mannose residues but cannot hydrolyze aryl α-D-mannopyranosides. They are inhibited by pyranose analogues such as 1-deoxymannojirimycin and kifunensine and are usually Ca^{2+}-dependent (for reviews, see refs *4*, *16*, and *17*). The α-1,2-mannosidases are distinct from the Class II (Family 38) α-mannosidases, which include Golgi α-mannosidase II and lysosomal α-mannosidases. The Class II α-mannosidases have different biochemical properties and catalytic mechanisms. They are Ca^{2+}-independent retaining glycosidases (*18*) capable of cleaving α-1,2-, α-1,3-, and α-1,6-linked mannose residues as well as aryl α-D-mannopyranosides and are inhibited by furanose analogues such as swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol.

Although several Class I α-1,2-mannosidases have been cloned and their enzymatic properties have been studied (*12*, *15*, *19*), their three-dimensional structure has not yet been determined, and little is known about their catalytic mechanism. Structure−function studies of this group of enzymes are important because Class I α-1,2-mannosidases are essential for the maturation of N-linked oligosaccharides to complex and hybrid structures that participate in a wide variety of biological processes in mammalian cells (*20*, *21*). In particular, there has been considerable interest in the development of inhibitors of processing glycosidases that have potential as antimetastatic and antiviral agents (*22*, *23*).

Since the development of expression systems that produce milligram quantities of the yeast α-1,2-mannosidase catalytic domain with properties similar to those of the endogenous enzyme (*24*, *25*), the yeast enzyme has served as a model to determine the structure and mechanism of action of Class I α-1,2-mannosidases. It was first shown that the yeast enzyme catalyzes removal of mannose with inversion of the anomeric configuration (*26*), an observation that was recently substantiated for a mammalian member of this enzyme family (*19*). Detailed chemical and mutagenesis studies of the role of cysteine residues (*25*) demonstrated that there are two disulfide bonds in the yeast α-1,2-mannosidase and that one of these disulfide bonds, between cysteine residues that are

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[1] Abbreviations: BMMY, buffered methanol complex; BSA, bovine serum albumin; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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conserved in all Class I α-1,2-mannosidases, is essential for enzyme activity.

The Class I α-1,2-mannosidases contain a region that is similar to the EF hand Ca^{2+}-binding motifs found in many different Ca^{2+}-dependent proteins (*27*). An EF hand motif consists of a 12-residue loop involved in Ca^{2+} binding flanked by α helices. The loop region is the most conserved part of the motif and corresponds to amino acids Asp^{121} to Glu^{132} in the yeast α-1,2-mannosidase. The Ca^{2+} dependence of the Class I α-1,2-mannosidases has previously been attributed to this putative Ca^{2+}-binding EF hand motif, but there is no experimental evidence demonstrating Ca^{2+} binding to this site.

Little is known regarding the catalytic mechanisms of processing glycosidases involved in N-glycan biosynthesis. Studies on the catalytic mechanisms of other glycosidases have shown that two acidic residues are usually involved in catalysis (for reviews, see refs *28*−*32*). For inverting glycosidases, one of the acidic amino acids acts as a base catalyst abstracting a proton from the incoming water molecule, and the other acts as an acid catalyst donating a proton to the leaving group. For retaining enzymes, one of the acidic residues acts as a nucleophile and the other as an acid/base catalyst. The two catalytic residues are usually conserved within each family of glycosidases, but the catalytic residues have not yet been identified in Class I α-1,2-mannosidases.

In the current work, it is demonstrated that the yeast α-1,2-mannosidase binds 1 Ca^{2+} ion/mol with high affinity and that the putative EF hand motif is not the Ca^{2+}-binding site. It is shown by site-directed mutagenesis that all of the invariant acidic residues are required for normal enzyme activity. Three of these residues (Asp^{275}, Glu^{279}, and Glu^{438}) are important for Ca^{2+} binding whereas three others (Asp^{86}, Glu^{132}, and Glu^{503}) are required for catalysis and include potential catalytic residues.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized at the Sheldon Biotechnology Centre (McGill University, Montréal, Canada). Restriction enzymes were from either Pharmacia Biotech, Inc., New England Biolabs, Gibco BRL, or Boehringer Mannheim. Trypsin inhibitor, bovine albumin (Fraction V), and bovine brain calmodulin (>98%) (phosphodiesterase 3′:5′-cyclic nucleotide activator) were from Sigma. Ovalbumin, as well as SP-Sepharose Fast Flow and Q-Sepharose Fast Flow ion exchangers, was from Pharmacia Biotech, Inc. [^{3}H]-labeled and unlabeled $Man_9GlcNAc$ oligosaccharide substrates were prepared as previously described (*24*). $^{45}CaCl_2$ (5−30 Ci/g) was purchased from ICN Biomedicals, Inc. Coomassie Brilliant Blue (R-250) and Chelex 100 resin (biotechnology grade 100−200 mesh) were from Bio-Rad Laboratories. See Blue prestained protein standard was from Novex. EGTA (≥99%) was from Fluka. Washed dialysis tubing with a molecular weight exclusion limit of 12 000−14 000 Da was from Gibco BRL. All solutions were prepared using water from a Milli-Q system with an Organex-Q cartridge. A 0.1 M (±0.5%) $CaCl_2$ standard solution was purchased from Fluka to prepare Ca^{2+}-containing buffers. Densitometry was performed using the Bio Image system from Millipore. All other chemicals were reagent grade.

