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STRUCTURE AND MECHANISM OF ACTION OF THE YEAST CLASS 1 α1,2-MANNOSIDASE INVOLVED IN N-GLYCAN BIOSYNTHESIS

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This thesis is submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The *Saccharomyces cerevisiae* α1,2-mannosidase that removes one specific mannose residue from $Man_9GlcNAc_2$ during the biosynthesis of N-glycans is a member of the Class 1 α1,2-mannosidase family conserved through eukaryotic evolution. These α1,2-mannosidases are type II membrane proteins with an N-terminal transmembrane domain and a large C-terminal catalytic domain. High levels of recombinant yeast α1,2-mannosidase catalytic domain were produced using *Saccharomyces cerevisiae* (0.6 mg/l) and *Pichia pastoris* (30 mg/l) expression systems, permitting further characterization of the structural and mechanistic properties of the enzyme. Peptide analysis was applied to localize the two disulfide bonds (Cys^{340}-Cys^{385} and Cys^{468}-Cys^{471}) and free thiol group (Cys^{485}). Atomic absorption analysis and equilibrium dialysis with $^{45}Ca^{2+}$ were used to show that the enzyme binds one Ca^{2+} ion with high affinity. It was also demonstrated by proton nuclear magnetic resonance spectroscopy that the α1,2-mannosidase hydrolyzes the substrate with inversion of the anomeric configuration. Site-directed mutagenesis studies were used to determine the importance of the cysteine residues, the invariant acidic residues, and the putative EF hand Ca^{2+}-binding motif. It was shown that the disulfide bond formed by the invariant Cys^{340} and Cys^{385} residues is essential for the structural integrity of the α1,2-mannosidase, while the second disulfide bond and free thiol are not necessary for enzyme activity. It was also demonstrated that the EF hand motif is not the site for Ca^{2+} binding, whereas Asp^{275}, Glu^{279}, and Glu^{438} are important for Ca^{2+} binding, and Asp^{86}, Glu^{132}, Glu^{503}, and Glu^{526} are likely active site residues. The yeast α1,2-mannosidase is the only Class 1 α1,2-mannosidase for which both structural and mechanistic properties have been characterized, and it can serve as a model for the other enzymes of the family.

RÉSUMÉ

L' α1,2-mannosidase de *Saccharomyces cerevisiae* qui élimine un seul résidu de mannose de l'oligosaccharide $Man_9GlcNAc_2$ durant la formation des N-glycannes est membre d'une famille d'α1,2-mannosidases (Classe 1) conservées durant l'évolution des eukaryotes. Ces α1,2-mannosidases sont des protéines transmembranaires de type II ayant une région N-terminale hydrophobe suivie d'un domaine catalytique. Le domaine catalytique de l'α1,2-mannosidase de levure fut produit en quantité importante sous forme sécrétée dans le milieu de *Saccharomyces cerevisiae* (0.6 mg/l) et de *Pichia pastoris* (30 mg/l). L'obtention de cette forme recombinante a permis d'étudier la structure et le mécanisme catalytique de l'α1,2-mannosidase. On a établi la localisation des deux ponts disulfure (Cys^{340}-Cys^{385} et Cys^{468}-Cys^{471}) et de la fonction thiol (Cys^{485}) par analyses peptidiques. On a utilisé l'absorption atomique et la dialyse à l'équilibre avec $^{45}Ca^{2+}$ pour démontrer que l'α1,2-mannosidase contient un site de rétention du Ca^{2+} de haute affinité. On a déterminé, en utilisant la spectroscopie de résonance magnétique nucléaire protonique, que l'hydrolyse du substrat par l'α1,2-mannosidase procède avec inversion de la configuration anomérique. Des études de mutagénèse dirigée ont été faites afin de déterminer l'importance des résidus de cysteine, des acides aminés carboxyliques invariants et de la séquence correspondante à une boucle EF. Il fut demontré que le pont disulfure entre les résidus invariants Cys^{340} et Cys^{385} est essentiel à l'intégrité de l' α1,2-mannosidase, mais que l'autre pont disulfure et la fonction thiol ne sont pas nécessaires à l'activité enzymatique. Les resultats obtenus démontrent que la boucle EF n'est pas le site de liaison du Ca^{2+}, mais les résidus Asp^{275}, Glu^{279}, et Glu^{438} sont nécessaires à la rétention du Ca^{2+} tandis que les résidus Asp^{86}, Glu^{132}, Glu^{503} et Glu^{526} sont impliqués dans le site actif de l'enzyme. Comme l'α1,2-mannosidase de levure est le seul membre de la famille dont la structure et le mécanisme catalytique ont été étudiés, les données obtenues servent de modèle pour les autres membres de cette famille.

TABLE OF CONTENTS

FOREWARD

The present thesis includes the text of three published papers and one manuscript submitted for publication. Each chapter has its own numeration of figures and tables. The references of all the chapters are provided at the end of the thesis. The list of abbreviations precedes Chapter 1.

CONTRIBUTION OF AUTHORS

All the work described in this thesis has been performed under the supervision of Dr. Annette Herscovics.

The work described in Chapters 2, 4, and 5 was performed by myself.

Chapter 3 of the thesis includes Dr. Barbara J. Gour-Salin as a co-author: The *Saccharomyces cerevisiae* processing α1,2-mannosidase is an inverting glycosidase. F. Lipari, B.J. Gour-Salin, and A. Herscovics (1995) **Biochem. Biophys. Res. Comm. 209,** 322-326. Dr. Barbara J. Gour-Salin helped in the analysis of the NMR data and assisted in the presentation of the results. My primary responsibility was in the preparation of the enzyme and substrate for the NMR experiment. I was involved in writing the entire manuscript and in the preparation of the figures.

PUBLICATION OF THE WORK PRESENTED IN THIS THESIS

The work presented in Chapters 2 to 4 of the thesis has been published in scientific journals. The work described in Chapter 5 has been submitted.

CHAPTER 2: Production, purification, and characterization of recombinant yeast processing α1,2-mannosidase. F. Lipari and A. Herscovics (1994) **Glycobiology 4**, 697-702.

CHAPTER 3: The *Saccharomyces cerevisiae* processing α1,2-mannosidase is an inverting glycosidase. F. Lipari, B.J. Gour-Salin, and A. Herscovics (1995) **Biochem. Biophys. Res. Comm. 209,** 322-326.

CHAPTER 4: Role of the cysteine residues in the α1,2-mannosidase involved in *N*-glycan biosynthesis in *Saccharomyces cerevisiae*. The conserved Cys^{340} and Cys^{385} residues form an essential disulfide bond. F. Lipari and A. Herscovics (1996) **J. Biol. Chem. 271**, 27615-27622.

CHAPTER 5: Effect of mutagenesis of the invariant acidic residues in the Class 1 α1,2-mannosidase from *Saccharomyces cerevisiae* on enzyme activity and calcium binding. F. Lipari and A. Herscovics (1998) submitted.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. *Saccharomyces cerevisiae* and *Pichia pastoris* expression systems were established to produce milligram amounts of recombinant yeast α1,2-mannosidase. This was the first Class 1 α1,2-mannosidase available in milligram quantities as a recombinant protein. Knowledge gained from the studies of the yeast α1,2-mannosidase can be applied to the other Class 1 enzymes.

2. The yeast α1,2-mannosidase was shown to be an inverting glycosidase by proton nuclear magnetic resonance analysis of the reaction process.

3. The yeast α1,2-mannosidase contains two disulfide bonds (Cys^{340}-Cys^{385} and Cys^{468}-Cys^{471}) and one free sulfhydryl group (Cys^{485}) as determined by peptide mapping. The Cys^{340}-Cys^{385} disulfide bond is essential whereas the other disulfide bond and free thiol group are not important for activity as demonstrated by site-directed mutagenesis of the cysteine residues.

4. It was shown that the yeast α1,2-mannosidase binds one Ca^{2+} ion with high affinity by atomic absorption analysis and equilibrium dialysis experiments.

5. Contrary to previous assumptions, it was demonstrated that the putative EF hand motif in the yeast α1,2-mannosidase is not the site for Ca^{2+} binding. Site-directed mutagenesis of essential residues of the EF hand motif had no effect on the Ca^{2+}-binding properties of the α1,2-mannosidase.

6. It was established that Asp^{275}, Glu^{279}, and Glu^{438} are important for Ca^{2+} binding. Mutation of any of these three residues results in the loss of Ca^{2+} binding in native polyacrylamide gels.

7. Asp^{86}, Glu^{132}, Glu^{503} and Glu^{526} are potential active site residues as determined by site-directed mutagenesis and kinetic analysis of the mutants.

ACKNOWLEDGMENTS

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I would also like to acknowledge the members of the lab. Dr. Pedro Romero is an exceptional scientist and his expertise was very helpful during my laboratory research. I am very happy to have worked alongside Barry Sleno who was always in a good mood. He also taught me how to handle the equipment that I used extensively throughout my studies, such as the gel electrophoresis units, the HPLC system, and the protein purification apparatus. As undergraduate students, Daniel Currie, Terry Poon, Denise Au Yeung, and Shin Numao performed some site-directed mutagenesis under my supervision. They also taught me the benefits of supervising. I thank the remaining members in the lab, both past and present, for their friendship and helpful advice.

I thank Dr. Ted Meighen and Dr. Robert Mackenzie for patiently taking time to advise me during my Ph.D. thesis. Their expertise in biochemistry was extremely helpful and some critical decisions would not have been made without their input.

Dr. Alex Bell is an excellent protein chemist and his advice was crucial during the work performed in Chapter 4.

Dr. Hugh Bennett and Susan James generously assisted me with the amino acid analysis and taught me the basics of this technique.

I thank Dr. Anne English for allowing us to perform the HPLC/mass spectrometry at Concordia University. I appreciated the help of George Tsaprailis and Craig Fenwick in performing the HPLC/mass spectrometry.

Dr. Joanne Turnbull and Dinesh Cristendat at Concordia University kindly provided advice on protein chemistry in general and helped with the circular dichroism experiments.

In Chapter 2, the YpJLO1 vector was supplied by Dr. Thierry Vernet at the Biotechnology Research Institute (Montréal , Québec, Canada).

In Chapter 3, Dr. Francoise Sauriol performed the NMR analysis.

In Appendix 1, Barry Sleno, Shin Numao, and Denise Au Yeung performed most of the technical work under my supervision.

The scholarships provided from the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche for my graduate studies were greatly appreciated.

LIST OF FIGURES AND SCHEMES

LIST OF TABLES

ABBREVIATIONS

BMGY, buffered glycerol-complex
BMMY, buffered methanol-complex
BSA, bovine serum albumin
CD, circular dichroism
CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CM, carboxymethyl
DMJ, 1-deoxymannojirimycin
DTNB, 5,5'-dithiobis(2-nitrobenzoate)
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
EGTA, ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
Endo H, endoglycosidase H
ER, endoplasmic reticulum
Gal, galactose
Glc, glucose
GlcNAc, N-acetylglucosamine
Gdn-HCl, guanidine hydrochloride
HPLC, high pressure liquid chromatography
Man, mannose
MES, 2-[N-morpholino]ethanesulphonic acid
MS, mass spectrometry
NMR, nuclear magnetic resonance
NTB, 2-nitro-5-thiobenzoate
NTSB, 2-nitro-5-thiosulfobenzoate
P, phosphate
PA, pyridylamino
PAGE, polyacrylamide gel electrophoresis
PCR, polymerase chain reaction
PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid]
PMSF, phenylmethylsulfonyl fluoride
PNGase F, peptide:N-glycosidase F
pNP-Man, ρ-nitrophenyl-α-D-mannopyranoside
SA, sialic acid

SDS, sodium dodecyl sulfate

TFA, trifluoroacetic acid

TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone

Tris, tris(hydroxymethyl)aminomethane

UGGT, UDP-glucose:glycoprotein glucosyltransferase

CHAPTER 1

General Introduction

Proteins of the secretory pathway in eukaryotic cells may be post-translationally modified by attachment and further elaboration of carbohydrates at specific asparagine residues. These asparagine-linked (N-linked) carbohydrates have been implicated in normal biological events such as protein folding and targeting, embryogenesis, and the immune response, and also in disease processes such as viral infection and metastasis.

The biosynthesis of N-linked oligosaccharides requires the action of many glycosyltransferases and glycosidases in the endoplasmic reticulum and Golgi apparatus. In general, biosynthesis occurs in three stages: 1) formation of a lipid-linked oligosaccharide and its transfer onto the protein; 2) trimming of the oligosaccharide by α-glucosidases and α-mannosidases; and 3) elaboration of the N-linked oligosaccharide by sequential addition of monosaccharides.

The essential participation of α1,2-mannosidases in the second stage of biosynthesis has indicated that these enzymes may be potential targets for antiviral and antimetastatic agents. In addition, there is growing evidence that the action of α1,2-mannosidases in the endoplasmic reticulum is an important step in the quality control of glycoprotein folding.

Characterization of various α1,2-mannosidases involved in N-linked oligosaccharide biosynthesis from yeast, insects, and mammals has established the existence of a family of homologous α1,2-mannosidases, which are classified as Class 1 α1,2-mannosidases. Although these enzymes play such a key role in N-glycan biosynthesis and are potential therapeutic targets, there was little information previously known about their structure and mechanism of action. The α1,2-mannosidases are Ca^{2+}-dependent enzymes with an N-terminal transmembrane domain and a large C-terminal catalytic domain that act only on terminal α1,2-linked mannose residues of the oligosaccharide substrate, but the number and order of mannose residues removed depends on the specific α1,2-mannosidase. The main reason for the lack of knowledge was the low availability of significant amounts of purified protein; therefore, a system was needed to obtain enough α1,2-mannosidase for further analysis. This thesis describes the production of the yeast Class 1 α1,2-mannosidase as a recombinant protein in milligram amounts and documents the information gathered on its structural and catalytic properties. The characterization of the yeast enzyme presented here serves as a model for the other members of the family of α1,2-mannosidases.

A: ROLE OF α1,2-MANNOSIDASES IN THE BIOSYNTHESIS OF ASPARAGINE-LINKED OLIGOSACCHARIDES

ASPARAGINE-LINKED OLIGOSACCHARIDES

Definition and general function

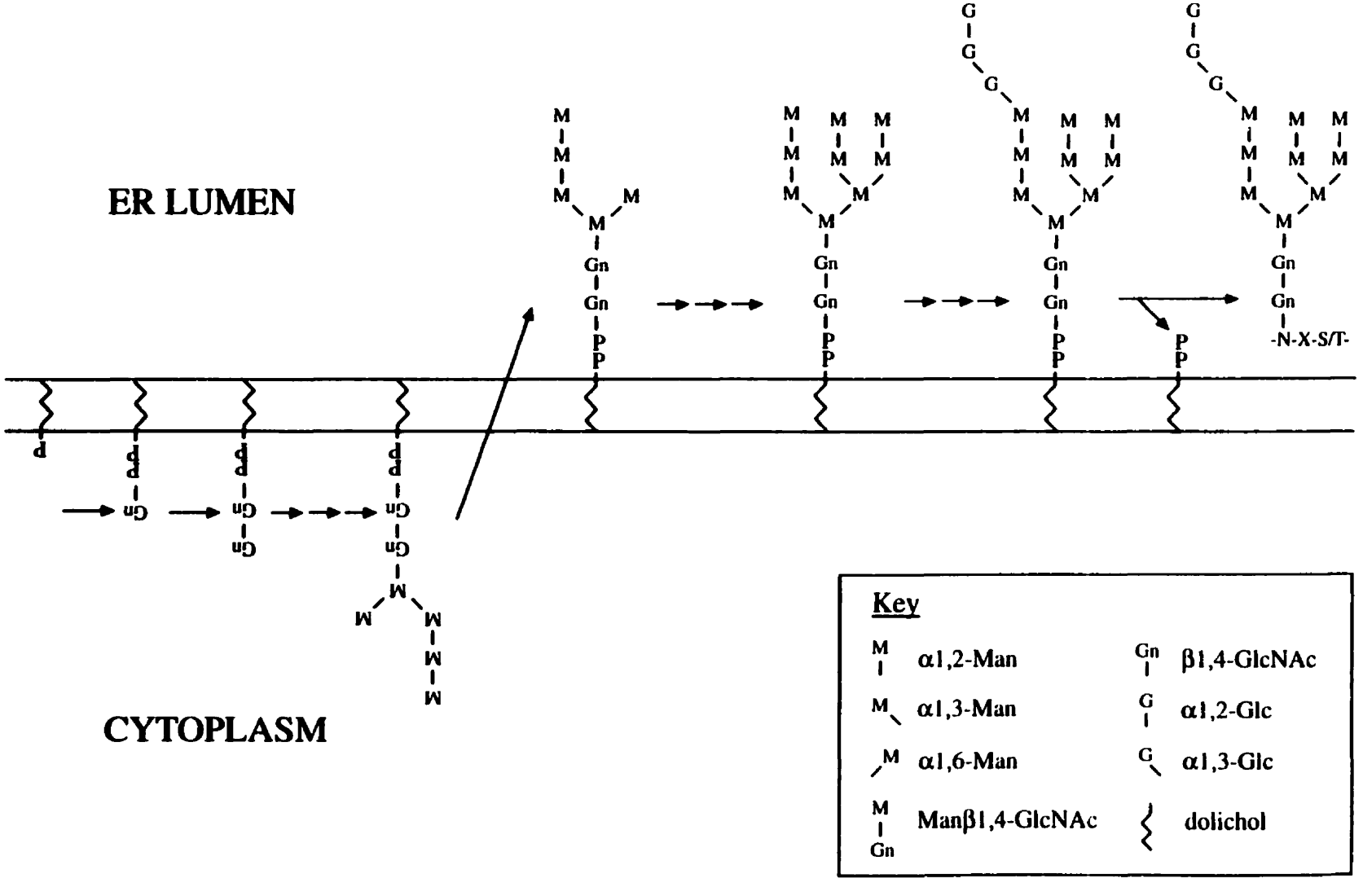
Attachment of asparagine-linked (N-linked) oligosaccharides at specific Asn-X-Ser/Thr consensus sites, where X is any amino acid except proline, is a post-translational modification occurring only in proteins of the secretory pathway of eukaryotic cells and is termed N-glycosylation. The oligosaccharides may also be referred to as N-glycans. Glycoproteins may carry one or more N-linked glycans. The structure of each glycan is highly variable and heterogeneity in the carbohydrate is often seen at a given site of a glycoprotein. Furthermore, if there are two or more N-linked oligosaccharides per glycoprotein, then each site may carry a different glycan. Therefore, N-glycosylation is a highly complex post-translational modification.

The functions of N-glycans are diverse and depend on the specific glycoprotein (reviewed by Varki, 1993; Fiedler and Simons, 1995; Dwek, 1996). The N-linked oligosaccharide(s) may be essential to the glycoprotein itself and may be required for folding, solubility, stability, targeting, protection from proteases, or bioactivity. In addition, the N-glycans at the cell suface can play an important role in cellular interactions including adhesion to other cells or to pathogens such as bacteria, viruses, or parasites.

Biosynthesis

The biosynthesis of N-glycans occurs in three stages (reviewed by Kornfeld and Kornfeld, 1985). i) In the first stage, the oligosaccharide is assembled by sequential addition of monosaccharides in specific linkages (α1,2-, α1,3-, etc.) to dolichyl phosphate (Fig. 1)(reviewed by Krag, 1998). The initial step is the attachment of GlcNAc-phosphate to dolichyl phosphate on the cytosolic side of the endoplasmic reticulum (ER). An N-acetylglucosamine and mannose residues are then added, and at one point during the assembly the oligosaccharide is translocated to the lumenal side of the ER. Currently, it is thought that the pentamannosyl oligosaccharide is the intermediate translocated (Krag, 1998). The remaining mannose and glucose residues are added, and then the glycan ($Glc_3Man_9GlcNAc_2$) is transferred to the newly formed polypeptide by the oligosaccharyltransferase complex (Silberstein and Gilmore, 1996).

