The *Saccharomyces cerevisiae* α1,2-mannosidase, which removes one specific mannose residue from ManαGlcNAC2 to form ManαGlcNAC2, is a member of a family of α1,2-mannosidases with similar amino acid sequences. The yeast α1,2-mannosidase contains five cysteine residues, three of which are conserved. Recombinant yeast α1,2-mannosidase, produced as the soluble catalytic domain, was shown to contain two disulfide bonds and one free thiol group using 2-nitro-5-thiosulfofenozoate and 5,5'-dithiobis(2-nitrobenzoate), respectively. Cys485 contains the free thiol group, as demonstrated by sequencing of labeled peptides following modification with [3H]I Harborman and high performance liquid chromatography/mass spectrometry tryptic peptide mapping. A Cys340-Cys385 disulfide was demonstrated by sequencing a purified peptide containing this disulfide and by tryptic peptide mapping. Cys468 and Cys471 were not labeled with [3H]ICH2COOH and a peptide containing these two residues was identified in the tryptic peptide map, showing that Cys468 and Cys471 form the second disulfide bond. The α1,2-mannosidase loses its activity in the presence of dithiothreitol with first order kinetics, suggesting that at least one disulfide bond is essential for activity. Mutagenesis of each cysteine residue to serine showed that Cys340 and Cys385 are essential for activity. In addition, they are inhibited by 1-deoxymannojirimycin and do not use N-trophenyl-α-D-mannopyranoside as substrate. The yeast α1,2-mannosidase has a very high specificity for removal of a single mannose residue on ManαGlcNAC2, whereas the mammalian enzymes can remove up to four mannose residues from ManαGlcNAC2 to form ManαGlcNAC2. The mammalian enzymes hydrolyze α-Man1,2-Man-OMe, whereas the yeast α1,2-mannosidase cannot hydrolyze this disaccharide. The smallest oligosaccharide substrate for the yeast α1,2-mannosidase is α-Man1,2-Man1,3α-O(CH2)8COOCH3, but it is a very poor substrate (Km = 9 mM). Recently, α1,2-mannosidases have also been cloned from *Drosophila melanogaster* (11), *Penicillium citrinum* (12), and *Aspergillus saitoi* (13), which have similar amino acid sequences to the yeast and mammalian enzymes.

The *Drosophila mas-1* gene encodes two α1,2-mannosidases (72.5 and 75 kDa) that differ in their N-terminal region and have the same topology as the yeast and mammalian α1,2-mannosidases. The *P. citrinum* and *A. saitoi* α1,2-mannosidase genes encode secreted proteins of 56–57 kDa with a cleavable signal peptide. Unlike the other members of this family, they do not contain an EF-hand Ca2+ binding consensus sequence and do not require Ca2+ for activity.

Little is known about the structure and mechanism of catalysis of any of the Class 1 α1,2-mannosidases (recently named Family 47 in the classification of glycosyl hydrolases in Release 34.0 of the SWISS-PROT Protein Sequence Data Bank), and the three-dimensional structure is not known. Until recently, a major difficulty has been the purification of sufficient enzyme for study. However, we can now produce milligram quantities of any of the catalytic domain of the yeast α1,2-mannosidase (14). The yeast processing α1,2-mannosidase is the first member of this family that can be produced in sufficient quantity to study its structure and its mechanism of catalysis, and we have shown recently that it is a glycosidase of the inverting type (15).

In the present work we demonstrate that the yeast α1,2-mannosidase has two disulfide bonds and one sulphydryl group in its catalytic domain. Their location is documented by peptide analysis, and their respective roles in enzyme activity is established by site-directed mutagenesis. Only one of these two disulfide bonds is essential for catalytic activity, and the free sulphydryl residue is not required.

---

*This work was supported in part by National Institute of Health Grant GM 31265. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a scholarship for graduate studies from the Natural Sciences and Engineering Research Council of Canada.

§ To whom correspondence should be addressed: McGill Cancer Centre, 3655 Drummond St., Montréal, Québec, Canada H3G 1Y6. Tel.: 514-398-3533; Fax: 514-398-6769; E-mail: annette@medcor.mcgill.ca.
Role of Cysteines in the Yeast Processing of α1,2-Mannosidase