Site-Directed Mutagenesis. Escherichia coli DH5α was used as the host for plasmid manipulations. Mutants for *S. cerevisiae* expression were prepared in the plasmid, pYH4, containing the *MNS1* gene in the YEp352 vector (*6*). Site-directed mutagenesis was performed using the U.S.E. mutagenesis kit from Pharmacia Biotech Inc. The selection primer, GAAAATACCGCATCAGCCGGCATTCGCCATTC, was used to convert a unique *Nar*I restriction site in the pYH4 plasmid to an *Nae*I restriction site. The following oligonucleotides were used as mutagenic primers: D86N, GGCTGGATTATCGTTAACTCAGTGGATACC; E132Q, GATGCCGAAGTTAACGTTTTTCAAACTACTATTAGAATGC; E214Q, GAATTCACTACGCTGCAGATGCAATTCAAATATCTGG; D275N, CCGGTTCGGATCTAGAGGTAATTCTTTTTATGAG; E279Q, CCGGTTCGGATCTAGAGGTGATTCTTTTTATCAGTATTTACTAAAAC; E435Q, CAACCTACAAAGGCCTCAAACGGTGGAATCG; E438Q, CCAGAAACGGTGCAGTCGATTATGTTCATG; E503Q, GGAAAGTTTCTGGCTAGCACAGACTTTAAAG; and E526Q, GTTGTTTTCAACACACAAGCTCATCCTTTTCC. The open reading frames of the mutants were sequenced by the dideoxy method (*33*) using either the T7 sequencing kit from Pharmacia Biotech, Inc., or the facilities at the Sheldon Biotechnology Centre with automated sequencing with the ALF express sequencer from Pharmacia Biotech, Inc., or the Applied Biosystems model 373A sequencer from Perkin-Elmer.

Mutants for expression in *P. pastoris* were prepared using the plasmid YpHMNS1, which contains the catalytic domain of the yeast α-1,2-mannosidase, beginning at amino acid 23, in the pHIL-S1 vector (Invitrogen) (*25*). The mutants D86N, E132Q, and E214Q were constructed by replacing an *Nco*I restriction fragment in YpHMNS1 by the equivalent fragment from the mutated genes in pYH4 (see above), using standard procedures. For the E435Q, E438Q, and E526Q mutants, a *Pfl*MI fragment was replaced in YpHMNS1. The D121N, D121A, E132V, D121A/E132V, D275N, E279Q, and E503Q mutants were constructed by the U.S.E. mutagenesis kit with the following oligonucleotides: D121N, GGATAAACGATGTTTTAAATTTTGATATTGATGCCG; D121A, GGATAAACGATGTGCTAGCTTTTGATATTGATGCCG; E132V, GATGCCGAAGTTAACGTTTTTGTAACTACTATTAGAATGC; and D121A/E132V, CGATGTTTTGGCTTTTGATATTGATGCCGAAGTTAACGTTTTTGTAACTACTATTAG. Oligonucleotides for D275N, E279Q, and E503Q were the same as those above. The mutated regions were sequenced as above.

Expression of Mutant α-1,2-Mannosidases in S. cerevisiae. An *S. cerevisiae* strain disrupted in both the vacuolar and endoplasmic reticulum α-mannosidases [strain 9.16a (*his4-619 mns1*::*URA3 ams1*::*LEU2*)] (*6*) was used to select *ura3* yeast cells by plating on a medium containing 5-fluoro-orotic acid and uracil, as described by Boeke et al. (*34*). The resulting YFL9.26 strain was transformed with the pYH4 plasmid, the YEp352 vector, or the YEp352 vector containing mutant *MNS1* genes by the lithium acetate method (*35*). Freshly transformed cells were grown overnight at 30 °C in 5 mL of a supplemented minimal medium without uracil, then 15 mL of YPD was added, and the cultures were grown to an A_{600} of 2−3. The cells were collected by centrifugation and resuspended in 200 μL of the following buffer: 10 mM PIPES (pH 6.5) containing 5 mM $CaCl_2$, 1 mM NaN_3, 2

μM leupeptin, 2 μM pepstatin, and 400 μM PMSF. The cells were broken by vortexing six times (30 s each) at 4 °C with 0.4 mL of glass beads. Supernatants were collected after a 5 min centrifugation at 2000*g*.

Production and Purification of α-1,2-Mannosidase Expressed in P. pastoris. The α-1,2-mannosidase catalytic domain, beginning at amino acid 23, from wild-type or mutants was expressed as a secreted protein in the *P. pastoris* strain KM71 (*his4*, *aox1*) obtained from Invitrogen. Transformation of the cells and growth conditions were described previously (*25*). The culture medium was concentrated using either a Diaflo Hollow Fiber Cartridge (Cartridge type H1P30-43, Amicon) or centrifugal filters with a nominal molecular weight limit of 30 000 (Millipore). In some cases, the wild-type and mutant α-1,2-mannosidases were purified essentially as described previously (*24*, *26*). The culture medium (0.4−4.2 L) was concentrated and then diafiltered with 10 mM succinate (pH 5.5) to obtain a final pH of 5.5. The solution then was loaded onto an SP-Sepharose column (8−20 mL) and eluted with a NaCl gradient (0−0.5 M). Fractions were collected into tubes containing a volume (about 5%) of 1.0 M potassium phosphate (pH 6.8) to attain the optimal pH of 6.8. The α-1,2-mannosidase was eluted between 0.1 and 0.3 M NaCl. The level of purity was assessed by Coomassie staining following SDS-PAGE. If further purification was necessary, the pooled fractions obtained from the SP-Sepharose column were dialyzed in 10 mM sodium phosphate (pH 6.8) and then loaded onto a Q-Sepharose column (1−4 mL). The α-1,2-mannosidase was eluted with a NaCl gradient (0−0.4 M) between 0.1 and 0.2 M NaCl. All of the purification procedures were performed at 4 °C with buffers containing 1 mM NaN_3.