FIGURE 1: Assembly of the dolichol-linked oligosaccharide precursor. For simplicity, only some intermediates formed during the biosynthesis of the dolichol-linked oligosaccharide are shown. The oligosaccharide is assembled by the sequential addition of monosaccharides and is then transferred onto the protein. The protein is represented by the N-glycosylation consensus sequence -N-X-S/T-. Three consecutive arrows indicates that more than one reaction occurs.


ER LUMEN
CYTOPLASM
-N-X-S/T-
Key
α1,2-Man
α1,3-Man
α1,6-Man
Manβ1,4-GlcNAc
β1,4-GlcNAc
α1,2-Glc
α1,3-Glc
dolichol

Although this initial stage has been essentially conserved through eukaryotic evolution, there are exceptions. For example, in Trypanosomatid parasites non-glucosylated carbohydrates ($Man_{6-9}GlcNAc_2$) are transferred (Parodi, 1993), and $Man_7GlcNAc_2$ can be transferred in F9 teratocarcinoma cells (Romero and Herscovics, 1986).

ii) The second stage of N-glycan biosynthesis has been partially conserved through evolution and involves processing α-glucosidases and α-mannosidases that modify the N-linked oligosaccharide in the ER and Golgi apparatus. In *Saccharomyces cerevisiae*, the three glucose residues and only one specific mannose residue are cleaved (Fig. 2), whereas in mammalian cells the three glucose residues and up to six mannose residues are removed (Fig. 3).

iii) The third stage of biosynthesis mainly involves the addition of monosaccharides to the preformed oligosaccharide by glycosyltransferases in the Golgi apparatus. In yeast, the process begins with the addition of an α1,6-linked mannose residue at a specific position of the $Man_8GlcNAc_2$ oligosaccharide (Fig. 2)(reviewed by Herscovics and Orlean, 1993). Further addition of mannose residues then results in two types of structures, depending on the glycoprotein (Fig. 2). The structure referred to as the mature core can have from 8 - 13 mannose residues, and in the other case, up to 200 mannose residues may be added to form mannan structures. In mammalian cells, N-acetylglucosaminyltransferase I adds a β1,2-linked N-acetylglucosamine residue to a specific mannose residue of the pentamannosyl structure (Fig. 3)(reviewed by Schachter, 1991). From this intermediate, two different types of carbohydrate structures are made, termed hybrid and complex (Fig. 3). Hybrid structures contain five mannose residues in addition to several other residues such as N-acetylglucosamine and galactose, whereas complex structures contain only three mannose residues and up to six oligosaccharide branches.

It is evident that the biosynthetic pathway of N-linked glycans is highly complex and variable when all organisms from yeast to mammals are considered. For the purpose of this thesis, only the biosynthetic intermediates and the enzymes involved in the second stage, also referred to as the processing pathway, are discussed further. The reactions catalyzed in mammalian cells are numbered in the text according to Fig. 3. The biochemical properties of the processing glycosidases have been reviewed (Moremen *et al.*, 1994; Herscovics, 1998). Significant progress has been made in the determination of the amino acid sequences for many enzymes in this processing pathway and, in many cases, homologous proteins from different organisms have now been cloned.

α-Glucosidases I and II: Following transfer of the $Glc_3Man_9GlcNAc_2$ oligosaccharide from the lipid onto the polypeptide, the terminal α1,2-linked glucose

FIGURE 2: The N-glycan processing pathway in *Saccharomyces cerevisiae*. Each number indicates a certain enzyme: (**1**) α-glucosidase I; (**2**) α-glucosidase II; (**3**) α1,2-mannosidase; (**4**) α1,6-mannosyltransferase. Examples of the two types of oligosaccharides made in yeast are shown. Three consecutive arrows indicates that more than one reaction occurs.

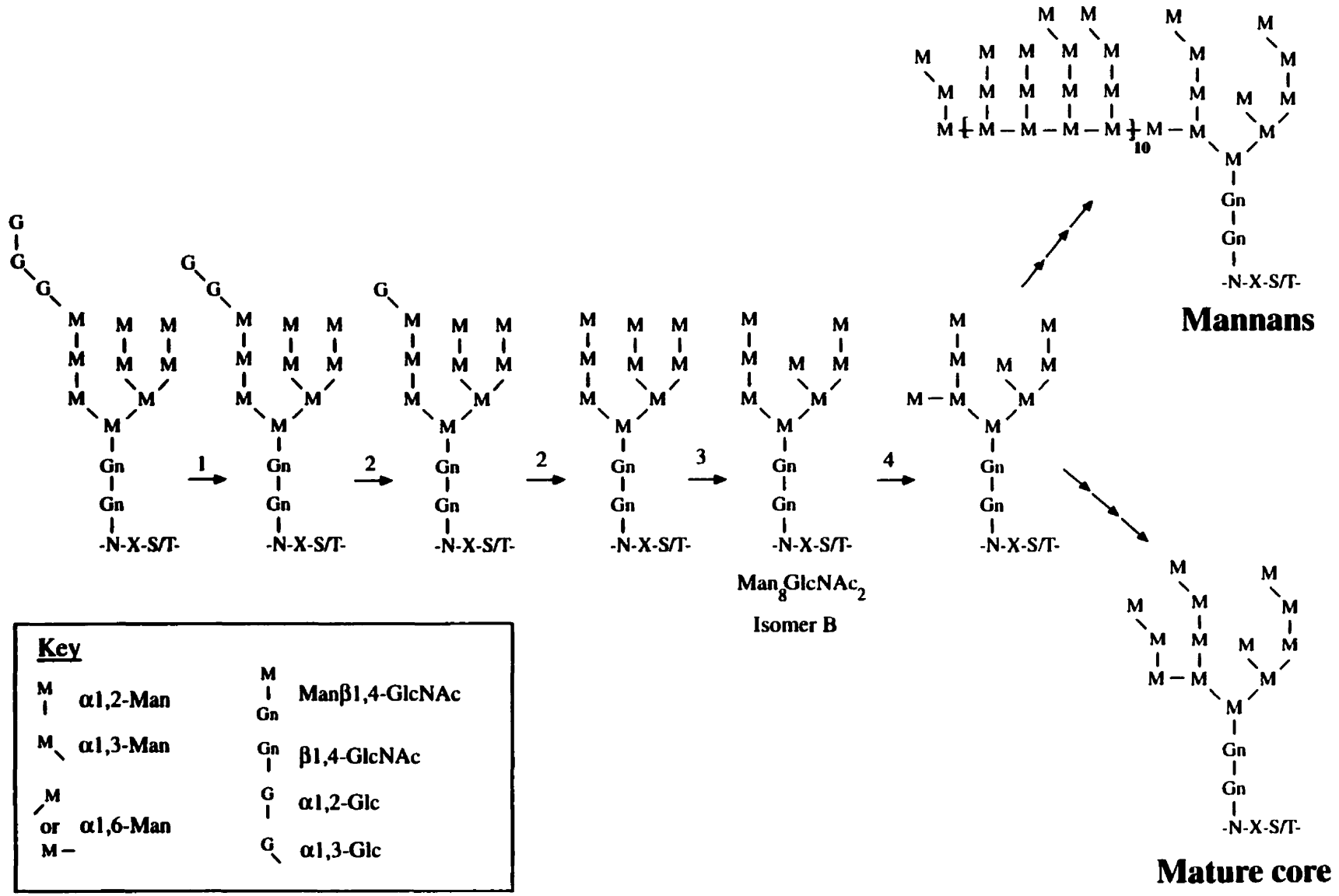
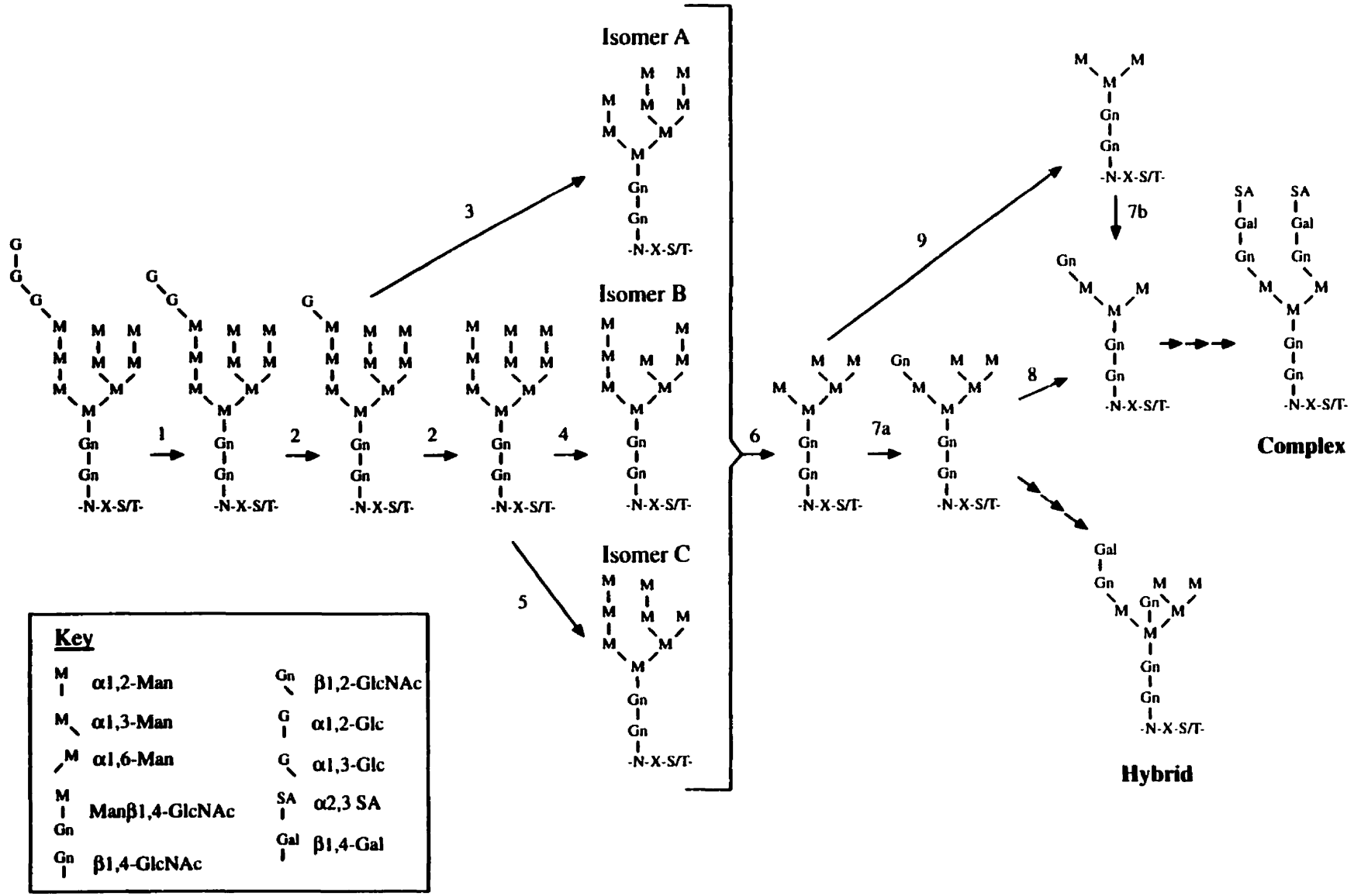

Mannans
Mature core
Man8GlcNAc2
Isomer B
-N-X-S/T-
1
2
2
3
4
Key
α1,2-Man
α1,3-Man
or α1,6-Man
Manβ1,4-GlcNAc
β1,4-GlcNAc
α1,2-Glc
α1,3-Glc

FIGURE 3: The N-glycan processing pathway in mammals. Each number indicates a certain enzyme: (**1**) α-glucosidase I; (**2**) α-glucosidase II; (**3**) endo-α-mannosidase; (**4**) α1,2-mannosidase (forms $Man_8GlcNAc_2$ isomer B); (**5**) α1,2-mannosidase (forms $Man_8GlcNAc_2$ isomer C); (**6**) α1,2-mannosidases (remove all α1,2-linked mannose residues); (**7a** and **7b**) N-acetylglucosaminyltransferase I; (**8**) α-mannosidase II; (**9**) α-mannosidase III. Examples of the two types of oligosaccharides made in mammals are shown. Three consecutive arrows indicates that more than one reaction occurs.


Isomer A
Isomer B
Isomer C
Complex
Hybrid
-N-X-S/T-
1
2
2
3
4
5
6
7a
7b
8
9
Key
α1,2-Man
α1,3-Man
α1,6-Man
Manβ1,4-GlcNAc
β1,4-GlcNAc
β1,2-GlcNAc
α1,2-Glc
α1,3-Glc
α2,3 SA
β1,4-Gal

residue is removed by α-glucosidase I (**1**). α-Glucosidase I was first cloned from a human hippocampus cDNA library (Kalz-Füller *et al.*, 1995). Very recently, a previously characterized yeast protein (Cwh41p), which was shown to be essential for cell wall integrity (Jiang *et al.*, 1996), was demonstrated to have significant similarity at the amino acid level to human α-glucosidase I (Romero *et al.*, 1997). Genetic and functional assays were used to show that yeast *CWH41* encodes α-glucosidase I (Romero *et al.*, 1997; Simons *et al.*, 1998).

The two remaining α1,3-linked glucose residues are removed by α-glucosidase II (**2**). Purification of the rat liver and mouse α-glucosidases demonstrated that the mammalian enzyme contains two subunits (α and β) and the cDNA sequences for these subunits from human (Trombetta *et al.*, 1996) and mouse (Arendt and Ostergaard, 1997) were cloned. It was demonstrated that the catalytic function is in the α subunit, while the β subunit may be required for ER localization. Sequence analysis indicated that the human α subunit shared amino acid sequence similarity to a yeast protein and its catalytic activity was established by gene disruption, but no homologue of the β subunit was found in yeast (Trombetta *et al.*, 1996). The α-glucosidase II α subunit is a member of Family 31 glycosidases, which includes lysosomal α-glucosidase, according to the glycosidase classification based on similarity in primary structure (Henrissat and Bairoch, 1996).

Endo-α-mannosidase: An alternate route for removal of glucose from the oligosaccharide involves endo-α-mannosidase (**3**) that releases the disaccharide Glcα1,3-Man. This is the only glycosidase involved in N-glycan biosynthesis that cleaves internal glycosidic bonds and *in vitro* studies have shown that the preferred substrates for this enzyme are $Glc_1Man_{4-9}GlcNAc$ (Lubas and Spiro, 1988). It was shown that the activity is not found in yeast, protozoa, and plants, but is present in mammals, birds, reptiles, and fish (Dairaku and Spiro, 1997). Hence, this processing reaction seems to be a late evolutionary event. The enzyme was recently cloned from rat liver and showed no similarity to any other known proteins (Spiro *et al.*, 1997).

α1,2-Mannosidases: The oligosaccharide containing 8 or 9 mannose residues and two N-acetylglucosamine residues is acted upon by α1,2-mannosidases. The extent and order of removal of α1,2-linked mannose residues varies depending on the organism, cell type, and individual N-linked oligosaccharide and may involve more than one α1,2-mannosidase.

In *S. cerevisiae* only one mannose is removed from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$ isomer B (Fig. 2) (Byrd *et al.*, 1982). This highly specific enzyme was the first α1,2-mannosidase involved in N-glycan biosynthesis to be cloned (Camirand *et al.*,

1991). $Man_8GlcNAc_2$ isomer B has been identified on ovalbumin (Ceccarini *et al.*, 1984) and human IgM (Cohen and Ballou, 1980) and α1,2-mannosidase activity producing isomer B has been characterized in the ER of mammalian cells (**4**) (Bischoff *et al.*, 1986; Rizzolo and Kornfeld, 1988; Weng and Spiro, 1993; Lal *et al.*, 1998), but no enzyme with this specificity has yet been cloned, other than the yeast α1,2-mannosidase. Formation of $Man_8GlcNAc_2$ isomer C from $Man_9GlcNAc_2$ has been reported to occur in the ER of mammalian cells (**5**) (Weng and Spiro, 1993), but an enzyme with this strict specificity has not yet been purified.

All other α1,2-mannosidases (**6**) characterized to date from insect, plant, chicken, and several mammalian sources are able to remove all four α1,2-linked mannose residues from $Man_9GlcNAc_2$; however, their substrate specificity and order of removal of mannose residues may vary and is not yet fully understood (Herscovics, 1998; Lal *et al.*, 1998). These enzymes form a family of homologous proteins classified as Class 1 α1,2-mannosidases and the current knowledge of their structural and biochemical properties, as well as their substrate specificity, will be explained in detail in a subsequent section.

α-Mannosidases and N-acetylglucosaminyltransferase I: $Man_5GlcNAc_2$, formed by the action of α-glucosidases and α-mannosidases, is a substrate for β1,2-N-acetylglucosaminyltransferase I (GnTI)(**7a**) that initiates the third phase of N-glycan biosynthesis in eukaryotes such as nematodes, insects, plants and vertebrates. $GlcNAcMan_5GlcNAc_2$ is then either modified by glycosyltransferases to form hybrid oligosaccharide structures or is trimmed by Golgi α-mannosidase II (**8**). α-Mannosidase II does not remove the mannose residues unless the terminal N-acetylglucosamine residue is present (Tulsiani *et al.*, 1982). This enzyme has been cloned from insects (Foster *et al.*, 1995; Jarvis *et al.*, 1997) and mammals (Moremen and Robbins, 1991; Misago *et al.*, 1995) and gene disruption of α-mannosidase II in mice has recently revealed the existence of an alternate biosynthetic route in mammalian cells (Chui *et al.*, 1997). Instead of the modification of $Man_5GlcNAc_2$ by GnTI, it is trimmed directly by α-mannosidase III (**9**) that removes two mannose residues from $Man_5GlcNAc_2$. GnTI then adds an N-acetylglucosamine residue to form $GlcNAcMan_3GlcNAc_2$ (**7b**), which is further modified to form complex oligosaccharides.

CHARACTERISTICS OF EUKARYOTIC α-MANNOSIDASES

As the α1,2-mannosidases involved in the second stage of N-linked oligosaccharide biosynthesis were initially characterized and purified from plants and animals, it became evident that they shared biochemical characteristics distinct from the

α-mannosidases (e.g. α-mannosidase II) that act later in the pathway (reviewed by Daniel *et al.*, 1994; Moremen *et al.*, 1994; Herscovics, 1998)). One method of distinguishing between the two classes of enzymes was by testing their sensitivity to inhibition by certain monosaccharide analogues. The α1,2-mannosidases are inhibited by the pyranose analogue, 1-deoxymannojirimycin (DMJ), but are not inhibited by the furanose analogue, swainsonine, whereas α-mannosidase II is inhibited by swainsonine, but not by DMJ. Another difference is their sensitivity to metal ions and their substrate specificity. The α1,2-mannosidases are Ca^{2+}-dependent and cannot hydrolyze aryl α-mannosides such as *p*-nitrophenyl-α-D-mannopyranoside (pNP-Man) or 4-methylumbelliferyl-α-mannoside, whereas the Ca^{2+}-independent α-mannosidases are not as specific for the linkage and can hydrolyze α1,2-, α1,3-, and α1,6-linked mannose residues and aryl α-mannosides.