EXPERIMENTAL PROCEDURES

Materials—Taq polymerase, endoprotease Asp-N (sequencing grade), and DTT were purchased from Boehringer Mannheim. Oligonucleotides were synthesized at the Sheldon Biotechnology Centre (McGill University, Montréal, Canada). Restriction enzymes were from either Pharmacia Biotech Inc., New England Biolabs, or Boehringer Mannheim. The Pichia expression kit was from Invitrogen and includes the Pichia pastoris strains GS115 and KM71 and the vector pHiL5. pHiL5 was from Fluka. Urea (≥99%) and iodoacetic acid were from ICN. DTNB, endoprotease Glu-C (sequencing grade) from Staphylococcus aureus V8, and TPCK-treated trypsin were from Sigma. Water for HPLC was obtained from a Barnstead nanopure water purification system or from Baxter. All other methods require water from a Milli-Q system with an Organex-Q cartridge. Acetonitrile suitable for chromatography was from BDH or Fisher. Trifluoroacetic acid (99% pure) was from Aldrich. Oligosaccharide substrates were obtained as solvent, monitoring the eluent at 230 nm. Five included peptide fractions were eluting between 44 and 48 min in fraction P3, and to a lesser extent, 1 ml of water was added, and the sample was lyophilized. One ml of water was added again and the sample was lyophilized and then stored at −80°C. CNBr-treated peptides were fractionated on a Sephadex G-50 column (1 × 120 cm) using 0.1 M or 0.2 M acetic acid as solvent, monitoring the eluent at 230 nm. Five included peptide fractions, P1–P5, were collected in their order of elution. Aliquots from each of these fractions were subjected to HPLC before and after reduction. A set of peptides eluting between 44 and 48 min in fraction P4, and to a

1 The abbreviations used are: DTT, dithiothreitol; BMGY, buffered methanol-complex; BSA, bovine se-

rium albumin; CM-Cys, S-carboxymethylcysteine; DTNB, 5,5'-dithio-

biuretic acid; Gdn-HCl, guanidine hydrochloride; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; NTBs, 2-nitro-

5-thiobenzoate; NTSB, 2-nitro-5-thiosulfobenzoate; PAGE, polyacryl-
amide gel electrophoresis; PCR, polymerase chain reaction; PIPES, piperezine-N,N'-bis[2-ethanesulfonic acid]; TPCK, L-1-tosylamido-

2-phenylethyl chloromethyl ketone.
lesser extent in fraction P2, were observed to shift upon reduction. None of the other fractions (P1, P3, or P4) contained peptides that changed elution position upon reduction.

For endoprotease Asp-N digestion, peptides were dried under a nitrogen stream and were then dissolved in 50 mM potassium phosphate (pH 6.0), 0.1 M Gdn-HCl at 1.25 µg/µl and treated with 0.8 µg of endoprotease Asp-N/100 µg of peptides for 16 h at 37 °C. Gdn-HCl was added to help solubilize the peptides. Glacial acetic acid was added to a final concentration of 10% (v/v) to stop the reaction, and the sample was stored at −20 °C.

For endoprotease Glu-C digestion, peptides were lyophilized and resuspended in 30 mM sodium phosphate (pH 7.8), containing 1.2 M urea and 2 mM EDTA. Urea was added to help solubilize the peptides. The peptides were digested at a concentration of 1.5 µg/µl with endoprotease Glu-C at a concentration of 13 µg/100 µg of peptides. The digestion was allowed to proceed for 24 h at 25 °C and terminated by the addition of glacial acetic acid to 10% (v/v). The sample was stored at −20 °C.

For trypsin digestion, recombinant α1,2-mannosidase was lyophilized and resuspended in 8 µM urea, 0.1 M potassium phosphate (pH 6.5) at a concentration of 5 µg/µl. The protein was denatured by sonication for 1 min, followed by incubation at 37 °C for 5 min. This was repeated three times. The solution was then incubated at 37 °C for 30 min. An equal volume of 0.1 M potassium phosphate buffer (pH 6.5) was then added before the addition of 1 µl of trypsin. TPCK-treated trypsin was prepared in 0.1 mM HCl and was added to yield a final concentration of 5 µg/100 µg of α1,2-mannosidase. The digestion was allowed to proceed at 30 °C for 3 h and stopped by freezing at −80 °C. For the reduced sample, 4 times the volume of 0.5 M Tris-HCl (pH 8.5) containing 0.375 M DTT was added to an aliquot of the trypsin-digested α1,2-mannosidase and the mixture was incubated at 37 °C for 10 min, then stored immediately at −80 °C.

For reduction of CNBr-treated and endoprotease Asp-N-digested peptides, peptide solutions were dried in a vacuum concentrator (Savant), then reconstituted with 150 µl of 50 mM DTT in 0.5 M Tris-HCl (pH 8.5) containing 6 mM Gdn-HCl (23). The mixture was incubated at 37 °C for 4 h. An identical sample lacking DTT was treated in the same way. The reaction was stopped by adding 15 µl of glacial acetic acid and 40 µl of 0.1% aqueous trifluoroacetic acid.

HPLC of Peptides—A Varian model 5020 HPLC system equipped with a reverse-phase C4 column (4.6 mm (inner diameter) × 25 cm, 10 µm, Vydac) was employed for peptide separations. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B contained 0.1% trifluoroacetic acid, 95% acetonitrile, and 5% water. The sample was injected, and solvent A was passed through the column for 5 min. The peptides were eluted with a linear gradient of 0–60% B over 60 min at a flow rate of 1 ml/min.