Atomic Absorption Spectrophotometry. Purified recombinant α-1,2-mannosidase (1.1 mL, 26 μM) was dialyzed for 16 h at 4 °C in 10 mM PIPES (pH 6.8). Then, 0.5 mL of the dialyzed enzyme was added to 0.5 mL of 2 mM EDTA containing 100 mM KCl, to obtain a final pH of 8.0. The enzyme solution was then analyzed for Ca^{2+} content on an atomic absorption spectrophotometer from Thermo Jarrell Ash (model Smith-Hieftje 11) at the Chemical Engineering Department (McGill University, Montréal, Canada). The Ca^{2+} standards (0−40 μM Ca^{2+}) were prepared under conditions identical to those of the enzyme sample. The determinations were done on duplicate samples.

Circular Dichroism. Circular dichroism experiments were performed at Concordia University (Montréal, Canada) using a Jasco J-710 spectropolarimeter. Samples (200 μL) contained 2 μM purified recombinant α-1,2-mannosidase in 8 mM PIPES (pH 6.8) and 10 mM EGTA with or without 10 mM Ca^{2+}. The protein solution was subjected to a temperature increase from 25 to 80 °C at 1 °C/min in a temperature-controlled cell. The CD spectrum (200−350 nm) was measured before and after the temperature gradient.

Equilibrium Dialysis. Equilibrium dialysis in $^{45}Ca^{2+}$-containing buffers was performed essentially as described previously by Potter et al. (*36*). All plasticware was washed with 10 mM EDTA and rinsed with deionized water. Samples (60−100 μL) of purified mutant and wild-type α-1,2-mannosidase (20−70 μM) were dialyzed in 9 mm dialysis capsules (InstruMed Inc.) against 0.6−1.0 L of buffer for 6−18 h at 30 °C. Depending on the experiment, one of the three following Chelex-treated (10 g/L) buffers was used for dialysis: (A) 10 mM PIPES (pH 6.8) containing 0.1 M KCl, (B) 0.1 M PIPES (pH 6.8), or (C) 0.1 M PIPES (pH 6.8) containing 0.2 mM EGTA. $^{45}Ca^{2+}$ and 0.1 M $CaCl_2$ were added to the buffers to obtain about 100 dpm/μL of $^{45}Ca^{2+}$ and 10−200 μM Ca^{2+}. Aliquots (10 μL) of the dialyzed solutions were added to 4 mL of scintillation fluid, and the radioactivity was measured in an LKB 1218 Rackbeta liquid scintillation counter. Aliquots (10 μL) were used to determine the concentration of α-1,2-mannosidase by A_{280} using an extinction coefficient of 115 200 M^{-1} cm^{-1}, as calculated according to Mach et al. (*37*). Duplicate samples were used for all measurements. The concentration of free Ca^{2+} in the buffers containing EGTA was determined using the MaxChelator program (*38*).

$^{45}Ca^{2+}$ Binding in Native Polyacrylamide Gels. $^{45}Ca^{2+}$ binding to α-1,2-mannosidase following electrophoresis in native PAGE gels was assayed essentially as previously described by Schibeci et al. (*39*). Duplicate gels were electrophoresed. One gel was soaked in 10 mM PIPES (pH 6.5) containing 100 mM KCl, 1 mM $MgCl_2$, 2% glycerol (v/v), and $^{45}Ca^{2+}$ (1 μCi/ml, 5 μM Ca^{2+}) for 30 min with gentle rotation. The gel was then washed for 5 min in the same buffer without $^{45}Ca^{2+}$. After three more washes (5 min each) in deionized water, the gel was dried and exposed to either a film (Kodak) or an imaging plate (Fuji). The volume of incubation or wash solutions was 7 times the volume of the gel. The other gel was treated in the same way, except no $^{45}Ca^{2+}$ was added and the gel was stained with Coomassie. The α-1,2-mannosidase bands in the Coomassie gel and the bands from the imaging plate were quantified by densitometry. The values were used to quantitate $^{45}Ca^{2+}$ binding to the proteins (autoradiogram densitometry unit/Coomassie densitometry unit).

Protein Analysis. The protein content of cellular extracts was determined by a modified Lowry method (*40*) with bovine serum albumin as the standard. The concentration of purified recombinant α-1,2-mannosidase was determined by absorbance at 280 nm. The amount of protein on gels was determined by densitometry following staining with Coomassie, using purified recombinant α-1,2-mannosidase as the standard. SDS-PAGE under reducing conditions was carried out according to Laemmli (*41*) using the Bio-Rad Mini-Protean II apparatus. Native PAGE gels (0.75 mm thickness) were prepared similarly to the SDS-PAGE gels with a 3% stacking gel and a 10% separating gel, except SDS and reducing agent were not added to the gels, samples, or the running buffer. For Western blotting, the proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell) and visualized by the ECL Western blotting detection system (Amersham) with affinity-purified polyclonal antibodies raised against recombinant yeast α-1,2-mannosidase (*42*). The antibodies were also pretreated with a cell extract from the *S. cerevisiae* strain YFL9.26 in order to reduce background.