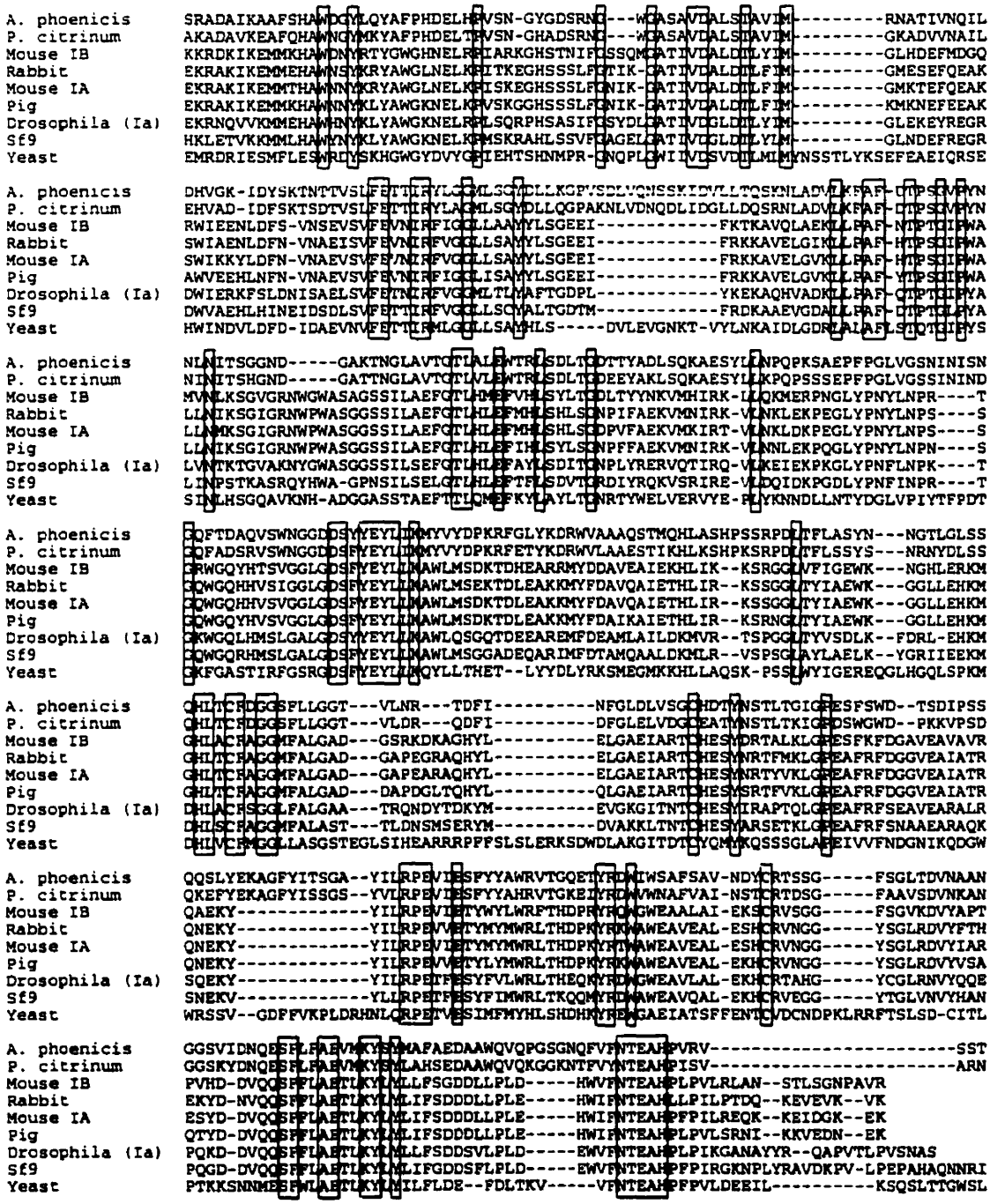
From primary amino acid sequences of cloned enzymes, it was observed that the α1,2-mannosidases have been conserved through eukaryotic evolution (reviewed by Moremen, *et al.*, 1994). This family of α1,2-mannosidases are classified as Class 1 α1,2-mannosidases and are distinct from the Class 2 α-mannosidases that include α-mannosidase II as well as cytosolic and lysosomal α-mannosidases. The Class 1 enzymes belong to glycosidase Family 47, whereas the Class 2 enzymes belong to Family 38 glycosidases (Henrissat and Bairoch, 1996).

CLASS 1 / FAMILY 47 α1,2-MANNOSIDASES

The primary structure of yeast, fungal, insect, and mammalian Class 1 α1,2-mannosidases have been determined (reviewed by Herscovics, 1998). They are either type II transmembrane proteins or possibly secreted enzymes containing a signal peptide as in the case of the fungal α1,2-mannosidases. The amino acid similarity observed among all the α1,2-mannosidases is within the large C-terminal catalytic domain of these enzymes (Schweden *et al.*, 1986; Forsee *et al.*, 1989; Schweden and Bause, 1989; Lipari and Herscovics, 1994; Schneikert and Herscovics, 1994; Lal *et al.*, 1998). As seen in Fig. 4 there are several areas with a high degree of identity and many invariant amino acids throughout the catalytic domains.

A summary of the current knowledge of the Class 1 α1,2-mannosidases for which the primary structure is known is provided in the following section. In addition, purified enzymes with properties very similar to the Class 1 α1,2-mannosidases are also presented.

FIGURE 4: Alignment of the catalytic domains of the Class 1/Family 47 α1,2-mannosidases. The catalytic domains of the α1,2-mannosidases were aligned by the Clustal Method using the Gene Jockey II software. Each sequence is identified according to the name most commonly used for the enzyme. The human α1,2-mannosidases IA and IB are not represented since they are over 90 % identical to the mouse α1,2-mannosidases IA and IB, respectively. The sequences presented begin at amino acid 41 for the *A. phoenicis* α1,2-mannosidase (Inoue *et al.*, 1995), amino acid 39 for *P. citrinum* (Yoshida and Ichishima, 1995), amino acid 179 for mouse IB (Herscovics *et al.*, 1994), amino acid 196 for mouse IA (Lal *et al.*, 1994), amino acid 200 for pig (Bieberich *et al.*, 1997), amino acid 175 for *Drosophila* (Ia) (Kerscher *et al.*, 1995), amino acid 196 for Sf9 (Kawar *et al.*, 1997), and amino acid 37 for yeast (Camirand *et al.*, 1991), according to the published primary sequences. The entire primary sequence is not available for the rabbit α1,2-mannosidase (Lal *et al.*, 1994). The invariant amino acids are indicated with a *box*.


A. phoenicis
P. citrinum
Mouse IB
Rabbit
Mouse IA
Pig
Drosophila (Ia)
Sf9
Yeast

Saccharomyces cerevisiae: The yeast α1,2-mannosidase is highly specific for the production of $Man_8GlcNAc_2$ isomer B from $Man_9GlcNAc_2$ (Jelinek-Kelly *et al.*, 1985). The gene for this enzyme (*MNS1*) encodes a 63 kDa protein with an N-terminal hydrophobic transmembrane domain and three potential N-glycosylation sites (Camirand *et al.*, 1991). Prior to its cloning, the enzyme was purified both as a soluble enzyme in which the transmembrane domain was proteolytically released during purification (Jelinek-Kelly and Herscovics, 1988), and as a transmembrane domain-containing enzyme solubilized by CHAPS (Ziegler and Trimble, 1991). The soluble and membrane form of the enzyme migrate at about 60 and 66 kDa, respectively, on non-reducing SDS-PAGE, although two bands of about 44 and 22 kDa are observed on reducing SDS-PAGE for both. The two bands observed upon reduction are due to a proteolytic clip, which suggested that at least one disulfide bond is present. The optimal pH for activity is 6.5-6.8 and α-Man1,2α-Man-OMe and pNP-Man are not substrates. A K_m of 0.2 mM for $Man_9GlcNAc$ was determined for the CHAPS-solubilized enzyme. The enzyme is inactivated upon incubation with metal chelators such as EDTA and EGTA and Ca^{2+} is required to completely restore activity after treatment with chelators. The apparent K_a for Ca^{2+} was demonstrated to be in the range of 10 μM for the CHAPS-solubilized enzyme. Activity is at least 50 % inhibited in the presence of 25 mM Tris or 50 μM DMJ. The protein contains about 4 kDa of N-linked oligosaccharides, suggesting that two to three of the potential N-glycosylation sites are used.

The yeast α1,2-mannosidase was recently localized to the ER by immunocytochemistry and immunoelectron microscopy (Burke *et al.*, 1996), which is consistent with the previous evidence for its localization in that compartment (Esmon *et al.*, 1984; Verostek *et al.*, 1993).

Disruption of the *MNS1* gene shows no obvious phenotype. Analysis of the oligosaccharide structures indicated that $Man_8GlcNAc_2$ isomer B is no longer formed in these cells, but addition of outer chain mannose residues to the core $Man_9GlcNAc_2$ oligosaccharide is not obstructed since there is little effect on the structures of N-glycans (Puccia *et al.*, 1993).

All other studies performed to date on the yeast α1,2-mannosidase are an integral part of this thesis.

Aspergillus phoenicis and Penicillium citrinum: Two similar α1,2-mannosidases believed to be secreted from the filamentous fungi, *Penicillium citrinum* (Yoshida *et al.*, 1993) and *Aspergillus phoenicis* (previously called *A. saitoi*) (Ichishima *et al.*, 1981), have very distinct biochemical properties compared to the other Class 1 α1,2-

mannosidases. These proteins are 56-57 kDa and are 70 % identical (Inoue *et al.*, 1995; Yoshida and Ichishima, 1995). They have a pH optimum of 5.0 and cannot hydrolyze pNP-Man. Although the *P. citrinum* α1,2-mannosidase is able to remove all four mannose residues from $Man_9GlcNAc_2$-PA, at lower enzyme levels the degradation is retarded at a specific isomer of $Man_7GlcNAc_2$-PA (Yoshida *et al.*, 1998). DMJ was shown to be a competitive inhibitor of the *P. citrinum* enzyme with a K_i = 105 μM (Yoshida *et al.*, 1994). It was reported that the fungal enzymes do not require Ca^{2+} for activity (Yoshida *et al.*, 1993; Yoshida and Ichishima, 1995).

An experiment to identify possible active site residues in the *P. citrinum* α1,2-mannosidase was performed (Yoshida *et al.*, 1994). It was initially demonstrated that chemical modification with a carboxyl specific reagent, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide, results in inactivation of the enzyme and that DMJ is able to partially protect the enzyme from this inactivation. Labeling of the enzyme with the carbodiimide in the presence of DMJ followed by peptide analysis resulted in the identification of a carboxylate residue (Asp^{375}) that was protected. The experiment suggests that this amino acid is in the active site and may be involved in binding to DMJ.

Mutagenesis of conserved acidic residues (Asp and Glu) was performed in the *A. phoenicis* enzyme (Fujita *et al.*, 1997). Five amino acids were found to be crucial for activity, but kinetic parameters (K_m and k_{cat}), which would have been helpful in determining potential functions for these residues, were not determined for the mutants.

Insects: The two insect α1,2-mannosidases cloned so far, one from *Spodoptera frugiperda* (Sf9 cells) (Kawar *et al.*, 1997) and the other from *Drosophila melanogaster* (Kerscher *et al.*, 1995), share 57% identity at the protein level.

The Sf9 α1,2-mannosidase cDNA encodes a protein of 75 kDa. *In vivo* expression of the cDNA produced α1,2-mannosidase activity against $Man_9GlcNAc$ that was inhibited by DMJ and inactivated by EDTA. Southern blot analysis indicated that there are two related α1,2-mannosidase genes in the Sf9 genome (Kawar *et al.*, 1997). Another α1,2-mannosidase was purified from baculovirus-infected *Spodoptera frugiperda* that has all the characteristics of a Class 1 α1,2-mannosidase (Ren *et al.*, 1995). The molecular mass is 63 kDa on SDS-PAGE and its activity is dependent on divalent cations, which is optimal for Ca^{2+} at pH 6.0. It is inhibited by DMJ (50 % at 20 μM), but not by swainsonine, and does not use pNP-Man as substrate. The preferred substrate is $Man_6GlcNAc_2$-ol, compared to $Man_{7-9}GlcNAc_2$-ol. The relationship between the purified and cloned Sf9 α1,2-mannosidases is not known and a further understanding of mannose trimming in this organism awaits the cloning of the second α1,2-mannosidase gene.

Upon characterization of a gene responsible for a developmental defect in *Drosophila melanogaster*, the *mas-1* gene encoding Class 1 α1,2-mannosidases was isolated from a genomic library (Kerscher *et al.*, 1995). It contains four exons and two promoters resulting in two transcripts that express two type II transmembrane proteins with different N-terminals termed MAS-Ia and MAS-Ib. Although it was claimed that the *Drosophila mas-1* gene encodes α1,2-mannosidase activity, no detail of the biochemical evidence was reported.

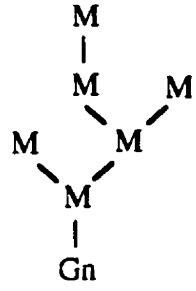
Mammals: Two different mouse and human α1,2-mannosidase cDNAs were isolated, which are now classified as α1,2-mannosidases IA and IB and share 64% identity at the amino acid level (Herscovics *et al.*, 1994; Lal *et al.*, 1994; Bieberich and Bause, 1995; Tremblay *et al.*, 1998). Both the human IA and IB proteins share over 90 % identity with the corresponding mouse proteins. The mouse and human α1,2-mannosidase IB genes contain 13 exons and span over 60 kbp of genomic DNA, and are located on chromosomes 3F2 and 1p13, respectively, which are syntenic regions (Campbell Dyke *et al.*, 1997; Tremblay *et al.*, 1998). The partial cDNA available for the rabbit α1,2-mannosidase (Lal *et al.*, 1994) and the cloned pig α1,2-mannosidase (Bieberich *et al.*, 1997) are most similar to the IA subclass (Herscovics, 1998).

Northern blot analysis of mouse and human tissues indicated different patterns of tissue-specific expression for α1,2-mannosidases IA and IB (Herscovics *et al.*, 1994; Lal *et al.*, 1994; Tremblay *et al.*, 1998). The mouse α1,2-mannosidase IA and IB genes are also differentially expressed during embryonic development (unpublished results, Herscovics, A.).

Immunolocalization studies have demonstrated that mouse α1,2-mannosidases IA (Lal *et al.*, 1994) and IB (Herscovics *et al.*, 1994) are in the Golgi of transfected L cells and COS 7 cells, respectively. Human α1,2-mannosidase IA has been localized to the Golgi of COS 1 cells (Bieberich and Bause, 1995), but the pig α1,2-mannosidase was found in the ER of COS 1 cells and pig hepatocytes (Roth *et al.*, 1990; Bieberich *et al.*, 1997). The determinants for the differential localization of the pig and other enzymes is still unknown; however, it is evident that both the IA and IB proteins can be located in the same cellular compartment.

Substrate specificity has been studied for the recombinant mouse α1,2-mannosidases IA and IB (Lal *et al.*, 1998), human α1,2-mannosidase IB (Tremblay *et al.*, 1998), and for the purified pig liver enzyme (Schweden and Bause, 1989; Bause *et al.*, 1992). All four enzymes readily remove three out of the four α1,2-linked mannose

residues of $Man_9GlcNAc$ with the accumulation of a specific isomer of $Man_6GlcNAc$ (shown below), followed by a slower conversion to $Man_5GlcNAc$.



The order of removal of mannose residues from $Man_9GlcNAc$ and $Man_8GlcNAc$ isomer B by recombinant mouse α1,2-mannosidases IA and IB was studied by NMR and HPLC (Lal *et al.*, 1998). Hydrolysis of $Man_9GlcNAc$ by α1,2-mannosidase IB resulted in the formation of equivalent amounts of $Man_8GlcNAc$ isomers A and C, whereas α1,2-mannosidase IA predominantly produced $Man_8GlcNAc$ isomer A (refer to Fig. 3 for structures). The same isomers of $Man_7GlcNAc$ and $Man_6GlcNAc$ were formed by both enzymes. $Man_8GlcNAc$ isomer B was quickly transformed to $Man_5GlcNAc$ by both enzymes and the intermediates produced during hydrolysis were the same.

The specificity of the mammalian α1,2-mannosidases IA and IB indicates that a third subclass of mammalian Class 1 α1,2-mannosidases exists that preferentially removes the α1,2-linked mannose residue of the middle arm, and in fact such activity has been characterized in mammalian tissues (see previous section, pages 11-12).

Miscellaneous: In addition to the Class 1 α1,2-mannosidases discussed above, there have been α1,2-mannosidases purified from mung bean and chicken that have typical properties of Class 1 enzymes. The partially purified mung bean seedling α1,2-mannosidase (Forsee, 1985; Szumilo *et al.*, 1986) has a pH optimum of 5.5-6.0. Inactivation by EDTA is reversed by Ca^{2+} and the enzyme is also inhibited by DMJ. It hydrolyzes four mannose residues from $Man_9GlcNAc$. The hen oviduct enzyme (Hamagashira *et al.*, 1996) has a molecular weight of 43 kDa on SDS-PAGE, a pH optimum of 6.5, and is inhibited by DMJ. The enzyme is inactivated by EDTA, and Ca^{2+} is required to restore activity. Substrate specificity studies showed that the α1,2-linked mannose hydrolyzed much more slowly by the mammalian α1,2-mannosidases IA and IB is also removed less efficiently by this enzyme.

BIOLOGICAL IMPORTANCE OF α1,2-MANNOSIDASES

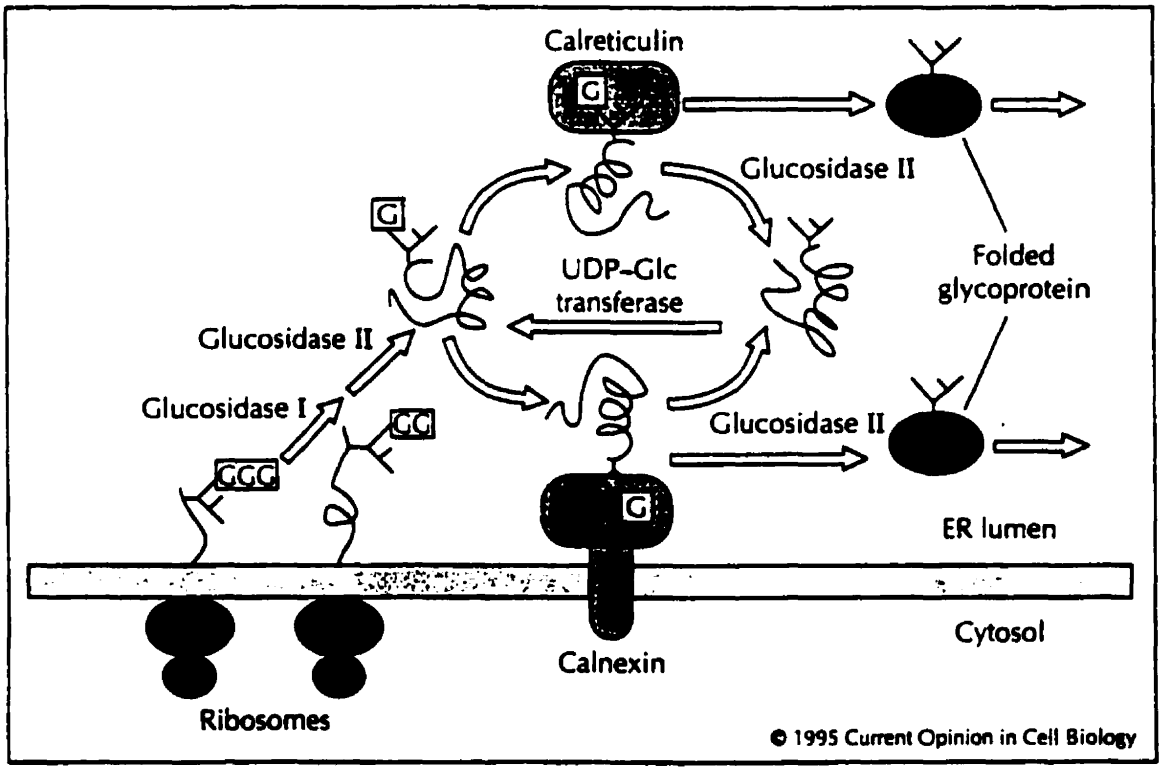
Role of α1,2-mannosidases in quality control of glycoprotein folding

The process of glycoprotein folding and quality control in the ER of mammalian cells has been under intense investigation since the discovery of molecular chaperones that bind the N-linked oligosaccharides on newly synthesized glycoproteins. The studies have demonstrated a role for calnexin, calreticulin, α-glucosidase II and UDP-glucose:glycoprotein glucosyltransferase (UGGT) (Fig. 5)(reviewed by Hammond and Helenius, 1995; Bergeron *et al.*, 1998; Vassilakos *et al.*, 1998). Calnexin and calreticulin are chaperones with lectin properties that bind to the N-linked oligosaccharide, preferably $Glc_1Man_9GlcNAc_2$, and probably also to the polypeptide portion. A cyclic process has been proposed whereby the chaperones bind transiently to the misfolded glycoproteins containing $Glc_1Man_9GlcNAc_2$, while α-glucosidase II removes the glucose residue and UGGT acts as a probe for denatured polypeptide and transfers the glucose back onto the incompletely folded glycoproteins. Upon acquiring a native conformation, the glycoprotein is no longer an efficient substrate for UGGT and is released from the cycle. Misfolded ER glycoproteins are exported to cytoplasmic proteosomes for degradation according to recent studies (reviewed by Kopito, 1997).