Microbore HPLC Electrospray Mass Spectrometry—5 µg of tryptic-digested α1,2-mannosidase (reduced or non-reduced) were fractionated on a microbore C18 column (1 mm (inner diameter) × 25 cm, 5 µm, Vydac), which was on-line with a Finnigan SSQ 7000 mass spectrometer equipped with an electrospray ionization source. A Hewlett Packard model 1090 HPLC system was used, and a discontinuous gradient was employed for elution: 5% B at 0 min, 33% B at 63 min, 60% B at 95 min, and 80% B at 105 min, using 0.05% trifluoroacetic acid/water as buffer A and 0.05% trifluoroacetic acid/acetonitrile as buffer B. The mass analysis was set at 5 s/scan with a range of 300-2500 m/z. The flow rate was 40 µl/min for the non-reduced sample and 30 µl/min for the reduced sample. For the non-reduced sample, mass data were collected 5 min after the gradient started. For the reduced sample, data collection started 10 min later.

Protein Analysis—The concentration of purified recombinant α1,2-mannosidase was quantitated using the absorbance at 280 nm as described (24) or the Micro BCA reagent kit from Pierce. Peptide concentration was determined using a modified Lowry method (25). SDS-PAGE under reducing conditions was carried out according to Laemmli (26) using the Bio-Rad Mini-PROTEAN II apparatus. For Western blotting, proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell) and visualized by the ECL Western blotting system (Amersham) using rabbit polyclonal antiserum raised against the purified soluble yeast α1,2-mannosidase (14) or against recombinant yeast α1,2-mannosidase (4).

Peptide Analysis—N-terminal sequencing was performed at Queen’s University (Kingston, Canada) on an Applied Biosystems model 473A sequenator equipped with an on-line microgradient phenylthiodantoin analysis system or at the Sheldon Biotechnology Centre (McGill University, Montréal, Canada) on a Beckman integrated microsequencing system, model PI2000E, equipped with an on-line HP1090 HPLC.

For amino acid composition analysis, peptides were subjected to constant boiling hydrochloric acid (about 6 n) vapor hydrolysis for 16–24 h at 110 °C using a Pico-Tag workstation (Waters), and amino acid analysis was performed on a Beckman 6300 series autoanalyzer. The amino acids were separated by ion-exchange chromatography, and ninhydrin post-column detection/quantitation was used.

RESULTS

Expression of S. cerevisiae α1,2-Mannosidase in P. pastoris—In order to obtain sufficient protein for structural analysis, the catalytic domain of the yeast α1,2-mannosidase was cloned downstream of the PHO1 signal peptide and strong alcohol oxidase (AOX1) promoter in the pHIL-S1 vector. The construct was introduced into the P. pastoris genome by homologous recombination. Expression from P. pastoris was induced with methanol, and the recombinant α1,2-mannosidase was secreted into the medium. Different clones expressed different amounts of α1,2-mannosidase, but the highest yield obtained using this system was 30 mg/liter of purified recombinant enzyme, which is 50 times more than was produced in S. cerevisiae as described previously (0.6 mg/liter) (14). Recombinant α1,2-mannosidase produced from S. cerevisiae had been shown to have similar properties as the native α1,2-mannosidase (14), and the recombinant α1,2-mannosidase from P. pastoris has the same specific activity and K_m as the enzyme produced in S. cerevisiae (data not shown).

Quantitation of Disulfides and Free Sulfhydryl Groups—The yeast α1,2-mannosidase contains five cysteine residues in its catalytic domain. NTSB in the presence of sodium sulfite was used to quantitate the number of disulfides plus free sulfhydryl groups. The protein was reduced with sodium sulfite releasing one thiol per disulfide bond, and NTSB reacts with free thiols to produce one NTB per free thiol. Reaction with α1,2-mannosidase yielded a value of 2.7 ± 0.4 NTB molecules/protein molecule. DTNB was used to quantitate free sulfhydryl groups. A value of 1.1 ± 0.1 sulfhydryl group/α1,2-mannosidase molecule was obtained. In addition, carboxymethylation with iodoacetate under denaturing conditions resulted in 0.85 CM-Cys/protein molecule as determined by amino acid composition. These results demonstrate that there is one free sulfhydryl group and two disulfide bonds in the yeast α1,2-mannosidase.