α-1,2-Mannosidase Assays. [^{3}H]mannose released from [^{3}H]Man_9GlcNAc was quantitated using the Concanavalin A method (*43*). Duplicate samples agreed within 10%. Depending on the experiment, one of the three following α-1,2-mannosidase assay protocols was used. (A) For cellular extracts, the assay mixtures contained 25−700 μg of protein in 10 mM PIPES (pH 6.5) containing 5 mM $CaCl_2$, 1 mM

NaN_3, 1 mg/mL BSA, 0.5% Triton X-100 (w/v), and 34 800 dpm of [^{3}H]$Man_9GlcNAc$ in a total volume of 40 μL. The incubation times at 37 °C were as follows: pYH4 (15−30 min), D86N (4−15 h), E132Q (3−7 h), E438Q (15 h), and E526Q (30−180 min). In each case, the enzyme activity was linear with time of incubation. The activity in cells transformed with wild-type α-1,2-mannosidase (pYH4) was about 280 ± 50 dpm/min/mg of protein. For mutants with no detectable activity (E214Q, D275N, E279Q, E435Q, and E503Q), the extracts were incubated for 7 h, additional cell extract was added (a 40 μL aliquot containing 200−550 μg of protein), and incubation was continued for another 15 h. Cell extracts containing 25 μg of protein were subjected to 8% SDS-PAGE and Western blotting. The α-1,2-mannosidase bands were quantitated by densitometry to compare enzyme activity for the same amount of protein. (B) To measure the specific activity or the kinetic parameters of the purified α-1,2-mannosidase, assays were performed as described previously (*24*) for 45 min or 2 h at 37 °C with 0.07−0.7 mM unlabeled $Man_9GlcNAc$, 17 400 dpm [^{3}H]Man_9-$GlcNAc$, 0.1 M PIPES (pH 6.5), 1 mM NaN_3, and 20 μg of BSA in a final volume of 20 μL. Under these conditions, enzyme activity was linear with time. The kinetic parameters were determined by Lineweaver−Burke analysis. (C) To assay for Ca^{2+} dependence, purified α-1,2-mannosidase was treated for 10 min at 4 °C with Chelex (10 mg/100 μL) in 0.1 M PIPES (pH 6.8). Duplicate enzyme samples (7 μM for E132Q or 0.1 μM for wild-type and D121N) were preincubated for 10 min at 37 °C with 5 mM EGTA and 0.01−3 mM Ca^{2+} in Chelex-treated (10 g/L) 0.1 M PIPES (pH 6.8) in a total volume of 17 μL. $Man_9GlcNAc$ was then added at a final concentration of 0.7 mM (wild-type and D121N) or 0.1 mM (E132Q) with 17 400 dpm of [^{3}H]Man_9-$GlcNAc$ to obtain a final volume of 20 μL. The mixtures were incubated for 7 min (wild-type and D121N) or 75 min (E132Q) at 37 °C.

RESULTS

Ca^{2+}-Binding Properties of Recombinant α-1,2-Mannosidase. It was previously shown that the yeast α-1,2-mannosidase is inhibited by EDTA and EGTA and that Ca^{2+} is able to restore the activity more efficiently than any other divalent cation tested (*2*, *3*), but the stoichiometry and affinity of the enzyme for Ca^{2+} were not determined. For this purpose, the catalytic domain of the yeast α-1,2-mannosidase was expressed in *P. pastoris* and purified from the medium. Atomic absorption analysis indicated that the enzyme was isolated with 0.96 ± 0.01 Ca^{2+} ion bound/molecule. This stoichiometry was further substantiated by equilibrium dialysis in buffer A containing 30, 90, or 200 μM Ca^{2+} that showed binding of about 0.9, 0.8, and 0.8 Ca^{2+} ion/molecule, respectively. To determine the dissociation constant for the Ca^{2+} ion, the enzyme was dialyzed in a buffer containing 0.2 mM EGTA and increasing concentrations of Ca^{2+} to obtain 0.1−5 μM free Ca^{2+} in the buffer. Scatchard analysis of the binding data (Figure 1) demonstrated that about 0.8 equiv of Ca^{2+} was bound per mole of enzyme with a K_D value of 4×10^{-7} M.

Role of Ca^{2+} in Thermostability. The possibility that the bound Ca^{2+} found associated with the yeast α-1,2-mannosidase plays a structural role was examined first by studying its effects on thermostability. The purified enzyme was incubated at 50 °C in the presence of 5 mM EGTA and increasing concentrations of Ca^{2+} (Figure 2). In the presence of 5 mM Ca^{2+} and 5 mM EGTA, the α-1,2-mannosidase was highly stable, with minimal loss of activity after 90 min of incubation. Decreasing the concentration of Ca^{2+} to 2.5 mM caused first-order inactivation of the α-1,2-mannosidase with a half-life of about 90−100 min. The half-life of the protein was further reduced to 60 min in the absence of Ca^{2+}.

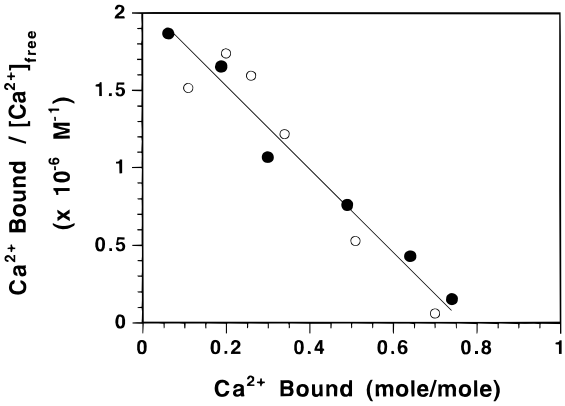

FIGURE 1: Scatchard analysis of Ca^{2+} binding to wild-type α-1,2-mannosidase. Equilibrium dialysis with purified recombinant α-1,2-mannosidase was performed using buffer C containing 10−200 μM Ca^{2+} according to Experimental Procedures. The data from two independent experiments are plotted, and linear regression was used to fit the best line.