There is accumulating evidence suggesting a role for α1,2-mannosidases in this quality control process. When yeast prepro α-factor is expressed in mammalian cells it acquires N-linked oligosaccharides in the ER, but it is quickly degraded and is not secreted as in yeast cells; however, when the cells are treated with the α1,2-mannosidase inhibitor, DMJ, the glycoprotein is stabilized in the ER (Su *et al.*, 1993). In another case, a mutant of human α_1-antitrypsin, which cannot fold into a functional conformation, is degraded. Exposure of the cells to DMJ substantially decreases the degradation of the glycoprotein (Liu *et al.*, 1997). Furthermore, in T cells, the failure of a T cell antigen receptor (TCR) subunit to enter the Golgi results in its degradation. Upon examination of the murine CD3δ and TCRα subunits, it was discovered that degradation of the CD3δ subunit is markedly inhibited when the cells are treated with DMJ, whereas there is no effect on the degradation of TCRα (Yang *et al.*, 1998). Hence, the results demonstrate that the action of α1,2-mannosidases in the ER of mammalian cells may be necessary for degradation of certain glycoproteins.

The quality control process for glycoprotein folding and degradation in *Saccharomyces cerevisiae* is still not well understood. Although α-glucosidase II is present, UGGT activity has not been detected (Fernandez *et al.*, 1994), which suggests that there is a different mechanism of quality control in this organism. A protein with

FIGURE 5: Quality control of glycoprotein folding. (Reprinted from "Quality control in the secretory pathway." C. Hammond and A. Helenius **Curr. Opin. Cell Biol. 7**, 523-529. Copyright 1995 Current Opinion in Cell Biology.)


Calreticulin
G
Glucosidase II
G
UDP-Glc
transferase
Folded
glycoprotein
Glucosidase II
Glucosidase I
GG
GGG
Glucosidase II
G
ER lumen
Cytosol
Calnexin
Ribosomes
© 1995 Current Opinion in Cell Biology

amino acid sequence similarity to calnexin was characterized, but its function as a lectin chaperone has not been established (Parlati *et al.*, 1995). There is one study indicating a possible involvement of the yeast Class 1 α1,2-mannosidase (Mns1p) in the degradation of misfolded ER glycoproteins. Analysis of a specific carboxypeptidase mutant, which is a labile glycoprotein that is proteolytically removed from the ER, was carried out in cells with a disrupted *MNS1* gene (Knop *et al.*, 1996). In *Δmns1* cells, the half life of the carboxypeptidase mutant is significantly increased compared to cells containing the α1,2-mannosidase, which suggests that mannose trimming may be important for degradation of misfolded glycoproteins. This result is also consistent with the evidence of α1,2-mannosidase involvement in degradation of misfolded ER glycoproteins in mammalian cells. Further knowledge of the mechanisms of folding and degradation of glycoproteins in yeast will be required in order to understand the role of the α1,2-mannosidase in this process.

Role of α1,2-mannosidases in development

There is direct evidence indicating a critical role of N-glycosylation during mammalian development. Upon disruption of the mouse gene, *MGAT1*, encoding GnTI, which is the enzyme that initiates N-glycan maturation, the formation of complex N-glycan structures is not observed and the mice do not live beyond day 10 of embryonic development (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). In addition, mutations in the human gene, *MGAT2*, that encodes N-acetylglucosaminyltransferase II, which is essential for the formation of complex N-glycans, causes Carbohydrate-Deficient Glycoprotein Syndrome Type II. Children with this disease have defective protein-bound carbohydrate and have severe abnormalities including retardation and susceptibility to multiple infections (Tan *et al.*, 1996).

The role of the processing α1,2-mannosidases in development has not been studied in mammals; however, the *Drosophila mas-1* gene encoding a Class 1 α1,2-mannosidase has been disrupted (Kerscher *et al.*, 1995). The insects show distinct developmental defects in the embryonic peripheral nervous system, eye, and wing. Furthermore, the expression of the gene in the developing embryo was also studied and a highly specific pattern of expression was observed. During the earlier stages of development the major tissues expressing *mas-1* transcripts are the central nervous system and the developing tracheal system. In late embryonic development, the gene is expressed mostly in the brain, peripheral muscles, and ventral nerve chord. The highly specific pattern of expression in the fly and the specific defects in the null mutant indicates that

the α1,2-mannosidase is highly regulated and its role is very specific in the developing organism. The mild phenotype in insects devoid of this gene suggests that another complementary α1,2-mannosidase is present so that complex N-glycans can still be synthesized.

α1,2-MANNOSIDASES AS POTENTIAL THERAPEUTIC TARGETS

The critical role of the processing glycosidases in the maturation of N-glycans in mammals indicates that they may be important targets for the development of drug therapy for diseases in which complex N-glycans have an important function (reviewed by Winchester and Fleet, 1992; Jacob, 1995). For example, α-glucosidase inhibitors such as N-butyldeoxynojirimycin are effective in blocking the maturation of N-glycans and have been shown to inhibit Human Immunodeficiency Virus (HIV) replication *in vitro* (Fischer *et al.*, 1996). α-Glucosidase inhibitors have been developed for clinical studies to test their effectiveness in the treatment of Aquired Immunodeficiency Syndrome (AIDS) (Jacob, 1995). Inhibition of α-mannosidase II has been shown to reduce both metastasis and tumor growth in animal models (Goss *et al.*, 1995). Phase I clinical trials on patients with advanced cancer have been reported for the α-mannosidase II inhibitor, swainsonine, but further studies are required to establish that the inhibitor is a useful anti-cancer agent (Goss *et al.*, 1997).

The first α1,2-mannosidase inhibitor synthesized was 1-deoxymannojirimycin (DMJ) (Fuhrmann *et al.*, 1984), and more recently the inhibitor, kifunensine, was isolated from an actinomycete (Kayakiri *et al.*, 1989; Elbein *et al.*, 1990). DMJ is a mannose analogue in which the ring oxygen is replaced by nitrogen. Kifunensine is a cyclic oxamide derivative of 1-aminodeoxymannojirimycin, with the added functional groups resulting in a much higher affinity (50-100 times) for the α1,2-mannosidases (Elbein *et al.*, 1990). These α1,2-mannosidase inhibitors block the formation of complex N-glycans in mammalian cells resulting in altered N-linked structures on glycoproteins at the cell surface where cellular interactions occur (reviewed by Elbein, 1991).

In vitro studies with α1,2-mannosidase inhibitors have demonstrated their potential for cancer therapy. DMJ has been shown to inhibit angiogenesis of bovine capillary endothelial cells (Nguyen *et al.*, 1992) and to partially block cell invasion of a reconstituted basement membrane by human melanoma cells (Seftor *et al.*, 1991). Furthermore, treatment with either DMJ or kifunensine increases the susceptibility of human erythroleukemia cells to natural killer cell lysis (Ahrens, 1993). Angiogenesis, metastasis, and immunomodulation are all critical events in cancer.

To develop more potent and specific inhibitors of the human α1,2-mannosidases involved in N-glycan processing, a knowledge of the active site topology and mechanism of catalysis of these enzymes would be very useful. Although two different human Class 1 α1,2-mannosidases have been cloned, there is no information available on their structure and catalytic mechanism. The yeast Class 1 α1,2-mannosidase shares significant amino acid similarity with the other members of the family and it is therefore a useful model for the characterization of the structural and mechanistic properties of the Class 1 α1,2-mannosidases.

Many other glycosidases have been extensively studied and they share some common aspects of structure and mechanism of catalysis. A review of these enzymes is provided in the next section.

B: GLYCOSIDASE STRUCTURE AND MECHANISM

Glycosidases catalyze the hydrolysis of glycosidic bonds and are classified as either exo- or endoglycosidases based on the position of the cleaved bond. Exoglycosidases remove one sugar from the non-reducing ends of carbohydrates, whereas endoglycosidases cleave at internal positions. Catalyzed hydrolysis of the glycosidic bond results in either net retention or inversion of the anomeric configuration. For example, if hydrolysis of the disaccharide, α-Man1,2α-Man-OMe, occurs with release of a β-mannose, then the α1,2-mannosidase is of the inverting type.

STRUCTURE

Several hundred glycosidases have been cloned, mostly from microorganisms, and many three-dimensional structures are available. Henrissat established a classification of glycosidases based on amino acid sequence similarity (Henrissat, 1991) and over 65 families are now classified. The catalytic domains of glycosidases within a family are alike in three-dimensional structure and exhibit the same stereospecificity. Different families with similar protein folds have been grouped into clans (Henrissat and Davies, 1997). For example, families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, and 53 all have the $(\alpha/\beta)_8$ barrel fold and are grouped into clan GH-A.

Analysis of the three-dimensional structures of glycosidases from many different families with distinct substrate specificity and stereospecificity resulted in only three different active site topologies (Davies and Henrissat, 1995; Henrissat and Davies, 1997). The active sites form either a pocket with buried catalytic residues, a cleft with exposed catalytic residues, or a tunnel. Exoglycosidases tend to have the pocket-like active site, where the shape of the pocket determines the substrate specificity. Endoglycosidases prefer the cleft, which favors random cleavage. The tunnel-shaped structure is advantageous for processive enzymes such as those that degrade cellulose, since many hydrolytic events can be performed without release of the polysaccharide.

CATALYTIC MECHANISMS

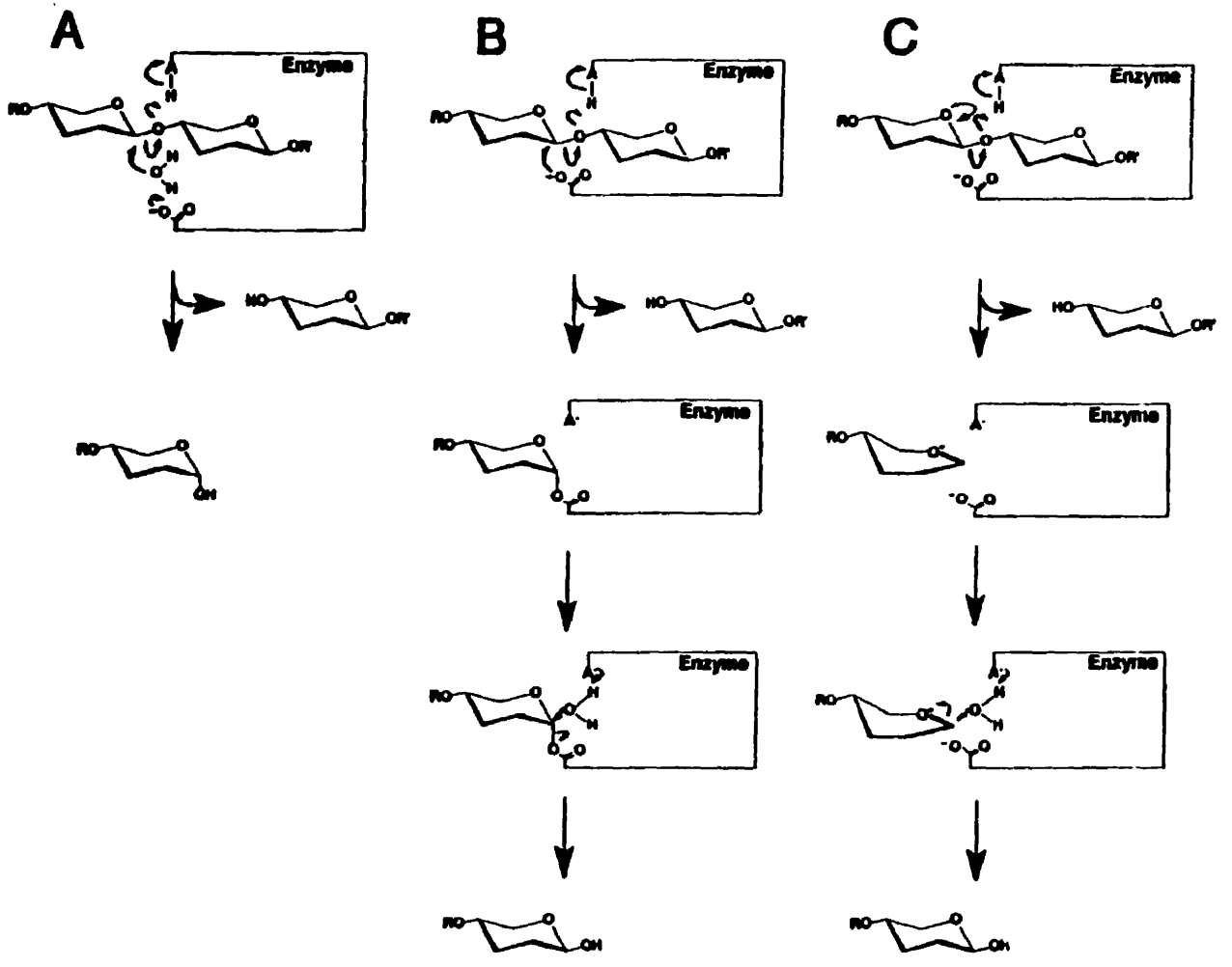
Before the availability of glycosidase primary and tertiary structures, the kinetics of substrate analogues lacking hydroxyl groups was investigated in order to determine their contribution to binding. In addition, binding of reversible and irreversible inhibitors was examined in order to understand the catalytic process (reviewed by Sinnott, 1987; Legler, 1990; Sinnott, 1990). The elucidation of many crystal structures coupled with

studies of active-site labeled glycosidases and kinetic analysis of mutants has resulted in a more complete picture of the catalytic process (reviewed by Svensson and Søgaard, 1993; McCarter and Withers, 1994; Davies and Henrissat, 1995). Both retaining and inverting enzymes hydrolyze the glycosidic bond with the assistance of two carboxylic acid residues at the active site (Fig. 6). In retaining glycosidases, a carboxylic acid group donates a proton to the oxygen of the leaving group while a nucleophilic carboxylate group attacks the glycosidic bond releasing the glycoside and forming a glycosyl-enzyme intermediate. The same group that donated the proton then abstracts a proton from an incoming water molecule forming a hydroxyl group that displaces the glycoside from the enzyme. Although there is considerable evidence for the occurrence of a glycosyl-enzyme intermediate in retaining enzymes, a mechanism involving an ion pair rather than a covalent intermediate cannot be discounted (McCarter and Withers, 1994). In inverting glycosidases, a concerted reaction occurs whereby the catalytic carboxylic acid donates a proton to the oxygen atom of the leaving group while the catalytic carboxylate abstracts a proton from the attacking water molecule. Analysis of the active site configuration of different glycosidases indicated that the distance between the two catalytic residues is about 5 Å for retaining enzymes, whereas it is about 9-10 Å for inverting enzymes (McCarter and Withers, 1994). The extra distance in the inverting enzymes is due to the simultaneous presence of a water molecule and the glycoside substrate during the reaction. Both retaining and inverting mechanisms operate by way of transition states with substantial oxocarbonium ion character. Distortion of the bound sugar ring promotes the formation of the oxocarbonium ion. Polar residues are located in hydrogen-bonding distance from the catalytic residues modulating their pK_a values.

Both aspartic and glutamic acid residues have been identified as catalytic groups and these catalytic residues are usually invariant within a family of glycosidases. Although almost all glycosidases studied have two carboxyl groups involved in catalysis, there are a few exceptions (Davies and Henrissat, 1995). For example, tyrosine instead of a carboxylate residue is thought to stabilize the transition state in a bacterial sialidase (Crennell *et al.*, 1993). For some retaining glycosidases that hydrolyze substrates with a C2 acetamido group adjacent to the cleaved bond, there is evidence that the acetamido functional group may be involved in the catalytic mechanism (e.g. goose lysozyme (Weaver *et al.*, 1995)).

Substrate binding occurs at distinct sugar subsites extending from the catalytic site and hydrogen bonding between planar, polar groups of the enzyme and hydroxyl groups of the monosaccharides determines specificity. Binding is also favored through

FIGURE 6: Mechanisms of inverting and retaining glycosidases. The inverting mechanism (A), and the retaining mechanisms either proceeding through a glycosyl-enzyme intermediate (B) or an oxocarbonium ion intermediate (C) are illustrated. HA and A- represent the catalytic acid in its acid or base form, respectively. (Reprinted with permission from "Mutational analysis of glycosylase function." B. Svensson and M. Søgaard **J. Biotechnol. 29**, 1-37. Copyright 1993 Elsevier Science Publishers B.V.)


A
B
C
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme
Enzyme

interaction with hydrophobic groups such as tryptophan and tyrosine and displacement of bound water molecules.

IDENTIFICATION OF CATALYTIC RESIDUES

The abundance of structural and mechanistic information on glycosidases has resulted in great improvements in methods to identify catalytic residues, even without the availability of a three-dimensional structure. Withers and his colleagues have developed methods to identify the catalytic nucleophile and acid/base catalyst in retaining glycosidases (Withers, 1995; Withers and Aebersold, 1995). The nucleophilic residue is labeled using mechanism-based inactivators, such as 2-deoxy-2-fluoro glycosides, that form a stable glycosyl-enzyme intermediate. After protease treatment of the inactivated enzyme, the labeled peptide is identified by HPLC and electrospray mass spectrometry. The acid/base catalytic residue is identified by mutation of invariant carboxylate residues and kinetic analysis of the mutants. Since the acid catalyst is required for the first step of the reaction (Fig. 6B, C), a substrate with a good leaving group will not significantly reduce this step of the reaction for the mutant enzyme; however, the second step of the reaction will not be affected by any substrate since the base catalyst abstracts a proton from water in all cases. Furthermore, replacement of the acid/base catalyst by alanine will generate a small opening at the active site and another nucleophilic anion that does not require base catalysis, such as azide, can attack the anomeric centre in the second step of the reaction.

It has also been possible to specifically label and identify the acid/base catalyst in some retaining enzymes using affinity labels. The acid/base catalysts in yeast α-glucosidase (Howard and Withers, 1998) and *Cellulomonas fimi* exoglucanase (Tull *et al.*, 1996) were modified with bromoketone α-C-mannoside and N-bromoacetyl cellobiosylamine, respectively. The labeled amino acid was identified by characterization of peptide digests using HPLC coupled to mass spectrometry.