Effect of DTT on α1,2-Mannosidase Activity—The importance of the disulfide bonds and the free sulfhydryl group in the yeast α1,2-mannosidase was investigated. It was found that purified recombinant α1,2-mannosidase loses about 90% activity when incubated for 9 h with 10 mM DTT (Fig. 1). The rate of inactivation clearly shows first order kinetics, suggesting that...
reduction of one of the two disulfide bonds was likely to cause the loss of enzyme activity. No change in the migration of the protein on SDS-PAGE was observed during this treatment with DTT. These results demonstrate that at least one disulfide bond is essential to maintain α,1,2-mannosidase activity. Treatment of the α,1,2-mannosidase with the sulfhydryl specific reagents N-ethylmaleimide, p-chloromercuribenzoate, iodoacetate, and iodoacetamide under native conditions did not affect enzyme activity (data not shown).

**Labeling the Sulfhydryl Group with 2-[3H]ICH2COOH**—The protein was labeled with radioactive iodoacetate to identify the cysteine containing the free sulfhydryl group. Labeling was carried out by adding Gdn-HCl and iodoacetate simultaneously to the dried protein in order to minimize disulfide exchange. The CNBr-digested protein was fractionated by gel filtration (Fig. 2). There was one major radioactive peptide fraction (C1) that contained about 43% of the radioactivity expected to be incorporated as CM-Cys. The radioactive peptides that were eluted between fractions 32 and 45 were larger than 10 kDa due to incomplete digestion with CNBr. The peptides in fractions 75 and 85 contained only about 10% of the recovered radioactivity.

Fraction C1 was treated with endoprotease Glu-C, under conditions in which the enzyme would cleave after both aspartic and glutamic acid residues (27), in order to obtain peptides containing only one cysteine residue according to the amino acid sequence. HPLC of the undigested peptides showed that all of the radioactivity was eluted with a peak at about 48 min (Fig. 3). HPLC of an aliquot of the endoprotease Glu-C-digested peptides showed radioactive fractions eluting with peaks at about 31 and 40 min. Preparative HPLC was carried out on the remaining digest, and the fractions expected to contain radioactivity were collected manually. About 64% of the radioactivity was eluted at about 31 min, about 30% was eluted at about 40 min, and 5% did not bind to the column. Fractions g1 and g2 were analyzed by N-terminal sequencing, collecting fractions for radioactivity measurement. The two fractions were found to contain the same radiolabeled peptide. The N-terminal sequence for the peptide in fraction g1 is shown in Table I. The peptide contains homoserine at its C terminus and was eluted from the column as a doublet due to the equilibrium between homoserine and homoserine lactone (28).

Fraction g3 was also analyzed by N-terminal sequencing (Table I) and contains the following CNBr/endoprotease Glu-C peptide: 466NTCDNDFKRLRTSLS-CM-Cys-ITLPTKKSNN-Hse496. There was no CM-Cys or radioactivity in cycles 3 or 6; hence, Cys468 and Cys471 did not react with iodoacetate. The only radioactivity seen was in cycles 20 and 21, corresponding to the dried protein in order to minimize disulfide exchange.

![Figure 2](image2.png)  
**Fig. 2.** Gel filtration of [3H]iodoacetate-labeled α,1,2-mannosidase digested with CNBr. α,1,2-Mannosidase (1.2 mg) was treated with [3H]ICH2COOH and was digested with a 500-fold molar excess (with respect to methionine) of CNBr as described under “Experimental Procedures.” The dried sample was dissolved in 0.1 M acetic acid and chromatographed on a Sephadex G-50 column (1 × 120 cm) in the same solvent. One ml fractions were collected, the A230 nm was measured (open circles), and 20 µl from each fraction were used to quantitate radioactivity (closed circles). Fraction C1 was pooled as indicated by the bar and subsequently digested with endoprotease Glu-C. Vp and Vt correspond to the elution position of BSA and tyrosine, respectively.

![Figure 3](image3.png)  
**Fig. 3.** HPLC of endoprotease Glu-C-digested peptides. Fraction C1, from Fig. 2 (about 75 µg of peptides) was treated with endoprotease Glu-C and fractionated by HPLC as indicated under “Experimental Procedures.” The elution was monitored at 206 nm (A), and an aliquot from each fraction was used to quantitate total radioactivity per fraction (hatched bars in B). An aliquot of undigested C1 was also applied to HPLC under the same conditions, and 0.5-ml fractions were collected. The total radioactivity in each fraction is plotted in B as white bars. Fractions subsequently analyzed by N-terminal sequencing are named g1, g2, and g3.
TABLE I  
N-terminal sequence of peptides g1 and g3