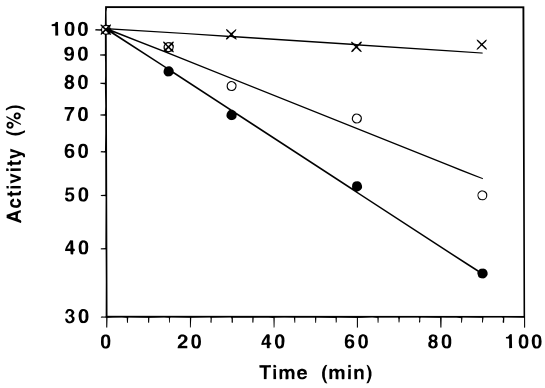

FIGURE 2: Role of Ca^{2+} in α-1,2-mannosidase thermostability. Purified recombinant α-1,2-mannosidase (2.5 μM) was incubated at 50 °C in 0.1 M PIPES (pH 6.8) containing 5 mM EGTA and 0 (●), 2.5 (○), or 5 (×) mM Ca^{2+}. Aliquots were removed at the indicated times and diluted 1/150 in 10 mM PIPES (pH 6.5) containing 1 mg/mL BSA and 1 mM NaN_3. α-1,2-Mannosidase activity was measured using 0.2 mM $Man_9GlcNAc$ according to assay protocol B in Experimental Procedures. The enzyme activity is plotted on a logarithmic scale as the percent of the activity before incubation at 50 °C.

Circular dichroism was used as a probe of α-1,2-mannosidase structure upon exposure of the enzyme to increased temperature. When the α-1,2-mannosidase was incubated with 10 mM EGTA, there was a dramatic decrease in the signal between 200 and 250 nm after exposure to a temperature gradient (25−80 °C), indicating unfolding of the α-1,2-mannosidase (data not shown). In contrast, minimal change in the spectrum was observed in the presence of 10 mM Ca^{2+} and 10 mM EGTA. These results show that Ca^{2+} binding to the α-1,2-mannosidase greatly reduces its susceptibility to thermal denaturation, indicating that Ca^{2+} plays a structural role.

Table 1: Characteristics of EF Hand Mutants

mutant	specific activity (%)[a]	$[Ca]_{50}$ (mM)[b]	Ca^{2+}/protein (mol/mol)[c]	K_D (μM)[d]
wild-type	100	0.2	0.7	0.4
D121A	100		0.7	0.4
D121N	100	0.2	0.8	
E132Q	0.1	0.2	0.7	
E132V	0.06		0.7	0.3
D121A/E132V	0.1		0.8	0.3

[a] α-1,2-Mannosidase activity was assayed using purified recombinant enzymes and 0.2 mM $Man_9GlcNAc$ according to assay protocol B in Experimental Procedures. The measure of 100% activity is 30 000 dpm/h/μg of α-1,2-mannosidase. [b] The concentration of Ca^{2+} needed in the presence of 5 mM EGTA to obtain 50% of maximum activity (see Figure 3A). [c] Number of Ca^{2+} ions/α-1,2-mannosidase molecule determined by equilibrium dialysis. For D121N and E132Q, equilibrium dialysis was performed using buffer B containing 30 μM Ca^{2+}. For wild-type, D121A, E132V, and D121A/E132V, the value presented is determined from the maximum binding observed in Figure 3B. For the wild-type enzyme, the maximum binding observed in five different determinations was in the range of 0.7−0.9 Ca^{2+} ions/α-1,2-mannosidase molecule. [d] The dissociation constants (K_D) were determined by equilibrium dialysis (Figure 3B) and Scatchard plots (not shown). For the wild-type enzyme, the K_D value obtained from two independent experiments was in the range of 0.3−0.5 μM.

Characterization of the EF Hand Mutants. An EF hand consists of a 12-residue loop sequence flanked by two α helices. The yeast α-1,2-mannosidase contains a sequence (^{121}DFDIDAEVNVF^{132}E) which is predicted to be an EF hand loop sequence upon comparison with the consensus sequence D−X−(D,N, or S)−(not I, L, V, F, Y, or W)−(D, E, N, S, T, or G)−(D, N, Q, G, H, R, or K)−(not G or P)−(L, I, V, M, or C)−(D, E, N, Q, S, T, A, G, or C)−X−X−(D or E)−(L, I, V, M, F, Y, or W) (*44*). It has been shown that the 1st and 12th positions are the most conserved amino acids of the consensus EF hand loop and that these residues are particularly important in coordinating Ca^{2+} in other proteins (*27*, *44*). To determine whether this motif is involved in Ca^{2+} binding in the yeast α-1,2-mannosidase, Asp^{121} and Glu^{132} were mutated and the Ca^{2+} binding and enzyme activity of the mutants expressed in *P. pastoris* were determined (Table 1). It was found that the D121N and D121A mutants retain complete enzyme activity whereas the E132Q, E132V, and D121A/E132V mutants have only 0.06−0.1% of the specific activity observed in the wild-type.