In contrast to retaining enzymes, the inverting glycosidases undergo a one step reaction with no glycosyl-enzyme intermediate formation (Fig. 6A), hence the technique used to trap the glycosyl-enzyme intermediate in retaining enzymes is not applicable to inverting glycosidases. There is only one case where a catalytic residue in an inverting glycosidase was identified using an irreversible inactivator. Labeling of soybean β-amylase with 2,3-epoxypropyl α-D-glucopyranoside resulted in modification of a glutamate residue (Nitta *et al.*, 1989), which was later shown to be the catalytic base residue by site-directed mutagenesis studies (Totsuka *et al.*, 1994) and crystallographic

determination of the structure (Mikami *et al.*, 1994). Instead, site-directed mutagenesis and kinetic analysis of mutants can be used to establish the role of putative catalytic residues in inverting enzymes. Mutation of a catalytic residue should result in very low or undetectable activity due to a dramatic decrease in k_{cat}, with a much smaller or no effect on substrate binding. For example, mutation of the catalytic base in soybean β-amylase resulted in no detectable enzyme activity, but substrate binding was not affected (Totsuka *et al.*, 1994). Mutation of the catalytic acid or base in glucoamylase from *Aspergillus niger* caused a 2000- or 60-fold decrease in k_{cat}, respectively (Frandsen *et al.*, 1994). The catalytic acid mutant showed little alteration in K_m, whereas the base mutant had a 3-fold increase in K_m. Mutagenesis of the catalytic acid in endoglucanase CelD from *Clostridium thermocellum* resulted in a 4000-fold decrease in k_{cat} and a minor decrease in K_m (2-fold), and mutation of the catalytic base lowered the k_{cat} by 1000-fold and increased the K_m 13-fold (Chauvaux *et al.*, 1992). Mutation of the catalytic acid or base in endoglucanase CenA from *Cellulomonas fimi* resulted in 22,000 or 34,000-fold lower k_{cat}, respectively (Damude *et al.*, 1995), yet the endoglucanase mutants bound to a cellobiose column as well as the wild type enzyme.

If substrates are available with leaving groups of varying potential, kinetic analysis with these substrates may aid in distinguishing the acid from the base catalyst in inverting glycosidases. For example, in the case of endoglucanase CenA from *Cellulomonas fimi* (Damude *et al.*, 1995) mutation of Asp^{252} resulted in a dramatic decrease in k_{cat} towards CM-cellulose, but no change of k_{cat} was observed towards 2,4-dinitrophenyl cellobioside. Since the dinitrophenyl group is a very good leaving group, acid catalysis was not necessary compared to CM-cellulose where a poor leaving group is present. This result is consistent with a role for this residue as acid catalyst. In contrast, mutation of Asp^{392} resulted in extremely low activity for both CM-cellulose and 2,4-dinitrophenyl cellobioside, supporting this residue as a base catalyst. In this case, the base catalyst is required to abstract the proton from the water molecule. In its absence, the water alone is a very poor nucleophile and the reaction is slow for any substrate.

In summary, a combination of active site labeling and site-directed mutagenesis followed by kinetic analysis of mutants can be applied to identify catalytic residues in retaining glycosidases, whereas only the site-directed mutagenesis approach has been successful for inverting enzymes. In Chapter 3 and Chapter 5, the stereospecificity of the yeast enzyme was determined and putative catalytic residues were identified by mutagenesis.

CHAPTER 2

Production of recombinant yeast α1,2-mannosidase

SUMMARY

The *Saccharomyces cerevisiae* processing α1,2-mannosidase which trims $Man_9GlcNAc$ to $Man_8GlcNAc$ has a lumenally oriented catalytic domain and an N-terminal transmembrane domain. To obtain sufficient protein to study the structure and mechanism of action of this enzyme the sequence encoding the catalytic domain was inserted downstream of the α-factor promoter and signal peptide in a high-copy vector for expression in *S. cerevisiae* as a secreted protein. Using oligosaccharide substrate ($Glc_1Man_9GlcNAc$ or $Man_9GlcNAc$), the medium of cells transformed with this plasmid showed an increase in α-mannosidase activity that was directly related to the increase in cell density, whereas no α-mannosidase activity was detected in cells transformed with vector alone. SDS-PAGE of the medium showed the presence of a doublet of 63 and 60 kDa that was revealed by Coomassie Blue staining and by Western blotting with antibodies to the endogenous solubilized α-mannosidase. The recombinant α-mannosidase was present in the medium at a level of ~ 1 mg/l and was purified in a single step by chromatography on S-Sepharose. High resolution ^1H NMR analysis of the $Man_8GlcNAc$ formed from $Man_9GlcNAc$ in the presence of the recombinant enzyme proved that it retained its specificity and removed only the α1,2-mannose residue of the α1,3 branch. Endoglycosidase H treatment decreased the molecular mass of both components of the doublet by about 5 kDa, showing that the heterogeneity is not due to differential N-glycosylation. EDTA inhibited the activity of the recombinant enzyme, but the inhibition was reversed by the addition of divalent cations. The K_m for the $Man_9GlcNAc$ substrate was 0.3 mM. These results demonstrate that the recombinant α1,2-mannosidase has the same properties as the endogenous processing enzyme.

INTRODUCTION

N-Glycosylation of proteins in *Saccharomyces cerevisiae* begins in the endoplasmic reticulum with the transfer of $Glc_3Man_9GlcNAc_2$ to Asn/X/Ser(Thr) of the growing polypeptide chains. The oligosaccharide precursor is first modified by glucosidases I and II that remove the three glucose residues and by a highly specific processing α1,2-mannosidase that removes one mannose residue to form a single isomer of $Man_8GlcNAc_2$. The oligosaccharide is then elongated by Golgi mannosyltransferases to form mature core oligosaccharides and the outer chains of mannans (Herscovics and Orlean, 1993). Although the early steps of the processing pathway are similar in all eukaryotes, several processing α1,2-mannosidases exist in mammalian cells (Moremen *et al.*, 1994), and additional mannose residues are removed to form $Man_5GlcNAc_2$, an essential intermediate in the synthesis of complex oligosaccharides.

The highly specific yeast processing α1,2-mannosidase has been purified both as an active soluble enzyme (Jelinek-Kelly *et al.*, 1985; Jelinek-Kelly and Herscovics, 1988) and with its transmembrane domain (Ziegler and Trimble, 1991). Its gene, *MNS1*, has been cloned (Camirand *et al.*, 1991) and shown to encode a type II membrane protein of 63 kDa with a short cytoplasmic tail, an N-terminal transmembrane domain, and a large C-terminal catalytic domain. *In vitro* translation in the presence of membranes demonstrated that the catalytic domain is lumenally oriented (Grondin and Herscovics, 1992). The catalytic region contains an EF hand calcium-binding consensus sequence and three N-linked glycosylation sites.

Recently, it was discovered that the *MNS1* gene is a member of a gene family that has been conserved through eukaryotic evolution (Herscovics *et al.*, 1994). The yeast processing α1,2-mannosidase exhibits significant similarity in amino acid sequence and in topology with rabbit, mouse and human α1,2-mannosidase cDNAs (Bause *et al.*, 1993; Herscovics *et al.*, 1994; Lal *et al.*, 1994). These mammalian cDNAs all encode type II membrane proteins of about 71-73 kDa. Although their N-terminal regions are quite different, the amino acid sequences of the C-terminal catalytic regions of the yeast and mammalian enzymes share distinct regions of identity and they all contain a similar calcium-binding consensus sequence. However, the mammalian enzymes do not have the same specificity as the yeast α-mannosidase. The purified rabbit and recombinant mouse α-mannosidases remove four mannose residues from $Man_9GlcNAc$ to yield $Man_5GlcNAc$ (Moremen *et al.*, 1994; Schneikert and Herscovics, 1994).

Although the mechanism of action of other types of glycosidases has been extensively investigated (Legler, 1990; Sinnott, 1990), there are no structure-function

studies on any of the processing α-mannosidases due to the difficulties in isolating and purifying a sufficient quantity of these low abundance membrane proteins. Therefore, an expression system must be employed to produce sufficient quantities of any of these enzymes. *S. cerevisiae* has proven to be a very useful system for expressing proteins due to its ease of manipulation and the availability of a variety of mutants (Romanos *et al.*, 1992). More specifically, fusion of the yeast α-factor signal peptide with heterologous proteins under the influence of a strong promoter has been used to secrete correctly processed proteins into the extracellular medium (Ernst, 1988; Bromme *et al.*, 1993; Vernet *et al.*, 1993). *S. cerevisiae* grows effectively in protein-free medium, hence a system whereby the recombinant protein is secreted into the medium is ideal, because the amount of contaminating proteins is greatly reduced. In the present work, we report an expression system for the production and purification of milligram quantities of the catalytic domain of the yeast processing α1,2-mannosidase as a protein secreted from *S. cerevisiae*, and show that the properties of the recombinant protein are similar to the previously purified endogenous enzyme.

RESULTS

Expression of yeast α-mannosidase

The sequence encoding the catalytic domain of the yeast processing α-mannosidase was isolated by PCR and cloned into the vector YpJLO1 to give the vector YpFL3 (Fig.1). The fusion protein contains a signal peptidase cleavage site and is under the control of the strong α-factor promoter. MKY 114 was used for expression because the vacuolar α-mannosidase (*AMS1*) is disrupted in this strain. α-Mannosidase activity in the medium from yeast transformed with YpFL3 increases continuously up to 73 h (Fig. 2A), but no α-mannosidase activity is detectable in the medium from cells transformed with the YpJLO1 vector alone. Increase in enzyme activity is directly related to increase in cell density. p-Nitrophenyl-α-D-mannopyranoside, a substrate for non-specific α-mannosidases such as the vacuolar yeast α-mannosidase (*AMS1*), was not hydrolyzed in the presence of medium from cells transformed with YpFL3 (data not shown).

Analysis of proteins in the medium by Western blotting indicates that there is a specific doublet of 63 and 60 kDa in the medium of YpFL3 transformed yeast, but not in cells transformed with the vector YpJLO1 (Fig. 2B). The band at about 200 kDa may represent aggregated mannosidase and is absent in some Western blots.

Purification of recombinant yeast α-mannosidase

S-Sepharose ion exchange chromatography, the first step used to purify the endogenous soluble mannosidase (Jelinek-Kelly and Herscovics, 1988), was enough to obtain highly purified recombinant mannosidase. Analysis of crude and purified enzyme by SDS-PAGE and Coomassie Blue staining indicates that the recombinant α-mannosidase is one of the major proteins in the medium and is highly purified in one step (Fig. 3A). Purification of a doublet of 63 and 60 kDa is better visualised on an 8% SDS-PAGE gel with less protein loaded (Fig. 3B, lane 1). The single chromatographic step gives about 45-60% yield with a 40 fold purification. About 0.6 mg of purified recombinant α-mannosidase per litre of medium was obtained.

Endoglycosidase H treatment

Fig. 3B illustrates that the doublet of 63 and 60 kDa is converted to a doublet of 58 and 55 kDa after Endoglycosidase H treatment. Therefore, the recombinant enzyme contains about 5 kDa of N-linked oligosaccharides and the occurrence of a doublet is not due to a difference in N-linked carbohydrates. Endoglycosidase H treatment gave the same result in the presence and absence of protease inhibitors.

FIGURE 1: Cloning of the catalytic domain of the α-mannosidase into the expression vector YpJLO1. The mannosidase (MNS1) consists of a transmembrane domain (T.M.D.) and a catalytic domain which contains a calcium-binding sequence (*black box*) and three putative N-glycosylation sites (*). Using PCR, a NotI/SacI fragment was generated of the sequence encoding the catalytic domain and cloned into the polylinker region of YpJLO1 to produce YpFL3. The amino acid sequence of the α-factor signal peptide (*hatched box*) and the following nine amino acids is shown to illustrate the primary structure of the N-terminal region.

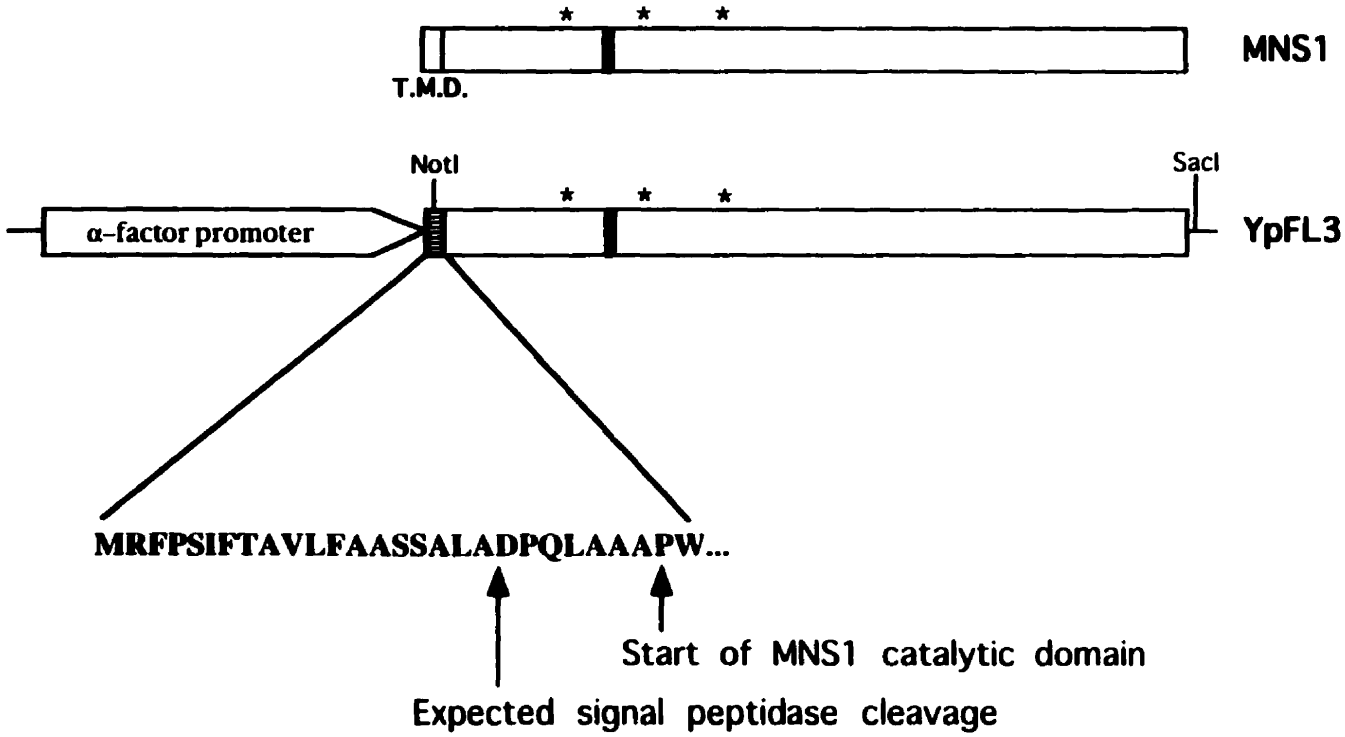
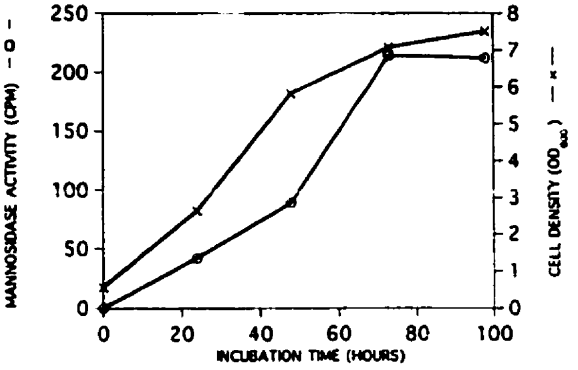

MNS1
T.M.D.
NotI
SacI
α-factor promoter
YpFL3
MRFPSIFTAVLFAASSALADPQLAAAPW...
Start of MNS1 catalytic domain
Expected signal peptidase cleavage

FIGURE 2: Time course of α-mannosidase secretion by yeast transformed with YpFL3. (A) Specific α-mannosidase activity was measured directly from the extracellular culture medium at the indicated times after addition of enriched medium. 1.5 µl of the medium was assayed with 22,800 cpm $Glc_1[^3H]Man_9GlcNAc$ for 1 h as described in Materials and methods. (B) 22 µl of 44-fold concentrated medium were subjected to 10% SDS-PAGE (non-reducing) and visualized by Western blotting. *Lanes 1* and 2 correspond to yeast containing YpJLO1 at 24 and 48 h, respectively. *Lanes 3* and *4* correspond to yeast containing YpFL3 at 24 and 48 h, respectively. Molecular masses of standards (kDa) are indicated in the *left margin*.

(A)


MANNOSIDASE ACTIVITY (CPM) - o -
CELL DENSITY (OD600) — x —
250
200
150
100
50
0
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
INCUBATION TIME (HOURS)

(B)

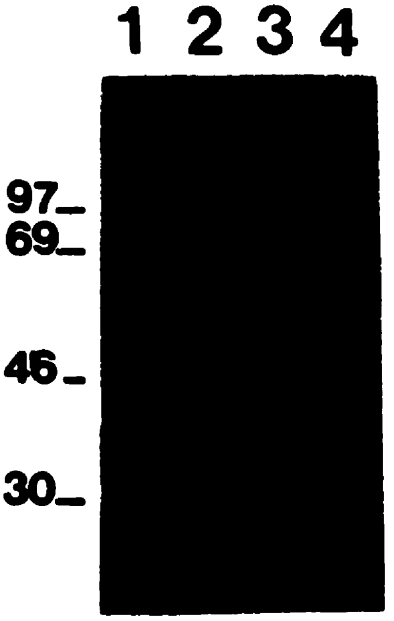
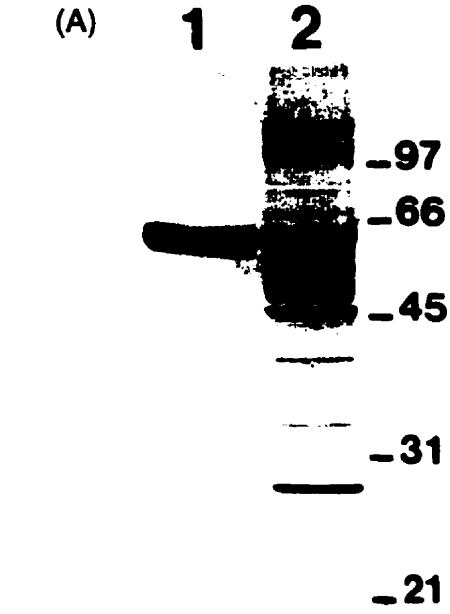
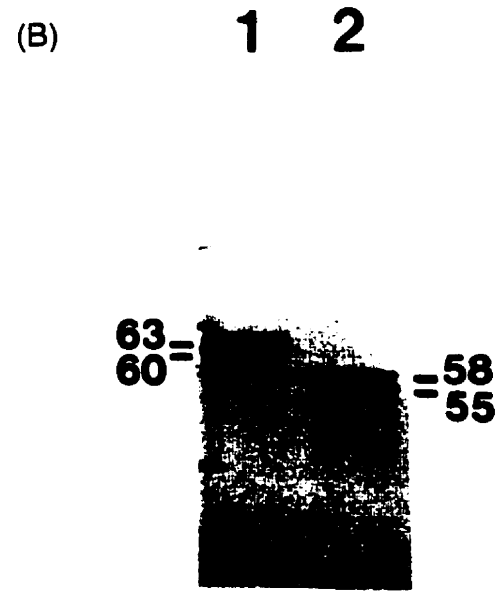

1 2 3 4
97
69
46
30

FIGURE 3: Purification of recombinant α-mannosidase and Endoglycosidase H treatment. (A) 10 μg of crude protein (2) and 2 μg of S-Sepharose pool (1) were applied to 10% SDS-PAGE (reducing) and visualized by Coomassie Blue staining. The molecular masses (kDa) of the standards is indicated in the *right margin*. (B) The purified recombinant α-mannosidase was treated with Endoglycosidase H according to Materials and methods. One microgram of untreated (1) and treated (2) enzyme was applied to 8% SDS-PAGE (reducing) and visualized by Coomassie Blue staining. Molecular masses (kDa) of the respective bands are indicated in the *margins*.


(A)
1
2
97
66
45
31
21


(B)
1
2
63
60
58
55

Characterization of the recombinant yeast α-mannosidase

The $Man_9GlcNAc$ substrate was exhaustively treated with the purified recombinant enzyme, and HPLC fractionation (Fig. 4A) demonstrated the formation of a single oligosaccharide product. (The small peak eluting after $Man_8GlcNAc$ is the unreacted $Man_9GlcNAc$.) ^1H NMR spectroscopy (Fig. 4B) of the oligosaccharide product indicated that it is the specific isomer of $Man_8GlcNAc$ that was also generated with the native α-mannosidase (Jelinek-Kelly *et al.*, 1985).