| Cycle no. | Amino acid | Radioactivity* | Picomoles |
|-----------|------------|----------------|-----------|
| Peptide g1 |            |                |           |
| 1         | CM-Cys     | 313            | 193       |
| 2         | Ile        | 80             | 100       |
| 3         | Thr        | 0              | 100       |
| 4         | Leu        | 0              | 145       |
| 5         | Pro        | 0              | 35        |
| 6         | Thr        | 0              | 55        |
| 7         | Lys        | 0              | 29        |
| 8         | Lys        | 0              | 65        |
| 9         | Ser        | 0              | 18        |
| 10        | Asn        | 0              | 39        |
| 11        | Asn        | 0              | 43        |
| Peptide g3 |            |                |           |
| 1         | Asn        | 0              | 50        |
| 2         | Thr        | 0              | 24        |
| 3         | —         | 0              | —         |
| 4         | Val        | 0              | 47        |
| 5         | Asp        | 0              | 72        |
| 6         | —         | 0              | —         |
| 7         | Asn        | 0              | 35        |
| 8         | Asp        | 0              | 57        |
| 9         | Pro        | 0              | 18        |
| 10        | Lys        | 0              | 11        |
| 11        | Leu        | 0              | 18        |
| 12        | —         | 0              | —         |
| 13        | —         | 0              | —         |
| 14        | Phe        | 0              | 19        |
| 15        | Thr        | 0              | 15        |
| 16        | Ser        | 0              | 8         |
| 17        | Leu        | 0              | 11        |
| 18        | Ser        | 0              | 7         |
| 19        | Asp        | 0              | 16        |
| 20        | CM-Cys     | 23             | 21        |
| 21        | Ile        | 18             | 11        |
| 22        | Thr        | 0              | 9         |
| 23        | Leu        | 0              | 8         |
| 24        | Pro        | 0              | 12        |
| 25        | Thr        | 0              | 5         |
| 26        | Lys        | 0              | 3         |
| 27        | Lys        | 0              | 5         |

* The eluent from the HPLC of the phenylthiohydantoin-amino acids was collected and the radioactivity in each cycle of Edman degradation was measured.

The dashes represent the absence of phenylthiohydantoin-amino acids in the indicated cycle. In this case cysteine residues were degraded and arginine residues were poorly recovered.

Isolation and Characterization of Disulfide-bonded Peptides—In order to isolate peptides containing a disulfide bond, the α,1,2-mannosidase was first treated with CNBr and the products were fractionated by Sephadex G-50 gel filtration chromatography as described under “Experimental Procedures.” Disulfide exchange was prevented by CNBr treatment under acidic conditions. Five peptide fractions (F1–F5) were pooled according to the pattern of absorbance at 230 nm. Aliquots from each peptide fraction were treated with or without DTT and analyzed by HPLC. Disulfide-containing peptides were identified by a change in their elution position upon reduction (data not shown). A set of peptides eluting between 44 and 48 min of HPLC were observed to shift upon reduction. These peptides were then digested with endoprotease Asp-N and were analyzed by HPLC before and after reduction with DTT (Fig. 4). Endoprotease Asp-N digestion was carried out at pH 6.0 in order to prevent disulfide rearrangement. Fraction a1 disappears upon reduction and fraction a2 is decreased by half. This residual absorbing material is a contaminant that is present in the buffer alone. Fractions a1 and a2 were collected manually and subjected to amino acid analysis. Fraction a1 did not contain any cysteine and did not correspond to any possible disulfide-containing peptide in the yeast α,1,2-mannosidase.

From amino acid composition and N-terminal sequencing results (Table II), it was determined that fraction a2 contains the following peptides, which are linked by a disulfide bond: 313DHLVCF-Hse342 and 383DCYQ-Hse388. These results demonstrate that there is a disulfide bond between Cys340 and Cys385.

HPLC/MS Peptide Mapping—In order to confirm the location of the disulfide bonds and the free sulphydryl group, HPLC/MS was used to analyze trypsin-digested α,1,2-mannosidase. Trypsin digestion was carried out at pH 6.5 in order to minimize disulfide exchange (29). Both reduced and non-reduced tryptic digests were analyzed by HPLC/MS. Some peaks...
could not be identified as tryptic peptides. Additionally, peaks after scan 850 contained peptides above 4000 Da and contained mixtures of peptides that could not be resolved. However, 77% of the amino acid sequence was confirmed. Longer incubation with trypsin was not useful, since there were too many peptides that could not be assigned to expected tryptic peptides.

A peptide with mass 4053.3, corresponding to the peptide in which Cys340 is bound to Cys385 (t16 + t19), was observed in the non-reduced sample (Fig. 5A and Table III). This peptide was not observed in the reduced sample, although the two peptides resulting from its reduction (2731.8, t16; 1322.9, t19) were only observed in the reduced sample (Fig. 5B and Table III).

The tryptic peptide containing Cys468 (t22) was observed in both the non-reduced and reduced digests (Fig. 5 and Table III). A peptide with a mass (5625.1) corresponding to residues 429–475 (t22) is observed in the non-reduced sample and in the reduced sample (5626.5) (Table III). This peptide elutes at scan number 1180 in both the reduced and non-reduced tryptic digests (data not shown). Trypsin does not cleave between Cys468 and Cys471; therefore, peptide t22 contains both cysteine residues. There is an increase in mass (1.4 mass units) upon reduction, which is within experimental error of the expected value of 2 mass units due to the loss of two protons.