The influence of Ca^{2+} concentration on enzyme activity was examined in the D121N and E132Q mutants and compared to that of the wild-type α-1,2-mannosidase (Figure 3A and Table 1). In all cases, the enzyme activity depended on the amount of Ca^{2+} added and reached a maximum level at about 3 mM Ca^{2+}. The concentration of Ca^{2+} needed to obtain 50% of maximal activity in the presence of 5 mM EGTA was very similar (0.2 mM) for the wild-type and the mutants. All EF hand mutants bound close to 1 Ca^{2+} ion/protein molecule as determined by equilibrium dialysis and had dissociation constants very similar to that of the wild-type enzyme (0.3−0.4 μM) (Figure 3B and Table 1). These results clearly demonstrate that mutation of the most essential residues of the EF hand-related sequence does not change the Ca^{2+}-binding characteristics of the enzyme.

Effects of Mutation of the Invariant Acidic Residues in Vivo. Alignment of all of the Class I α-1,2-mannosidase catalytic domains indicates that there are nine invariant acidic residues in this enzyme family. Because acidic residues have been shown to be directly involved in catalysis in all inverting glycosidases studied to date and are also invariant within a given family of glycosidases (*29*, *31*), the role of these residues in α-1,2-mannosidase activity was examined in vivo. The invariant acidic amino acids were mutated in the pYH4 high-copy yeast-expression plasmid containing the *MNS1* gene (*6*). Each acidic group was replaced by the corresponding amido group, and the mutants were expressed in an *S. cerevisiae* strain completely lacking α-mannosidase activity to test for complementation of the defect. The corresponding amido amino acid was chosen for its steric similarity to the acidic residue and because it is the most conservative replacement. All of the mutants were expressed at appreciable

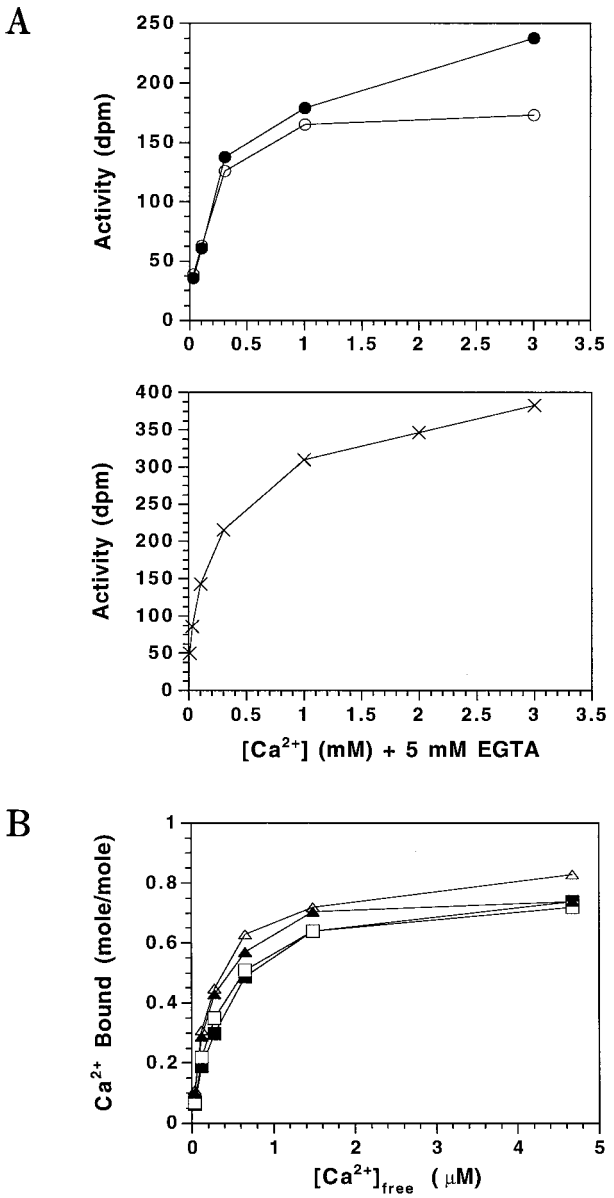

FIGURE 3: Effect of EF hand mutations on α-1,2-mannosidase activity and on Ca^{2+} binding. (A) Purified wild-type (●), D121N (○), and E132Q (×) α-1,2-mannosidases were assayed in the presence of 5 mM EGTA and increasing amounts of Ca^{2+} according to assay protocol C in Experimental Procedures. (B) The amount of Ca^{2+} bound to purified wild-type (■), D121A (□), E132V (▲), and D121A/E132V (△) with increasing concentration of Ca^{2+} was determined by equilibrium dialysis using buffer C and 10−180 μM Ca^{2+} according to Experimental Procedures.