The recombinant enzyme is stable at -20°C in the culture medium for at least one month. The purified enzyme is stable for at least four months at 4°C in 10 mM phosphate buffer, pH 6.8.

Optimum activity for the recombinant enzyme is observed in 0.1 M PIPES buffer, pH 6.5, 1 mg/ml BSA, and 37°C. Under these conditions the K_m is 0.3 mM.

The recombinant enzyme is greatly inhibited by 0.5 mM EDTA, but its activity is regained to different extents by the addition of 2 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} (Table I).

FIGURE 4: Specificity of recombinant α-mannosidase. The products of α-mannosidase hydrolysis of $Man_9GlcNAc$ were analysed by HPLC (A) and the oligosaccharide product was subjected to ^1H NMR analysis (B) as described in Materials and methods.

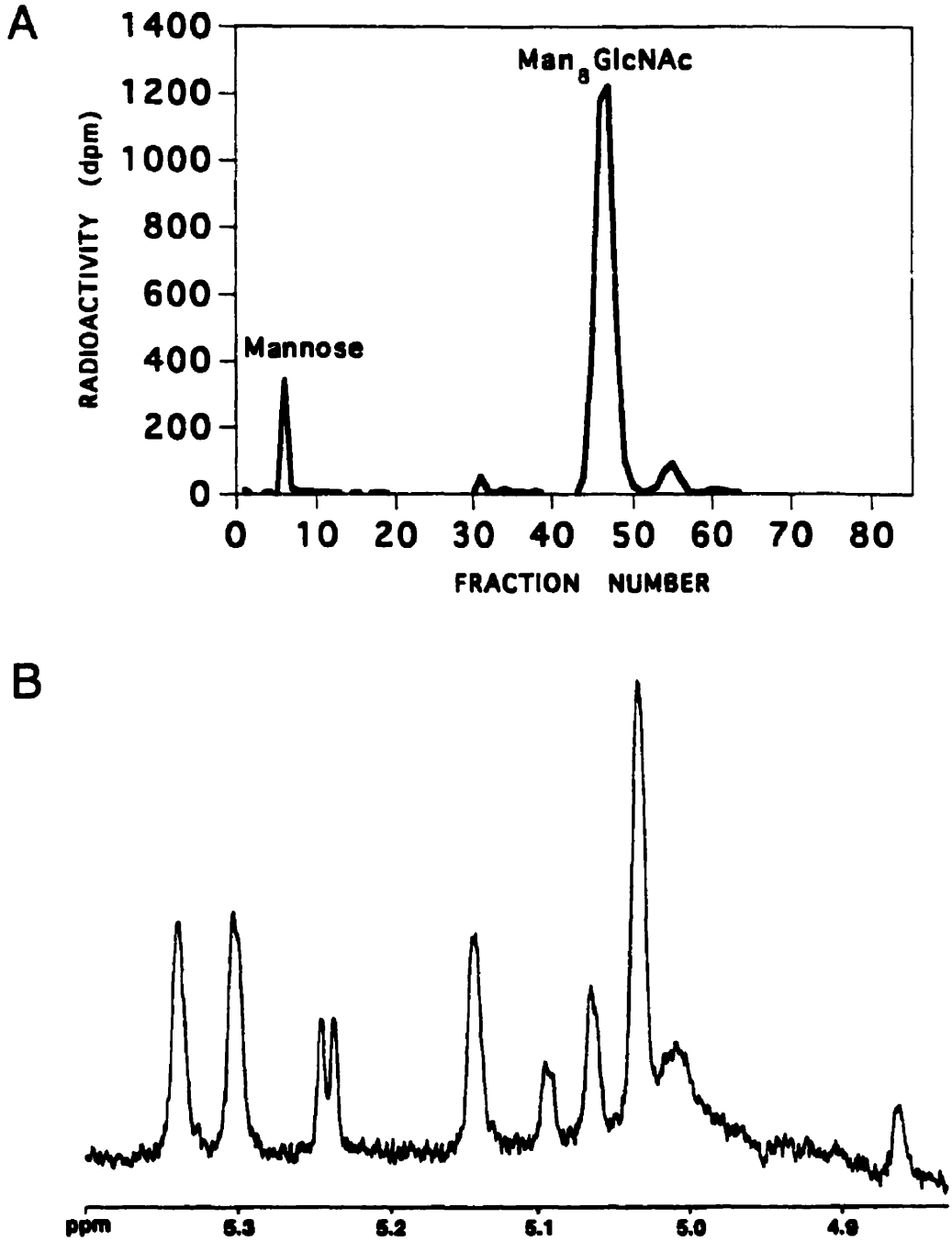

A
1400
1200
1000
800
600
400
200
0
RADIOACTIVITY (dpm)
Man8GlcNAc
Mannose
0 10 20 30 40 50 60 70 80
FRACTION NUMBER
B
ppm
5.3
5.2
5.1
5.0
4.9

TABLE I

Effects of divalent cations on recombinant α-mannosidase

Cations (2 mM)	Activity (%)[a]	
	-EDTA	+EDTA (0.5 mM)
None	100	7
Ca^{2+}	106	100
Mg^{2+}	93	55
Mn^{2+}	75	79
Co^{2+}	78	95
Zn^{2+}	80	86

[a] Assays were according to Materials and methods. Activity (%) is the relative amount of [^{3}H]mannose released during the 2 h incubation compared to the control with no EDTA and no divalent cations.

DISCUSSION

The system described to produce recombinant yeast processing α-mannosidase yields milligram quantities of secreted enzymatically active protein. Since the α-mannosidase is a type II membrane protein naturally located in the yeast secretory pathway, replacement of the transmembrane domain by a cleavable signal sequence favors the translocation of the recombinant protein into the endoplasmic reticulum, subsequent signal peptidase cleavage, and secretion of the catalytic domain into the medium. It is the major protein in the medium and is easily purified in one step. It consists of a doublet of 63 and 60 kDa. The largest member of the doublet has an apparent molecular mass on SDS-PAGE of about 58 kDa after Endoglycosidase H treatment. The expected molecular weight of the recombinant protein is 61.5 kDa, which is close to that observed for the deglycosylated protein. NMR analysis of the oligosaccharide product indicates that the recombinant yeast processing α1,2-mannosidase retains its specificity for one specific mannose residue of $Man_9GlcNAc$. The recombinant enzyme is also inhibited by EDTA, and all the activity is regained with the addition of calcium, as previously observed for the purified endogenous α-mannosidase (Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991). The pH optimum of 6.5 is the same as that of the endogenous α-mannosidase purified with and without the transmembrane domain intact (Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991). Under optimum conditions the recombinant enzyme has a K_m of 0.3 mM, which is very close to the K_m of 0.2 mM determined for the transmembrane form of the endogenous enzyme (Ziegler and Trimble, 1991). Therefore, the results demonstrate that the recombinant α-mannosidase has very similar characteristics to the endogenous enzyme.

Since the endogenous yeast α-mannosidase is most likely located in the endoplasmic reticulum (Esmon *et al.*, 1984), it is not normally susceptible to oligosaccharide modifications that occur in the Golgi apparatus. These possible modifications include the addition of mannose residues to N-linked glycans to form mature core oligosaccharides ($Man_{8-13}GlcNAc_2$) and large mannans with as many as 200 mannose residues, the phosphorylation of N-linked oligosaccharides, and the elongation of O-linked glycans to five mannose residues (Herscovics and Orlean, 1993). In contrast to the ER localized endogenous α-mannosidase, the recombinant enzyme exits the ER and is therefore susceptible to modifications in the secretory pathway. The endogenous α-mannosidase was shown to have about 4 kDa of N-linked oligosaccharides (Ziegler and Trimble, 1991) corresponding to three N-linked core oligosaccharides. The recombinant enzyme has only about 5 kDa of N-linked oligosaccharides. Assuming that all three possible N-glycosylation sites are used for the recombinant enzyme as observed for the

endogenous one, it appears that the oligosaccharides on the recombinant enzyme are not susceptible to significant elongation, as has been observed for other recombinant proteins expressed in yeast (Petersen *et al.*, 1993). It seems likely that the core oligosaccharides of the recombinant enzyme have undergone some maturation with the addition of only a few mannose and/or mannosyl phosphate residues.

The recombinant α-mannosidase is present as two differently migrating forms in the extracellular medium, which are not due to differences in N-linked glycosylation. The size difference may be explained by differential O-glycosylation or by proteolytic degradation. Time course studies suggest that proteolytic degradation is the most likely cause, since extended incubation of the yeast culture resulted in a decreased intensity of the 63 kDa band with respect to the 60 kDa band and the addition of a mixture of protease inhibitors causes an enrichment in the 63 kDa band (data not shown). Expression of the enzyme in the protease deficient yeast strains *kex2*, *yap3* , *pep4*, and *prb1* (Jones, 1991) still resulted in a doublet on SDS-PAGE of 63 and 60 kDa. *Kex2* and *yap3* are mutants defective in proteases of the secretory pathway, whereas *pep4* and *prb1* are mutants deficient in vacuolar proteases.

Since the recombinant α-mannosidase retains the same characteristics as the endogenous enzyme, it is useful to study the structure and mechanism of action of this class of α1,2-mannosidases. The expression system used in the present work may provide a desirable method for producing the catalytic domains of other glycosidases or glycosyltransferases with type II membrane topology which are naturally located in the secretory pathway.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Endoglycosidase H, New England Biolabs (Beverly, MA); Taq polymerase and Concanavalin A, Boehringer/Mannheim (Laval, Québec, Canada); restriction enzymes, New England Biolabs (Beverly, MA) and Gibco BRL (Gaithersburg, MD); CentriCell 20 centrifugal ultrafilters (molecular weight cut-off of 30 000), Polysciences Inc. (Warrington, PA); oligonucleotides were prepared at the Sheldon Biotechnology Center, McGill University, on a Gene-assembler Plus from Pharmacia according to the manufacturer's instructions; S-Sepharose, Pharmacia LKB Biotechnology (Baie d'Urfé, Québec, Canada). All other chemicals were reagent grade.

Plasmid construction

The vector YpJLO1 was a gift from Thierry Vernet and Jean Chatellier (Biotechnology Research Institute (NRC), Montréal, Québec, Canada). It was derived from the shuttle vector pVT100-U (Vernet *et al.*, 1987) and contains the α-factor promoter and signal peptide sequence followed by a multiple cloning site for insertion of proteins to be secreted. The plasmid pBS9.5 contains the whole open reading frame for the *MNS1* gene (Grondin and Herscovics, 1992) The following oligonucleotides were used to isolate the DNA sequence encoding the catalytic domain (nucleotides 67 to 1651 or amino acids 23 to 549) of the mannosidase from pBS9.5 by PCR: 1) a sense 5' oligonucleotide TAT*GCGGCCGC*TCCATGGTACGAACACTTTGAGAG containing a NotI site and 2) a 3' antisense oligonucleotide GGT*GAGCTC*CTACAACGACCAACCTGTG containing a SacI site. Preparative PCR was carried out in a final volume of 50 µl containing 0.2 mM of each dNTP, 0.5 µM of each oligonucleotide primer, 2.5 units TAQ Polymerase, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1 mg/ml gelatin, overlaid with 50 µl of mineral oil. 20 automated step cycles were conducted as follows: 1 min at 94°C, 1 min at 37°C, and 6 min at 72°C. The specific PCR product was then digested with NotI and SacI, and ligated into the NotI/SacI sites of the YpJLO1 vector to produce the vector YpFL3 (Fig. 1). Dideoxy DNA sequencing (Sanger *et al.*, 1977) of the entire NotI/SacI insert showed that the sequence was as expected.

Host strains and culture conditions

Escherichia coli DH5α was used as the host for all plasmid manipulations. The vectors YpJLO1 and YpFL3 were transformed as described (Ito *et al.*, 1983) into the yeast

strain MKY 114 (Matα ams1::LEU2, ura 3-52, leu2-3,112, his4-619) which was generously provided by M. Kuranda (Massachusetts Institute of Technology, Cambridge, MA). Freshly transformed yeast cells were grown at 30°C for 48 h in supplemented minimal medium without uracil, and then four times the volume of enriched medium containing 4% (w/v) casamino acids, 2% (w/v) dextrose, 0.67% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% (w/v) ammonium sulfate, and 50 mM MES at pH 6.3 (Ernst, 1986).

Purification of recombinant yeast α-mannosidase

After a 48 h incubation in enriched medium, the yeast culture was centrifuged to separate the cells from the extracellular medium. Sodium azide was added to a final concentration of 1 mM and the crude medium was kept at 4°C during concentration or at -20°C for storage. Centrifugal ultrafilters were used to concentrate the medium up to 350-fold. In previous work (Jelinek-Kelly and Herscovics, 1988) three chromatography steps were used to purify the endogenous α-mannosidase, the first of which was S-Sepharose ion exchange chromatography. This same first step was enough to purify the recombinant α-mannosidase.

Protein concentrations were determined using a modified method of Lowry (Markwell *et al.*, 1981) or using the Micro BCA reagent kit from PIERCE (Rockford, IL).

α-Mannosidase assays

Specific α-mannosidase activity was assayed with [^{3}H]mannose-labelled $Glc_1Man_9GlcNAc$ or $Man_9GlcNAc$ as substrates using the Concanavalin A and polyethyleneglycol precipitation method (Con A method)(Herscovics and Jelinek-Kelly, 1987). The method for preparation of [^{3}H]mannose-labelled $Man_9GlcNAc$ from rat liver also yields substantial amounts of [^{3}H]mannose-labelled $Glc_1Man_9GlcNAc$ (Jelinek-Kelly *et al.*, 1985), which is as effective as substrate of the yeast processing α-mannosidase as $Man_9GlcNAc$. All buffers and assay mixtures contained sodium azide at a final concentration of 1 mM.

Assay mixtures for monitoring columns and for measuring activity in the crude medium consisted of 5,000-22,800 cpm [^{3}H]mannose-labelled $Glc_1Man_9GlcNAc$, 1 mg/ml BSA, and 10 mM potassium phosphate buffer, pH 6.8, in a total volume of 40 µl.

To study the role of divalent cations, about 6 ng of purified α-mannosidase were incubated for 2 h with 0.2 mM unlabelled $Man_9GlcNAc$ and 10,000 cpm labelled Man_9-GlcNAc, 0.1 M PIPES, pH 6.5, and 20 µg BSA in a final volume of 20 µl. Unlabelled

$Man_9GlcNAc$ was prepared from soya bean agglutinin as previously described (Bhattacharyya *et al.*, 1988). The concentration of labelled $Man_9GlcNAc$ added is insignificant compared to unlabelled substrate. The amount of [^{3}H]mannose released was then measured as above by the Con A method. Divalent cations were added to yield a final concentration of 2 mM as chloride salts. EDTA was present at a final concentration of 0.5 mM.

For the determination of K_m, the assay was performed as above for studies on divalent cations, except that the incubation time was 45 min. Substrate concentrations used were 0.052, 0.072, 0.10, 0.20, 0.30, and 0.54 mM. All plots of product versus time were linear up to 45 min. The K_m was determined by Lineweaver-Burke analysis.

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) using the Bio-Rad Mini-Protean II apparatus. Protein bands were visualized with Coomassie Blue staining. For Western blotting proteins were transferred onto pure nitrocellulose membrane (Schleicher and Schuell, Keene, NH) and visualized by the ECL Western blotting detection system (Amersham, Amersham, U.K.) using rabbit polyclonal antiserum raised against the purified soluble yeast α-mannosidase.

Preparation of $Man_8GlcNAc$ for ^1H NMR

Unlabelled $Man_9GlcNAc$ (1 mg) was incubated at 37°C with 100,000 cpm [^{3}H]mannose-labelled $Man_9GlcNAc$, BSA (1 mg/ml), and 15 ng of purified recombinant enzyme in a total volume of 100 µl of 10 mM potassium phosphate, pH 6.8. The mixture was incubated for a total of 65 h with 30, 30, and 45 ng of recombinant mannosidase added at 3, 22, and 29 h, respectively. Four microliters of the sample were analysed by HPLC as described previously (Jelinek-Kelly *et al.*, 1985). The rest of the sample was applied to a Bio-Gel P6 column (1x109 cm) and eluted with water. The labelled oligosaccharide-containing fractions were pooled and lyophilized. D_2O was added and the sample lyophilized. This exchange with D_2O was repeated five times. The dry sample was exposed to P_2O_5 for 6 days and then analysed by high resolution ^1H NMR at 25°C as previously described (Jelinek-Kelly *et al.*, 1985).

Endoglycosidase H treatment

Two µg purified recombinant enzyme were treated with 50 munits of Endoglycosidase H for 24 h at 37°C in 0.5% SDS, 1% β-mercaptoethanol, 0.05 M sodium citrate, pH 5.5. As a control, incubation was done without Endoglycosidase H.

TRANSITION: CHAPTER 2 TO 3

In Chapter 2, it was demonstrated that the catalytic domain of the yeast α1,2-mannosidase is produced in milligram amounts as a secreted recombinant enzyme from *S. cerevisiae*. Replacement of the transmembrane domain by the α-factor signal peptide results in the translocation of the catalytic domain of the enzyme into the endoplasmic reticulum, where it is then proteolytically clipped away from the signal peptide and secreted into the medium. Highly purified protein is obtainable in one simple purification step and the recombinant enzyme has very similar characteristics as the endogenous α1,2-mannosidase. The *S. cerevisiae* expression system is therefore suitable for providing recombinant α1,2-mannosidase for studying its structure and mechanism of catalysis.

In Chapter 3, the first study on the catalytic mechanism of the recombinant α1,2-mannosidase is presented.

CHAPTER 3

The yeast α1,2-mannosidase is an inverting glycosidase

Reprinted from "The *Saccharomyces cerevisiae* processing α1,2-mannosidase is an inverting glycosidase." F. Lipari, B.J. Gour-Salin, and A. Herscovics **Biochem. Biophys. Res. Comm. 209,** 322-326.

SUMMARY

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.

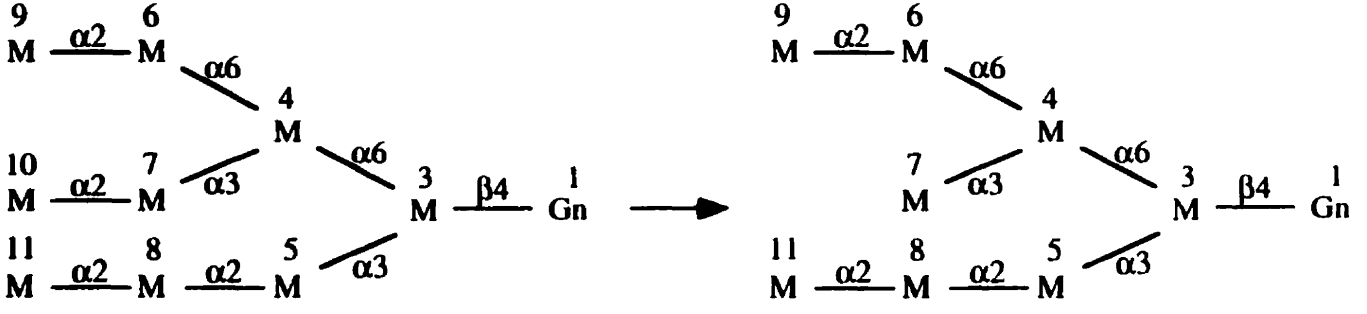
INTRODUCTION

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 (Moremen *et al.*, 1994). Complex carbohydrates have been implicated in a variety of diseases (Brockhausen, 1993), 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 (Winchester and Fleet, 1992). 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 Moremen *et al.*, 1994). 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 Elbein, 1991; Moremen *et al.*, 1994). Such inhibition affects important cellular recognition processes, such as angiogenesis (Nguyen *et al.*, 1993), neutrophil adhesion to endothelial cells (Sriramarao *et al.*, 1993), and natural killer cell targeting (Ahrens, 1993). In addition, preventing mannose trimming can interfere with ER degradation of abnormal glycoproteins (Su *et al.*, 1993), 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 Sinnott, 1990), 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 (Moremen *et al.*, 1994). 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 (Jelinek-Kelly and Herscovics, 1988; Camirand *et al.*, 1991)

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) (Lipari and Herscovics, 1994). 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") (Wang *et al.*, 1994). The present work reports the stereochemical course of hydrolysis of $Man_9GlcNAc$ by the recombinant yeast α1,2-mannosidase using ^1H NMR spectroscopy (Eveleigh and Perlin, 1969; Sinnott, 1990; Gebler *et al.*, 1992).