Mutagenesis of Cysteine Residues—In order to determine the role of each disulfide bond and of the free sulphydryl residue in the yeast α,1,2-mannosidase, each cysteine was individually mutated to serine. The plasmids were transformed into P. pastoris, and clones were screened for α,1,2-mannosidase expression using Western blotting and α,1,2-mannosidase assay. Although a significant number, 25, of transformants were screened for each mutant, recombinant α,1,2-mannosidases containing C340S or C385S mutations were never detected in the medium. Western blotting of cellular extracts showed minimal or no α,1,2-mannosidase in cells transformed with C340S or C385S mutant plasmids in contrast to cells transformed with the wild type plasmid (data not shown). The C468S, C471S, and C485S mutants were all expressed in the medium and were enzymatically active. However, the C468S and C471S mutations resulted in a reduction in the amount of α,1,2-mannosidase found in the medium, whereas the C485S mutant was expressed at a similar level as the wild type protein. In order to compare the relative specific activity of the mutant α,1,2-mannosidases to the wild type enzyme, aliquots of medium containing equal α,1,2-mannosidase found in the medium, whereas the C485S mutant was expressed at a similar level as the wild type protein. Several clones expressing the C468S and C471S mutant enzymes had about 50% of the specific activity present in the wild type enzyme. The Km values were 0.7, 0.7, and 0.4 mM for the C468S, C471S, and C485S mutants, respectively, compared to 0.3 mM for the wild type enzyme. The C471S and C485S mutants were incubated with labeled Man9GlcNAc, and the products were analyzed by HPLC as described previously (14). The results indicate that the mutant α,1,2-mannosidases retained the same specificity as the wild type α,1,2-mannosidase (data not shown).

**TABLE III**

Cysteine-containing peptides identified by HPLC/MS of reduced and non-reduced trypsin digest of yeast α,1,2-mannosidase

| Treatment<sup>a</sup> | Peptide no.<sup>b</sup> | Amino acid nos. | MH<sup>c</sup> | Cysteine<sup>d</sup> |
|----------------------|------------------------|-----------------|-------------|-----------------|
| NR                  | t16                    | 335–360         | 4053.3      | Cys340 → Cys385 |
| R                   | t16                    | 380–390         | 4052.5      |                 |
| R                   | t19                    | 335–360         | 2731.8      | Cys340          |
| R                   | t19                    | 380–390         | 1322.9      | Cys385          |
| NR                  | t22                    | 429–475         | 5625.1      | Cys468 → Cys471 |
| R                   | t22                    | 429–475         | 5626.5      | Cys468, Cys471  |
| NR                  | t23                    | 479–492         | 1554.3      | Cys385          |
| R                   | t23                    | 479–492         | 1554.2      | Cys385          |

<sup>a</sup> Non-reduced (NR) or reduced (R).
<sup>b</sup> Peptides are numbered according to the amino acid sequence from the N to C terminus.
<sup>c</sup> Expected mass calculated from average molecular weights.
<sup>d</sup> An arrow indicates a disulfide bond, whereas a comma between two Cys residues indicates that the two amino acids are present in the same tryptic peptide.
Role of Cysteines in the Yeast Processing α1,2-Mannosidase

In the present work, we have shown using several methods that the yeast α1,2-mannosidase contains two disulfide bonds and one free thiol group in its catalytic domain, and we have identified their position in the primary sequence of the enzyme. Cys\(^{465}\) was found to contain the free thiol group by sequencing of labeled peptides following modification with radioactive iodoacetate and by HPLC/MS tryptic peptide mapping. The presence of a disulfide bond between Cys\(^{340}\) and Cys\(^{385}\) was demonstrated directly by sequencing a purified peptide containing this disulfide and by HPLC/MS mapping of tryptic peptides. The existence of the other disulfide bond between Cys\(^{468}\) and Cys\(^{471}\) was deduced from several observations. First, quantitation with DTNB and NTSB clearly demonstrated the presence of two disulfide bonds and one free thiol. Second, Cys\(^{468}\) was the only residue labeled with iodoacetate and no CM-Cys was formed from Cys\(^{468}\) and Cys\(^{471}\) following carboxymethylation. Third, a tryptic peptide (peptide \(t_{22}\)) containing only Cys\(^{468}\) and Cys\(^{471}\) was identified by HPLC/MS peptide mapping. Because there is no cleavable trypsic site between these two residues, no large effect is observed upon reduction of this peptide. However, the fact that this peptide containing both Cys\(^{468}\) and Cys\(^{471}\) was identified by HPLC/MS, in conjunction with the assignment of the other cysteine residues, supports the conclusion that the second disulfide bond is present between Cys\(^{468}\) and Cys\(^{471}\). Finally, a peptide containing Cys\(^{468}\) or Cys\(^{471}\) disulfide bonded to any of the other cysteine residues was never isolated by isolating disulfide-containing peptides or by HPLC/MS peptide mapping.