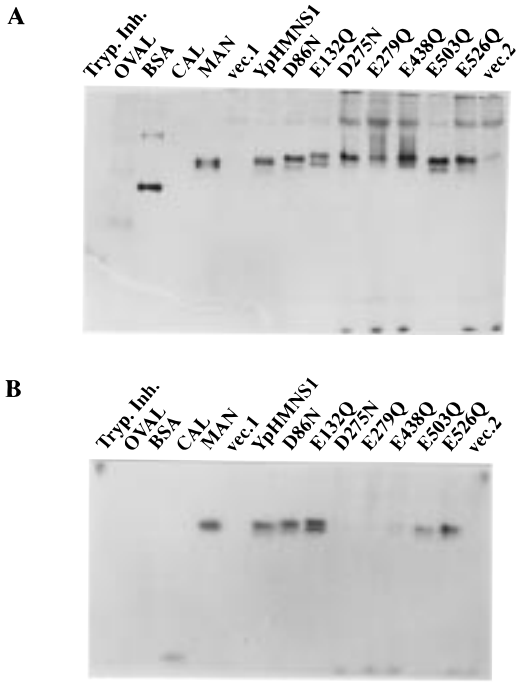

FIGURE 4: Role of the invariant acidic residues in Ca^{2+} binding to recombinant α-1,2-mannosidase. *P. pastoris* clones transformed with wild-type (YpHMNS1) or mutant α-1,2-mannosidases were induced with methanol in BMMY medium for 4 days as previously described (*25*). The medium was concentrated 20−60-fold, and an aliquot containing 2−3 μg of α-1,2-mannosidase/lane was subjected to PAGE on native gels. For cells transformed with the vector (pHIL-S1), aliquots similar to those used from cells expressing D86N (vec.1) or D275N (vec.2) were subjected to PAGE. One gel was stained with Coomassie (A) and the other (B) was incubated with $^{45}Ca^{2+}$ and exposed to film according to Experimental Procedures. $^{45}Ca^{2+}$ binding was quantitated as indicated in Experimental Procedures and is comparable (D86N, E132Q, E503Q, and E526Q) to the wild-type, <0.1% (D275N and E279Q), or <10% (E438Q). The $^{45}Ca^{2+}$ blot was performed at least twice for all of the mutants with similar results. The following were used as controls: 2 μg of trypsin inhibitor (Tryp. Inh.), 2 μg of ovalbumin (OVA), 2 μg of bovine albumin (BSA), 0.2 μg of calmodulin (CAL), and 2 μg of purified recombinant α-1,2-mannosidase (MAN).

levels as shown by Western blotting of cellular extracts (data not shown). With the E214Q, D275N, E279Q, E435Q, and E503Q mutants, there was no detectable α-1,2-mannosidase activity. Measurable α-1,2-mannosidase activity was detected in cells transformed with the D86N, E132Q, E438Q, and E526Q mutants, but the enzyme activity was less than 2% of that observed in cells transformed with the wild-type enzyme.

Ca^{2+} Binding to Invariant Acidic Residue Mutants. Because the putative EF hand is not the site of Ca^{2+} binding and acidic residues are often involved in binding this divalent cation (*45*), the possibility that some of the invariant acidic residues required for enzyme activity in vivo may be important for Ca^{2+} binding was explored. The binding of $^{45}Ca^{2+}$ to the mutants secreted into the medium of *P. pastoris* was evaluated by autoradiography following electrophoresis in native polyacrylamide gels (Figure 4). Because the level of expression of the mutants varied, the amounts of the medium were adjusted to obtain comparable amounts of α-1,2-mannosidase on the gels, and different amounts of the medium obtained from cells transformed with the pHIL-S1 vector were also loaded (vec.1 and vec.2) for comparison.

Table 2: Kinetic Parameters of Acidic Residue Mutants

mutants	specific activity (%)[a]	K_m (mM)[b]	k_{cat} (s^{-1})
wild-type	100	0.5	12
D86N	0.1	0.2	0.007
E132Q	0.1	0.07	0.005
E132V	0.06	0.3	0.005
E526Q	7	4	5

[a] Specific activity of the α-1,2-mannosidases was assayed using purified recombinant enzymes and 0.2 mM $Man_9GlcNAc$ as a substrate (see Table 1). [b] The kinetic parameters were determined according to assay protocol B in Experimental Procedures.

The α-1,2-mannosidase appears as a doublet following staining of the gels with Coomassie, and no significant protein is observed in the medium obtained from cells transformed with the vector alone. It is evident from the results shown in Figure 4 that D86N, E132Q, E503Q, and E526Q mutants retained an affinity for Ca^{2+} similar to that of the wild-type enzyme whereas the D275N, E279Q, and E438Q mutants showed little or no significant binding of Ca^{2+}. Furthermore, the D275N, E279Q, and E438Q mutants migrated slightly differently than the wild-type enzyme and the other mutants, with trailing of protein clearly seen above the doublets. Western blot analysis of the gels confirmed that the trailing is due to the α-1,2-mannosidase (data not shown). The activity of the mutants in the medium was similar to the activity of the mutants expressed intracellularly in *S. cerevisiae* (data not shown). The E214Q and E435Q mutants were not secreted into the medium at a level sufficient to perform the Ca^{2+}-binding assay. These results demonstrate that Asp^{275}, Glu^{279}, and Glu^{438} are required for normal Ca^{2+} binding. It was also found that $^{45}Ca^{2+}$ binding to wild-type α-1,2-mannosidase was lost upon electrophoresis in 8 M urea whereas calmodulin that has functional EF hands still bound $^{45}Ca^{2+}$ under the same denaturing conditions (data not shown).

Kinetic Characterization of Invariant Acidic Residue Mutants. Because mutation of Asp^{86}, Glu^{132}, Glu^{503}, or Glu^{526} greatly inhibits enzyme activity in vivo without affecting Ca^{2+} binding, the kinetic properties of these mutants were examined following their purification from the medium of *P. pastoris* (Table 2). Mutation of Asp^{86} resulted in a dramatic decrease (1750-fold) in k_{cat} and a 2.5-fold decrease in K_m. Mutation of Glu^{132} also greatly decreased k_{cat} (2300-fold) and decreased K_m (2−7-fold). In contrast, mutation of Glu^{526} resulted in an 8-fold increase in K_m and only a 2-fold decrease in k_{cat}, suggesting that this mutation mostly affected substrate binding. The E503Q mutant produced by *P. pastoris*, which was inactive in *S. cerevisiae* in vivo, was also inactive in vitro.