Scheme 1: Structures of the oligosaccharide substrate and product in the reaction catalyzed by the yeast α1,2-mannosidase. Each sugar residue is numbered.



$Man_9GlcNAc$ $Man_8GlcNAc$

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 (Lipari and Herscovics, 1994). 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 (Mach *et al.*, 1992). 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 (Romero *et al.*, 1994). 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 $Man_9GlcNAc$ (2 mM, 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 2 min for 96 min 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 (Gebler *et al.*, 1992). The time course for the hydrolysis of $Man_9GlcNAc$ to $Man_8GlcNAc$ (Scheme 1) by the recombinant yeast α1,2-mannosidase is shown in Fig. 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 Fig. 1. These signals have been characterized previously (Byrd *et al.*, 1982; Jelinek-Kelly *et al.*, 1985). Fig. 1A depicts the spectrum of the substrate before addition of the α1,2-mannosidase. Six min after addition of the enzyme, the appearance of the hemiacetal anomeric proton of β-D-mannose at δ 4.89 is observed (Fig. 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 min (Fig. 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 min after the addition of enzyme (Fig. 1E), all the starting oligosaccharide has been converted to product. After 134 min (Fig. 1F) the equilibrium anomeric ratio of D-mannose has been established. Fig. 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 Fig. 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, Fig. 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 (Mikami *et al.*, 1994), glucoamylase from *Aspergillus awamori* (Aleshin *et al.*, 1992), and endocellulase E2 from *Thermomonospora fusca* (Spezio *et al.*, 1993), 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 general base catalyst

FIGURE 1: Partial ^1H NMR spectra of the anomeric region taken at different times of incubation of $Man_9GlcNAc$ with the yeast α1,2-mannosidase. ^1H 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.

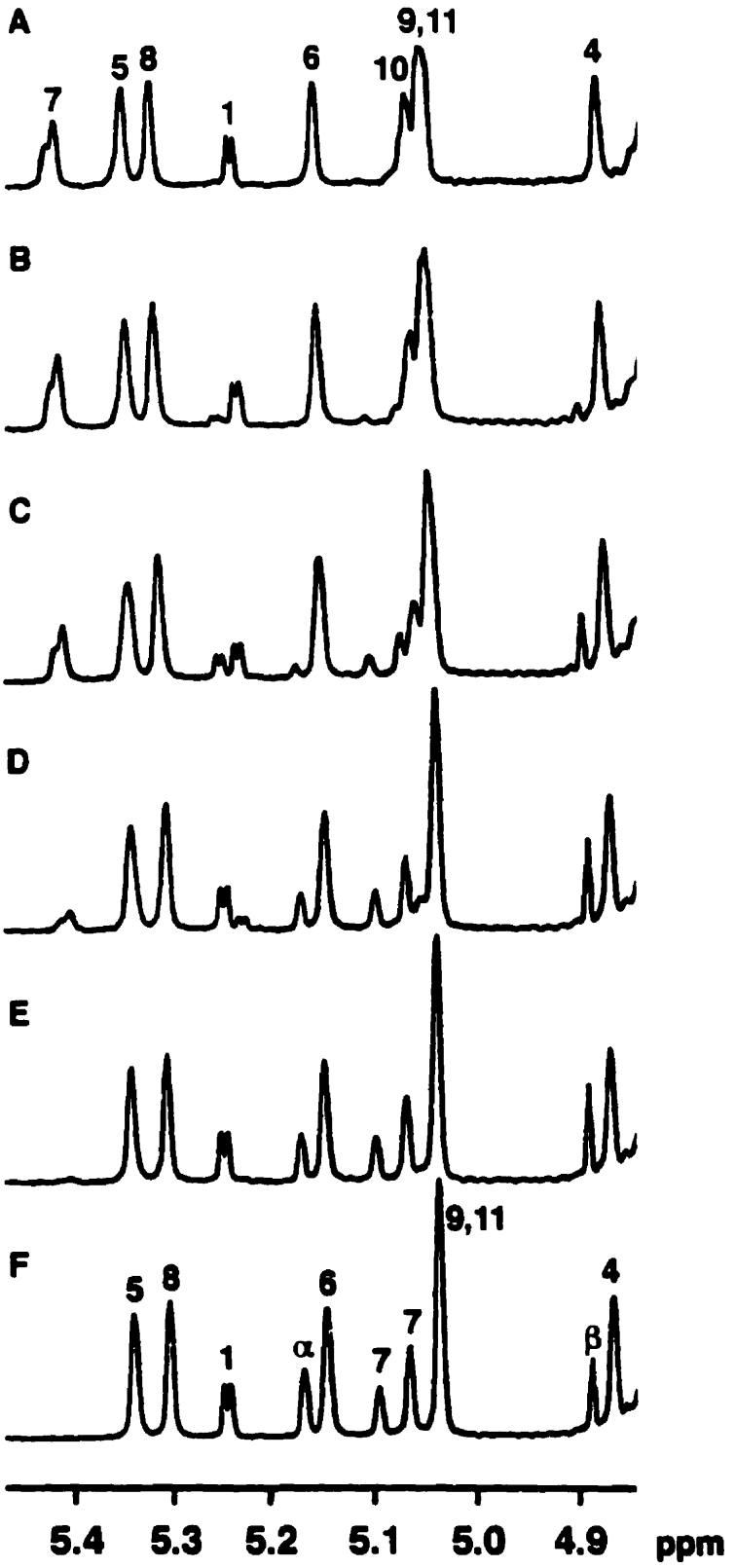
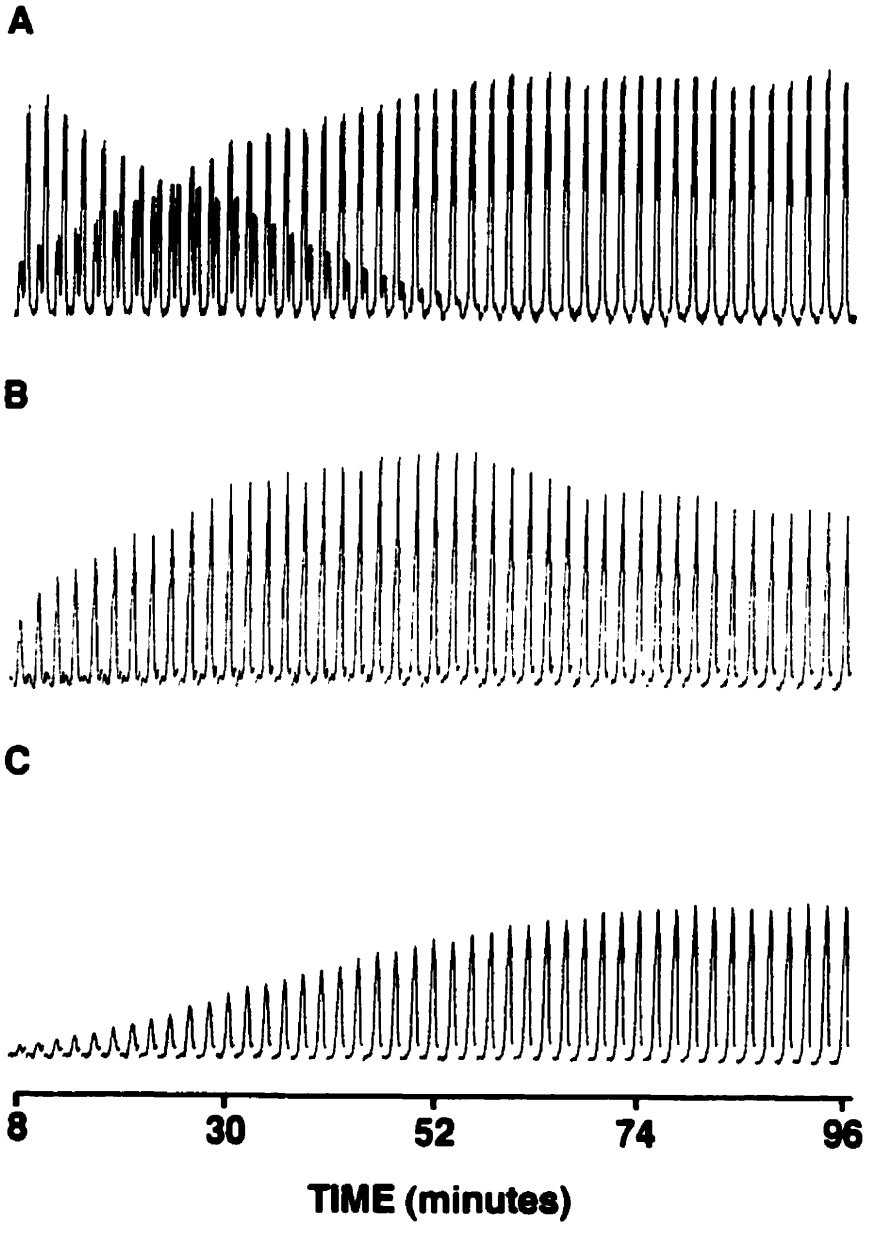

A
9,11
5
8
7
1
6
10
4
B
C
D
E
9,11
F
5
8
6
4
7
1
α
7
β
5.4
5.3
5.2
5.1
5.0
4.9
ppm

FIGURE 2: Time course of α1,2-mannosidase action on $Man_9GlcNAc$. Individual signals from the spectra of Fig. 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.


A
B
C
8
30
52
74
96
TIME (minutes)

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 (Wang *et al.*, 1994). 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.* (1992) 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.

TRANSITION: CHAPTER 3 TO 4

In Chapter 3, it was demonstrated that the yeast α1,2-mannosidase is an inverting glycosidase, producing a β-mannose residue from $Man_9GlcNAc$. Since glycosidases act by one of two distinct mechanisms, retaining or inverting, it was important to know the stereochemistry of the α1,2-mannosidase as a first step in understanding its mechanism of catalysis.

In Chapter 4, the disulfide bonds are localized and the role of the cysteine residues is elucidated by site-directed mutagenesis. In addition, a *P. pastoris* expression system for the production of recombinant yeast α1,2-mannosidase is described.

CHAPTER 4

Role of the cysteine residues in the yeast α1,2-mannosidase

SUMMARY

The *Saccharomyces cerevisiae* α1,2-mannosidase, which removes one specific mannose residue from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$, 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-thiosulfobenzoate and 5,5'-dithiobis(2-nitrobenzoate), respectively. Cys^{485} contains the free thiol group, as demonstrated by sequencing of labeled peptides following modification with $[^3H]ICH_2COOH$ and by high performance liquid chromatography/mass spectrometry tryptic peptide mapping. A Cys^{340}-Cys^{385} disulfide was demonstrated by sequencing a purified peptide containing this disulfide and by tryptic peptide mapping. Cys^{468} and Cys^{471} were not labeled with $[^3H]ICH_2COOH$ and a peptide containing these two residues was identified in the tryptic peptide map, showing that Cys^{468} and Cys^{471} 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 Cys^{340} and Cys^{385} are essential for production of recombinant enzyme, whereas Cys^{468}, Cys^{471}, and Cys^{485} are not required for production and enzyme activity. These results indicate that the sensitivity to dithiothreitol is due to reduction of the Cys^{340}-Cys^{385} disulfide. Since Cys^{340} and Cys^{385} are conserved residues, it is likely that this disulfide bond is important to maintain the correct structure in the other members of the α1,2-mannosidase family.

INTRODUCTION

The processing α1,2-mannosidase present in the endoplasmic reticulum of *Saccharomyces cerevisiae* removes one specific mannose residue from $Man_9GlcNAc_2$ to form $Man_8GlcNAc_2$ during the formation of *N*-linked oligosaccharides (Jelinek-Kelly *et al.*, 1985; Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991; Burke *et al.*, 1996). Its gene (*MNS1*) encodes a type II membrane protein of 63 kDa with no significant cytoplasmic tail, an N-terminal transmembrane domain and a large C-terminal catalytic domain (Camirand *et al.*, 1991). The yeast α1,2-mannosidase exhibits significant similarity in amino acid sequence and topology with α1,2-mannosidases cloned from rabbit, mouse, and human (71-73 kDa) that are essential for the formation of complex and hybrid *N*-linked oligosaccharides (Bause *et al.*, 1993; Herscovics *et al.*, 1994; Lal *et al.*, 1994). The yeast and mammalian proteins are about 35% identical in amino acid sequence in their C-terminal catalytic regions. Based on sequence homology and common properties (Moremen *et al.*, 1994), these enzymes were grouped as Class 1 α1,2-mannosidases. They all contain an EF hand Ca^{2+}-binding consensus sequence and require Ca^{2+} for activity. In addition, they are inhibited by 1-deoxymannojirimycin and do not use ρ-nitrophenyl-α-D-mannopyranoside as substrate. The yeast α1,2-mannosidase has a very high specificity for removal of a single mannose residue on $Man_9GlcNAc_2$, whereas the mammalian enzymes can remove up to four mannose residues from $Man_9GlcNAc_2$ to form $Man_5GlcNAc_2$. The mammalian enzymes hydrolyze α-Man1,2α-Man-OMe, whereas the yeast α1,2-mannosidase cannot hydrolyze this disaccharide. The smallest oligosaccharide substrate for the yeast α1,2-mannosidase is α-Man1,2α-Man1,3α-$O(CH_2)_8COOCH_3$, but it is a very poor substrate (K_m = 9 mM)(Scaman *et al.*, 1996). Recently, α1,2-mannosidases have also been cloned from *Drosophila melanogaster* (Kerscher *et al.*, 1995), *Penicilium citrinum* (Yoshida and Ichishima, 1995), and *Aspergillus saitoi* (Inoue *et al.*, 1995) 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 Ca^{2+} binding consensus sequence and do not require Ca^{2+} 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. Up to recently, a major difficulty has been the purification of sufficient enzyme for study. However, we can now produce milligram quantities of the catalytic domain of the yeast α1,2-mannosidase (Lipari and Herscovics, 1994). 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 (Lipari *et al.*, 1995).

In the present work we demonstrate that the yeast α1,2-mannosidase has two disulfide bonds and one sulfhydryl 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 sulfhydryl residue is not required.

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, 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 pHIL-S1. CNBr 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. TFA (HPLC/Spectro Grade, Sequanal Quality) was from Pierce or TFA (99% pure) was from Aldrich. Oligosaccharide substrates were obtained as previously described (Lipari and Herscovics, 1994). All other chemicals were reagent grade.

Plasmid Construction and Site-directed Mutagenesis

Escherichia coli DH5α and DH10 were used as the host for plasmid manipulations. The plasmid pBS9.5 contains the whole open reading frame of the *MNS1* gene in pBluescript (Grondin and Herscovics, 1992). The following oligonucleotides were used to isolate the DNA sequence encoding the catalytic domain (nucleotides 64-1651 corresponding to amino acids 22-549) of the α1,2-mannosidase from pBS9.5 by PCR: (i) a sense 5' oligonucleotide ATA*CTCGAG*TGCCATGGTACGAACACTTTG containing an XhoI site and (ii) a 3' antisense oligonucleotide GGT*GGATCC*CTACAAC GACCAACCTGTG containing a BamHI site. Preparative PCR was carried out as previously described (Lipari and Herscovics, 1994). The specific PCR product was digested with XhoI and BamHI, and ligated into the XhoI/BamHI sites of the pHIL-S1 vector to produce the plasmid YpHMNS1. Cysteine to serine mutations were accomplished by the unique site elimination procedure (Deng and Nickoloff, 1992)(U.S.E. Mutagenesis kit, Pharmacia) using the plasmid YpHMNS1 and the following oligonucleotides: GACCACCTCGTAAGCTTTATGGG for the C340S mutation , GGGATAACTGACACTAGTTATCAAATGTACAAGC for C385S, CTTT GAAAATACTAGTGTTGATTGTAATGACCC for C468S, CTGTGTTGATTCTAAT GACCCAAAATTAAGG for C471S, and GGCGGTTCACTAGTTTAAGTGATTCTAT

for C485S. The plasmids YpHC340S, YpHC385S, YpHC468S, YpHC471S, and YpHC485S were thus constructed from the wild type YpHMNS1 plasmid. The entire coding region of all the constructs was sequenced by the dideoxy method (Sanger *et al.*, 1977).

Production of Recombinant α1,2-Mannosidase

The secreted recombinant α1,2-mannosidase lacking the transmembrane domain was produced using two different yeast expression systems, one in *S. cerevisiae* as already described (Lipari and Herscovics, 1994) and the other in *P. pastoris*. BglII-digested plasmids were transformed by the spheroplast method or by electroporation into the *P. pastoris* strains GS115 (*his4*) or KM71 (*his4, aox1*) as described in the *Pichia* Expression Kit manual obtained from Invitrogen. Histidine-independent transformants were selected and subsequently screened for methanol utilization when the GS115 strain was used. Use of the KM71 strain does not require selection for methanol utilization. BMGY and BMMY media were prepared according to the *Pichia* Expression Kit manual. His^+, Mut^- yeast cells were grown at 30°C (250 - 300 rpm) for 48 h in BMGY medium (buffered with 100 mM potassium phosphate, pH 6.0), and then centrifuged and resuspended with BMMY medium (buffered with 100 mM potassium phosphate, pH 6.0) in 20-40% of the original volume, and incubated for an additional one to four days at 30°C (250-300 rpm). If the cells were induced for more than two days, methanol was added to a final concentration of 0.5% (v/v) after two days of induction. Cells were separated from the medium by centrifugation. In order to screen for clones expressing α1,2-mannosidase, 5 μl of medium were assayed for α1,2-mannosidase activity. The assay mixture contained 8700 dpm $[^3H]Glc_1Man_9GlcNAc$, 1 mg/ml BSA, 0.1 M PIPES (pH 6.5), and 1 mM sodium azide in a total volume of 20 μl and was incubated for 1 h at 37°C. In order to screen for intracellular expression of recombinant enzyme, the cells were vortexed four to five min at 4°C in the presence of glass beads in 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM sodium azide. Glass beads, buffer, and cells were added in a ratio of 1:1:0.5. The glass beads were allowed to settle and aliquots of the supernatants were analyzed by Western blotting.

Recombinant α1,2-mannosidase was purified by S-Sepharose chromatography or by sequential S-Sepharose and Q-Sepharose chromatography as previously described (Lipari *et al.*, 1995).

α1,2-Mannosidase Assays

Unless otherwise indicated, enzyme samples were diluted in 10 mM PIPES (pH 6.5) containing 1 mg/ml BSA and 7.5 µl were assayed under standard conditions with 0.2 mM $Man_9GlcNAc$ (17400 dpm [^{3}H]$Man_9GlcNAc$) as previously described (Lipari and Herscovics, 1994). K_m values for the mutant and wild type enzymes present in the medium were determined as described previously (Lipari and Herscovics, 1994).

Quantitation of Disulfide Bonds and Free Sulfhydryl Groups

The disulfide bonds were quantitated using 2-nitro-5-thiosulfobenzoate, which was synthesized from DTNB (Thannhauser *et al.*, 1984). The reaction was carried out in a solution of pH 9.5 containing 0.2 M Tris-HCl, 0.1 M sodium sulfite, 3 mM EDTA, 3M guanidine thiocyanate, and 0.5 mM NTSB. 100 µl of the reagent solution were added to the protein solution (7 to 15 µl containing 26-147 µg of α1,2-mannosidase). The reaction was monitored at 412 nm until a constant absorbance reading was achieved (45-90 min).