Treatment of the yeast α1,2-mannosidase with DTT clearly shows that at least one of the two disulfide bonds is essential to maintain its activity. This conclusion is supported by the mutagenesis studies demonstrating that neither the disulfide bond between Cys\(^{468}\) and Cys\(^{471}\) nor the free thiol group on Cys\(^{468}\) is essential for enzyme activity. This idea is supported by difficulties encountered in purifying the C471S mutant, most likely due to traces of protease activity. The second disulfide bond is therefore essential for the protein to acquire its proper conformation.

From alignment of the amino acid sequences of the known members of the α1,2-mannosidase family (Fig. 7), it is observed that Cys\(^{468}\) and Cys\(^{471}\) have been conserved through evolution. This observation indicates that this disulfide bond may also play an essential role for these enzymes, and it supports the conclusion that the yeast enzyme requires formation of the Cys\(^{468}\)-Cys\(^{471}\) disulfide bond to fold properly.

Although the Cys\(^{468}\)-Cys\(^{471}\) disulfide bond is not essential for enzyme activity, the binding affinity for the substrate and the specific activity of the C468S and C471S mutants decreased 2-fold compared to the wild type enzyme. It seems, therefore, that this disulfide bond stabilizes the enzyme. This idea is supported by difficulties encountered in purifying the C471S mutant, most likely due to traces of protease activity. The above observations, coupled with the fact that Cys\(^{468}\) is conserved in all the members of the family (Fig. 7), suggest that the region corresponding to Cys\(^{340}\) and Cys\(^{385}\) in the yeast enzyme is likely to be important for proper structure and catalytic activity in other members of the family.

Fig. 6. Expression of wild type and mutant α1,2-mannosidases in *P. pastoris*. The KM71 *P. pastoris* strain was transformed with either pHIL-S1, YpHMNS1, YpHC468S, YpHC471S, or YpHC485S. Transformed clones were grown in BMGY medium for 2 days, then induced in BMMY medium for 2 days. The medium was diluted 1/8 to transform the yeast. The medium was diluted 1/8 to transform the yeast. The medium was diluted 1/8 to transform the yeast. The medium was diluted 1/8 to transform the yeast. The medium was diluted 1/8 to transform the yeast. The medium was diluted 1/8 to transform the yeast. The medium was diluted 1/8 to transform the yeast.

| Human IA | Mouse IA | Rabbit IA | Mouse IB | Drosophila | A. Saitoi | P. citrinum | S. cerevisiae |
|----------|----------|-----------|----------|------------|-----------|-------------|--------------|
| TYIAEKWIRGL-...-HGLMHTPFNAGFMFL-A---AEPEAGMQHYESL | TYIAEKWIRGL-...-HGLMHTPFNAGFMFL-A---AEPEAGMQHYESL | TYIAEKWIRGL-...-HGLMHTPFNAGFMFL-A---AEPEAGMQHYESL | VFTGEMKWL-...-HGLMHTPFNAGFMFL-A---AEPEAGMQHYESL | TYLLASNYRTG-...-LQGSSMLYGTS-FOGSSFLIGT-...-VLNR-TOFDHF | TYLLASNYRTG-...-LQGSSMLYGTS-FOGSSFLIGT-...-VLNR-TOFDHF | TYLLASNYRTG-...-LQGSSMLYGTS-FOGSSFLIGT-...-VLNR-TOFDHF | WYGERQPLQHQLLEAGGMTHSLAEGSGL-...-SHGGLASSTEGSHLEARRPFQ |

Fig. 7. Alignment of the α1,2-mannosidase family. The present results demonstrate that mutation of Cys\(^{385}\) does not affect activity, showing that the free sulhydryl group is not required for activity of the yeast α1,2-mannosidase. This conclusion is only in apparent disagreement with a previous study showing inactivation of the rabbit α1,2-mannosidase.
with $\rho$-chloromercuribenzoate (30). This inactivation was only observed in the presence of very low Ca\(^{2+}\) concentration (0.01 mM), and normal enzyme activity was observed following $\rho$-chloromercuribenzoate treatment in the presence of 2 mM Ca\(^{2+}\). Furthermore, the location of the free sulfhydryl group is not conserved between the yeast enzyme and other members of the family and cysteine residues have not been implicated in the catalytic mechanism of any glycosidases to date. Two acidic residues are usually directly involved in catalysis for both inverting and retaining enzymes (31).