DISCUSSION

In the present work, the Ca^{2+}-binding characteristics of the yeast-processing α-1,2-mannosidase were determined. It was found by atomic absorption analysis and equilibrium dialysis that the yeast α-1,2-mannosidase binds 1 Ca^{2+} ion/mol with high affinity ($K_D \approx 10^{-7}$ M). It was also demonstrated that the predicted EF hand-like sequence is not the site of Ca^{2+} binding. Mutagenesis of the two most essential residues, Asp^{121} and Glu^{132}, in the loop consensus sequence of the putative EF hand has no effect on the Ca^{2+}-binding properties of the enzyme. On the other hand, all nine

invariant acidic residues found in Class I α-1,2-mannosidases are necessary for enzyme activity in vivo. Mutagenesis of five of these residues (Glu^{214}, Asp^{275}, Glu^{279}, Glu^{435}, and Glu^{503}) completely prevents complementation of an α-1,2-mannosidase deficient *S. cerevisiae* strain, and mutagenesis of the other four residues (Asp^{86}, Glu^{132}, Glu^{438}, and Glu^{526}) results in very low levels of α-1,2-mannosidase activity compared to that observed following complementation with the wild-type enzyme. Studies on recombinant yeast α-1,2-mannosidase produced in *P. pastoris* demonstrated that at least three of the invariant acidic residues (Asp^{275}, Glu^{279}, and Glu^{438}) are required for normal $^{45}Ca^{2+}$ binding. These residues may be involved directly in Ca^{2+} binding by coordinating the Ca^{2+} ion, or they may be essential to maintain the conformational integrity of the enzyme. Whether Glu^{214} and Glu^{435} are also important for Ca^{2+} binding could not be studied because insufficient protein was produced for the $^{45}Ca^{2+}$-binding assay.

The yeast α-1,2-mannosidase catalytic domain was isolated with tightly bound Ca^{2+} that is essential for enzyme activity. Although it was shown previously that other divalent cations could partially reverse the inhibitory effects of chelating agents (*2*, *3*, *24*), the present results demonstrate that Ca^{2+} is an integral component of the enzyme. Evidence using CD spectroscopy was obtained to indicate that Ca^{2+} protects the yeast α-1,2-mannosidase from thermal denaturation. However, removal of Ca^{2+} completely inhibited enzyme activity but caused no change in the CD or fluorescence spectra or in V8 protease sensitivity (data not shown) at normal temperatures (25−37 °C). In addition, the Ca^{2+}-depleted enzyme is highly stable at normal temperatures, and no loss of activity was observed after equilibrium dialysis at 30 °C under conditions where there is no Ca^{2+} bound (data not shown). Therefore, any conformational change induced by Ca^{2+} depletion is minor under these conditions, suggesting that Ca^{2+} may have other functions under physiological conditions. In a previous study, it was shown that the rabbit Class I α-1,2-mannosidase also has a high affinity for Ca^{2+} and that Ca^{2+} is required for substrate binding (*46*).

Ca^{2+} binding may have different functions depending on the specific enzyme. In some cases (e.g., staphylococcal nuclease and phospholipase A_2), Ca^{2+} is directly involved in binding to one or two oxygen atoms of the substrate (*47*, *48*). In other enzymes, such as xylanase (*49*), β-glucanase (*50*), and proteinase K (*51*), Ca^{2+} is located at a distance from the active site and is not directly involved in catalysis, but it helps stabilize the enzyme structure and imparts resistance to proteases or to thermal denaturation. Sometimes, as observed for endoglucanase CelD (*52*), α-amylase (*53*), and gelatinase B (*54*), the Ca^{2+} ion is located close to the catalytic center where it is essential to maintain the active conformation of the enzyme without necessarily coordinating the substrate.

Four of the mutants (D86N, E132Q, E503Q, and E526Q) were found to retain a high affinity for Ca^{2+}, but their enzyme activity is greatly inhibited. Kinetic analysis showed that the catalytic efficiency (k_{cat}) of the D86N and E132Q(V) mutants is greatly reduced (about 2000-fold) and that their substrate affinity is increased 2−7-fold (Table 2). Two conserved acidic amino acids are usually involved in catalysis by glycosidases (for reviews, see refs *28*−*32*), and the changes observed in the behavior of D86N and E132Q are typical for mutants of catalytic residues in inverting glycosidases (*55*−*59*). In contrast, the catalytic efficiency of the E526Q mutant was much less affected while its affinity for substrate was greatly reduced. It is possible, therefore, to exclude Glu^{526} as a residue directly involved in catalysis and to suggest that Asp^{86}, Glu^{132}, and Glu^{503} are potential residues involved in catalysis.

While this work was in progress, it was shown by mutagenesis of the α-1,2-mannosidase from *Aspergillus saitoi* that five conserved acidic residues corresponding to Asp^{275}, Glu^{279}, Glu^{435}, Glu^{438}, and Glu^{503} in the yeast α-1,2-mannosidase are crucial for enzyme activity (*60*). These results are in agreement with the present study, but the Ca^{2+} binding and kinetic properties of the *A. saitoi* mutants were not studied.

In view of the amino acid sequence similarity between the yeast and mammalian Class I α-1,2-mannosidases, it is likely that the results obtained with the yeast enzyme apply to other members of the family. The yeast and mammalian α-1,2-mannosidases have recently been crystallized, and preliminary X-ray crystallographic data have been obtained (*61*, *62*). Determination of the three-dimensional structure of these enzymes in conjunction with the present mutagenesis studies will be required to determine the exact Ca^{2+}-binding site and the amino acid residues involved in catalysis.

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