This study combines results from protein chemistry and mutagenesis in order to elucidate the role of the cysteine residues in the yeast $\alpha_1,2$-mannosidase. It is the only member of the family for which this type of study is possible, since it is the only Family 47 $\alpha_1,2$-mannosidase that has been produced in sufficient quantity as a recombinant enzyme.

**Acknowledgments**—We thank Dr. Anne English, George Tsaprailis, and Craig Fenwick at Concordia University (Montréal, Canada) for performing the HPLC/MS analysis. We thank Dr. Hugh Bennett and Susan James for performing the amino acid analysis. We thank Dr. Alex Bell for help in the analysis of N-terminal sequencing and amino acid composition data and for helpful discussion.

**REFERENCES**

1. Jelinek-Kelly, S., Akiyama, T., Saunier, B., Tkacz, J. S., and Herscovics, A. (1985) *J. Biol. Chem.* 260, 2253–2257
2. Jelinek-Kelly, S., and Herscovics, A. (1988) *J. Biol. Chem.* 263, 14757–14763
3. Ziegler, F. D., and Trimble, R. B. (1991) *Glycobiology* 1, 605–614
4. Burke, J., Lipari, F., Igdoura, S., and Herscovics, A. (1996) *Eur. J. Cell Biol.* 70, 298–305
5. Camirand, A., Heysen, A., Grondin, B., and Herscovics, A. (1991) *J. Biol. Chem.* 266, 15120–15127
6. Bause, E., Bieberich, E., Rolfs, A., Volker, C., and Schmidt, B. (1993) *J. Biochem.* 217, 525–540
7. Herscovics, A., Schneikert, J., Athanassiadis, A., and Moremen, K. W. (1994) *J. Biol. Chem.* 269, 9864–9871
8. Lal, A., Schutzbach, J. S., Forsee, W. T., Neame, P. J., and Moremen, K. W. (1994) *J. Biol. Chem.* 269, 9872–9881
9. Moremen, K. W., Trimble, R. B., and Herscovics, A. (1994) *Glycobiology* 4, 113–125
10. Scaman, C. H., Lipari, F., and Herscovics, A. (1996) *Glycobiology* 6, 265–270
11. Kerscher, S., Albert, S., Wucherpfennig, D., Heisenberg, M., and Schneuwly, S. (1995) *Dev. Biol.* 168, 613–626
12. Yoshida, T., and Ichishima, E. (1995) *Biochim. Biophys. Acta* 1263, 159–162
13. Inoue, T., Yoshida, T., and Ichishima, E. (1996) *Biochem. Biophys. Acta* 1255, 141–145
14. Lipari, F., and Herscovics, A. (1994) *Glycobiology* 4, 697–702
15. Lipari, F., Garcia-Salinas, B. J., and Herscovics, A. (1995) *Biochem. Biophys. Res. Commun.* 209, 322–326
16. Grondin, B., and Herscovics, A. (1992) *Glycobiology* 2, 369–372
17. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Chem.* 64, 81–88
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
19. Thannhauser, T. W., Konishi, Y., and Scheraga, H. A. (1984) *Anal. Biochem.* 138, 181–188
20. Jocelyn, P. C. (1987) *Methods Enzymol.* 143, 44–57
21. Carr, S. A., Barr, J. R., Roberts, G. D., Anumula, K. R., and Taylor, P. B. (1990) *Methods Enzymol.* 193, 508
22. Villa, S., DeFazio, G., and Canosi, U. (1989) *Anal. Biochem.* 177, 161–164
23. Schmelzer, C. H., Harris, R. J., Butler, D., Yedinak, C. M., Wagner, K. L., and Burton, L. E. (1993) *Arch. Biochem. Biophys.* 302, 484–489
24. Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) *Anal. Chem.* 64, 81–88
25. Markwell, M. A. K., Haas, S. M., Tolbert, N. E., and Bieber, L. L. (1981) *Methods Enzymol.* 72, 296–303
26. Laemmli, U. K. (1970) *Nature* 227, 680–685
27. Sorensen, S. B., Sorensen, T. L., and Breddam, K. (1991) *FEBS Lett.* 294, 195–197
28. Gross, E. (1986) *Methods Enzymol.* 11, 238–241
29. Robertson, J. G., Adams, G. W., Medzikradskey, K. P., Burlingame, A. L., and Villafranca, J. J. (1994) *Biochemistry* 33, 11563–11575
30. Forsee, W. T., Palmer, C. F., and Schutzbach, J. S. (1989) *J. Biol. Chem.* 264, 3869–3876
31. Wang, Q., Graham, R. W., Trimbur, D., Warren, R. A. J., and Withers, S. G. (1994) *J. Am. Chem. Soc.* 116, 11594–11595