The free sulfhydryl groups were quantitated using 5,5'-dithiobis(2-nitrobenzoate) (Jocelyn, 1987). The protein was prepared in 50 mM sodium phosphate (pH 7.8) containing 2% SDS and 1 mM sodium azide, and then heated at 50°C for 30 min. 50 µl of protein solution, containing 73-165 µg of α 1,2-mannosidase, were added to 50 µl of reagent containing 1 mM DTNB, 6 mM EDTA, 50 mM sodium phosphate (pH 7.8) and 1mM sodium azide and the absorbance was monitored at 412 nm for 20 min.

Carboxymethylation with 2-[^{3}H]ICH_2COOH

Recombinant α1,2-mannosidase (1.2 mg) in 2 mM sodium phosphate (pH 6.8), 1 mM sodium azide was lyophilized. Reaction with iodoacetic acid was carried out according to Carr *et al.* (1990), with a few modifications. Iodoacetic acid (recrystallized in chloroform) was added to a final concentration of 0.1 M in 0.5 M Tris-HCl (pH 8.2), containing 2 mM EDTA and 6 M Gdn-HCl. The pH was adjusted to 8.2 with ammonium hydroxide and the solution was purged with nitrogen. 500 µCi of dried 2-[^{3}H]ICH_2COOH (172 mCi/mmole, Amersham) were added to 2.4 ml of this solution and this was then added to the lyophilized protein. The reaction was carried out on ice in the dark under a nitrogen atmosphere for 140 min. 3 ml of 1% acetic acid were added to stop the reaction, the sample was extensively dialyzed against 1% acetic acid (six changes of 1.0 liter), and lyophilized. Reaction with unlabeled 0.1 M iodoacetate was performed as above.

Cleavage of α1,2-Mannosidase (Scheme 1)

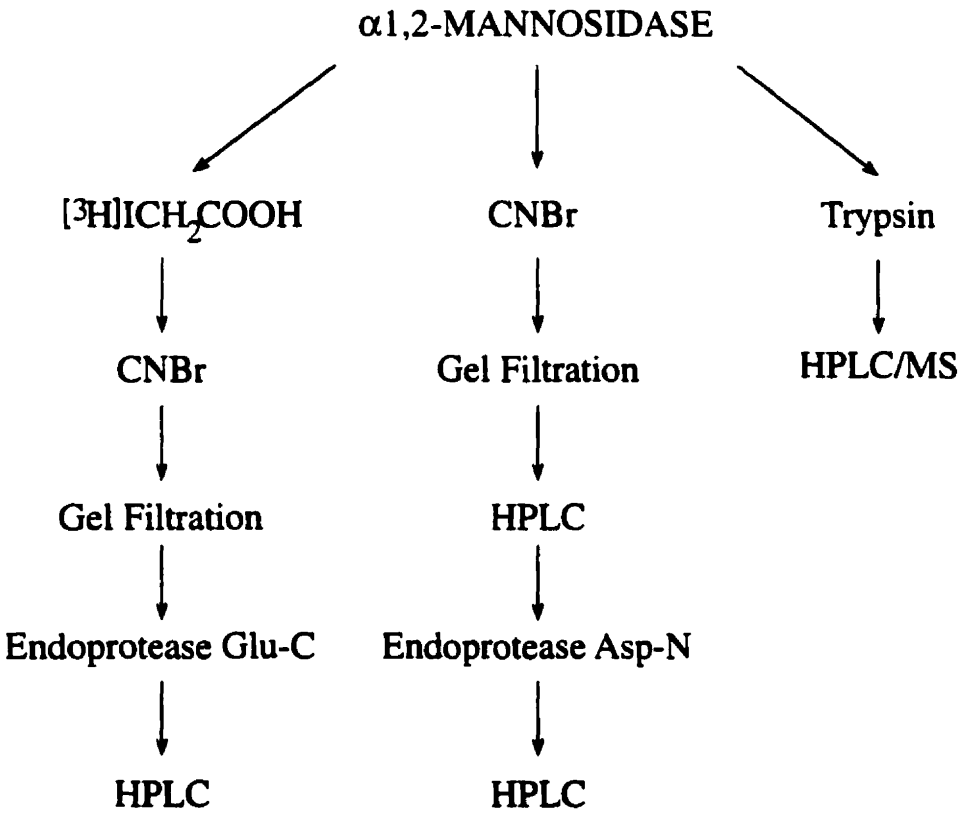
Lyophilized α1,2-mannosidase was treated with a 100 or 500 fold molar excess (with respect to methionine content) of CNBr in 6 M Gdn-HCl/0.2 M HCl (Villa *et al.*, 1989). The reaction was allowed to proceed for 24 h in the dark at room temperature, 1 ml of water was added, and the sample was lyophilized. 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 (1x120 cm) using 0.1 M or 0.2 M acetic acid as solvent, monitoring the eluent at 230 nm. Five included peptide fractions, P_1-P_5, 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 P_3, and to a lesser extent in fraction P_2, were observed to shift upon reduction. None of the other fractions (P_1, P_4, or P_5) contained peptides which 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 per 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 per 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 8M 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 per 100 μg of α1,2-mannosidase. The digestion was allowed to proceed at 37°C for 5 h and stopped by freezing at -80°C. For the reduced sample, four times the volume of 0.5 M Tris-HCl (pH 8.5) containing

Scheme 1: Cleavage of α1,2-mannosidase.



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 M Gdn-HCl (Schmelzer *et al.*, 1993). 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 glacial acetic acid and 40 µl of 0.1% aqueous TFA.

HPLC of Peptides

A Varian model 5020 HPLC system equipped with a reverse-phase C4 column (4.6 mm (inner diameter) x 25 cm, 10 µm, Vydac) was employed for peptide separations. Solvent A was 0.1% TFA in water and solvent B contained 0.1% TFA, 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 trypsin-digested α1,2-mannosidase (reduced or non-reduced) were fractionated on a microbore C18 column (1 mm (inner diameter) x 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% TFA/water as buffer A and 0.05% TFA/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 (Mach *et al.*, 1992) or the Micro BCA reagent kit from Pierce. Peptide concentration was determined using a modified Lowry method (Markwell *et al.*, 1981). SDS-PAGE under reducing conditions was carried out according to Laemmli (1970) using the Bio-Rad Mini-Protean II apparatus. For Western

blotting, proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell) and visualized by the ECL Western blotting detection system (Amersham) using rabbit polyclonal antiserum raised against the purified soluble yeast α1,2-mannosidase (Lipari and Herscovics, 1994) or against recombinant yeast α1,2-mannosidase (Burke *et al.*, 1996).

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 (PTH) analysis system or at the Sheldon Biotechnology Centre (McGill University, Montréal, Canada) on a Beckman integrated microsequencing system, model PI2090E, equipped with an on-line HP1090 HPLC.

For amino acid composition analysis, peptides were subjected to constant boiling hydrochloric acid (about 6N) 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/L of purified recombinant enzyme, which is 50 times more than was produced in *S. cerevisiae* as previously described (0.6 mg/L) (Lipari and Herscovics, 1994). Recombinant α1,2-mannosidase produced from *S. cerevisiae* had been shown to have similar properties as the native α1,2-mannosidase (Lipari and Herscovics, 1994) 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).

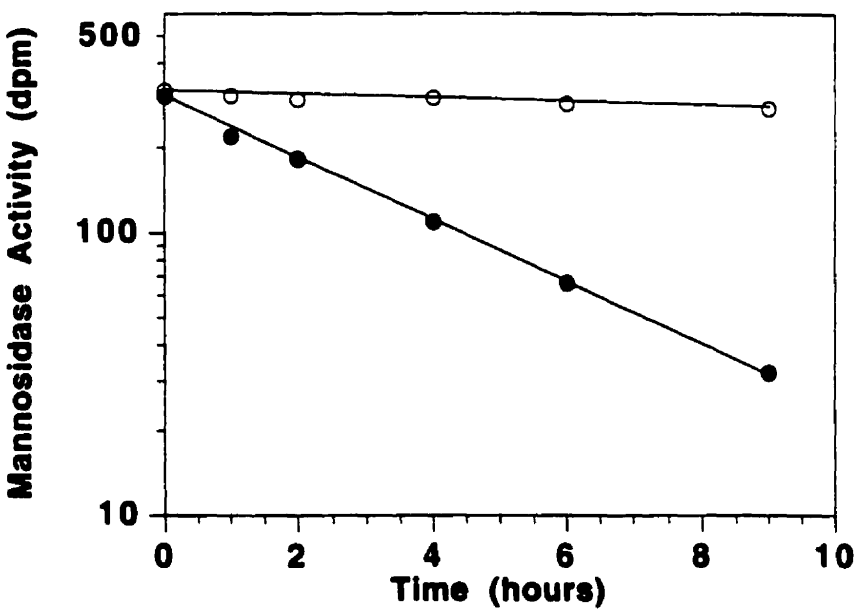
Quantitation of Disulfides and Free Sulfhydryl Groups

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 per protein molecule. DTNB was used to quantitate free sulfhydryl groups. A value of 1.1 ± 0.1 sulfhydryl group per α1,2-mannosidase molecule was obtained. In addition, carboxymethylation with iodoacetate under denaturing conditions resulted in 0.85 CM-Cys per 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

FIGURE 1: Effect of DTT on α1,2-mannosidase activity. Purified recombinant α1,2-mannosidase (5.6 μM) was incubated at 30°C with (*closed circles*) or without (*open circles*) 10 mM DTT in 0.1 M sodium phosphate (pH 8.0) containing 1 mM sodium azide. Aliquots were removed at different time intervals and diluted 1/560 to measure α1,2-mannosidase activity as indicated under "Experimental Procedures". The α1,2-mannosidase activity was plotted on a logarithmic scale.


Mannosidase Activity (dpm)
500
100
10
0
2
4
6
8
10
Time (hours)

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 α1,2-mannosidase activity. Treatment of the α1,2-mannosidase with the sulfhydryl specific reagents N-ethylmaleimide, ρ-chloromercuribenzoate, iodoacetate, and iodoacetamide under native conditions did not affect enzyme activity (data not shown).

Labeling the Sulfhydryl Group with 2-[^{3}H]ICH$_2$COOH

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 (C_1) 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 C_1 was treated with endoprotease Glu-C, under conditions in which the enzyme would cleave after both aspartic and glutamic acid residues (Sorensen *et al.*, 1991), 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 as a doublet at about 31 min, about 30% was eluted at about 40 min, and 5% did not bind to the column. Fractions g_1 and g_2 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 g_1 is shown in Table I. The radioactivity elutes with the CM-Cys in the first cycle and the peptide was identified as: ^{485}CMCys-ITLPTKKSNNHse^{496}. The peptide contains homoserine at its C-terminal and was eluted from the column as a doublet due to the equilibrium between homoserine and homoserine lactone (Gross, 1967).

Fraction g_3 was also analyzed by N-terminal sequencing (Table I) and contains the following CNBr/endoprotease Glu-C peptide:

FIGURE 2: Gel filtration of [^{3}H]iodoacetate-labeled α1,2-mannosidase digested with CNBr. α1,2-Mannosidase (1.2 mg) was treated with [^{3}H]ICH_2COOH 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 (1x120 cm) in the same solvent. One ml fractions were collected, the A_{230nm} was measured (*open circles*), and 20 µl from each fraction were used to quantitate radioactivity (*closed circles*). Fraction C_1 was pooled as indicated by the bar and subsequently digested with endoprotease Glu-C. V_o and V_t correspond to the elution position of BSA and tyrosine, respectively.

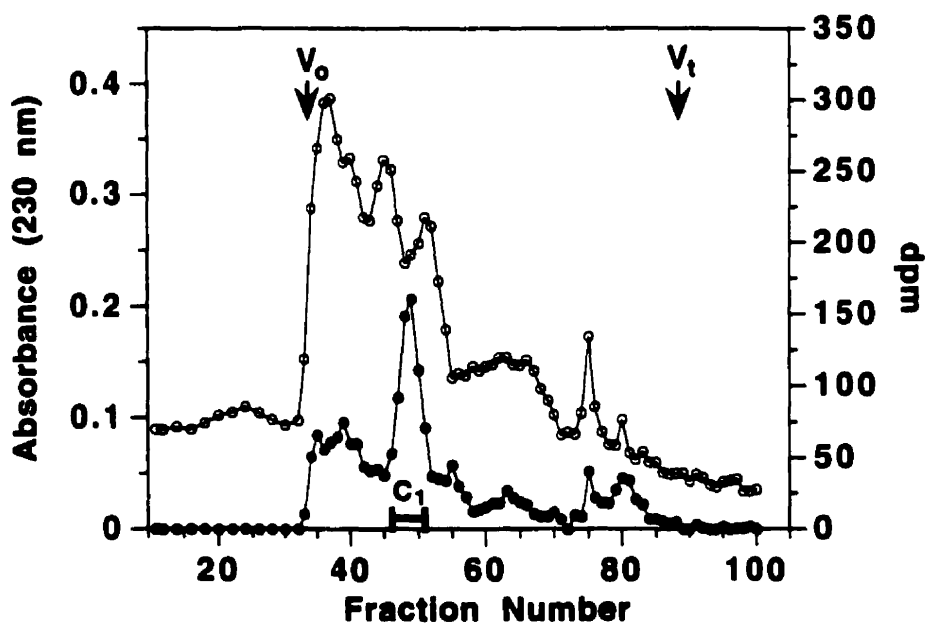

Absorbance (230 nm)
dpm
Fraction Number
V_o
V_t
C_1
0
0.1
0.2
0.3
0.4
0
50
100
150
200
250
300
350
20
40
60
80
100

FIGURE 3: HPLC of endoprotease Glu-C-digested peptides. Fraction C_1 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 C_1 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 g_1, g_2, and g_3.

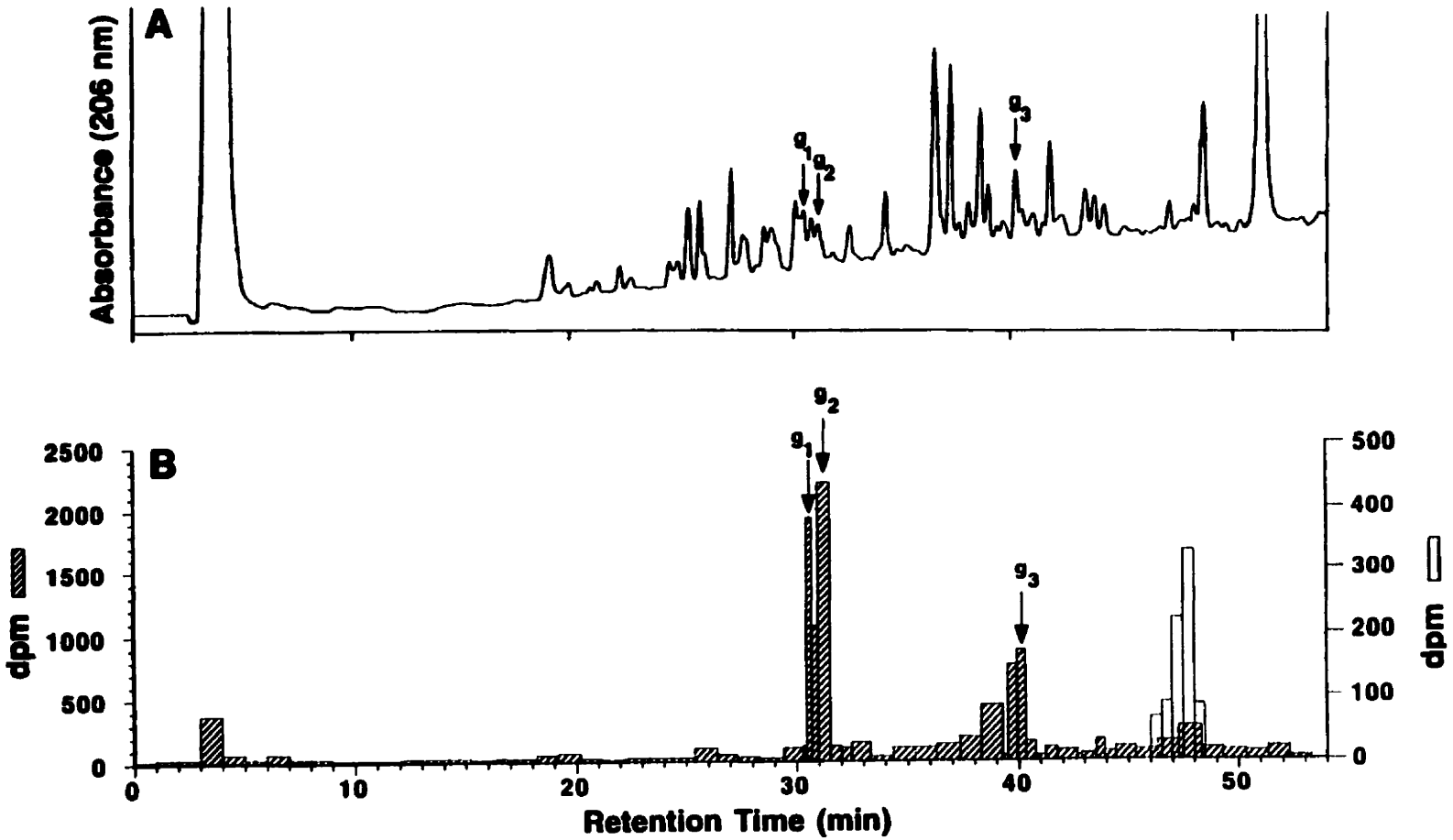

A
Absorbance (206 nm)
g1
g2
g3
B
dpm
2500
2000
1500
1000
500
0
500
400
300
200
100
0
dpm
0
10
20
30
40
50
Retention Time (min)

TABLE I

N-terminal sequence of peptides g_1 and g_3

Fractions g_1 and g_3 from HPLC of endoprotease Glu-C-digested peptides (see Fig. 3) were analyzed by N-terminal sequencing.

Cycle no.	Amino acid	Radioactivity [a]	Picomoles
		dpm	
Peptide g_1			
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 g_3			
1	Asn	0	50
2	Thr	0	24
3	- [b]	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

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

[b] 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.

^{466}NTCVDCNDPKLRRFTSLSD-CMCys-ITLPTKKSNNHse^{496}. There was no CM-Cys or radioactivity in cycles 3 or 6, hence, Cys^{468} and Cys^{471} did not react with iodoacetate. The only radioactivity seen was in cycles 20 and 21, corresponding to the CM-Cys residue. These data show that fraction g_3 is a partially digested peptide also containing carboxymethylated Cys^{485}.

Isolation and Characterization of Disulfide-bonded Peptides

In order to isolate peptides containing a disulfide bond, the α1,2-mannosidase was first treated with CNBr and the products were fractionated by Sephadex G-50 gel filtration chromatography as described in Experimental Procedures. Disulfide exchange was prevented by CNBr treatment under acidic conditions. Five peptide fractions (P_1-P_5) 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 a_1 disappears upon reduction and fraction a_2 is decreased by half. This residual absorbing material is a contaminant that is present in the buffer alone. Fractions a_1 and a_2 were collected manually and subjected to amino acid analysis. Fraction a_1 did not contain any cysteine and did not correspond to any possible disulfide-containing peptide in the yeast α1,2-mannosidase. From amino acid composition and N-terminal sequencing results (Table II), it was determined that fraction a_2 contains the following peptides which are linked by a disulfide bond: ^{336}DHLVCFHse^{342} and ^{383}DTCYQHse^{388}. These results demonstrate that there is a disulfide bond between Cys^{340} and Cys^{385}.

HPLC/MS Peptide Mapping

In order to confirm the location of the disulfide bonds and the free sulfhydryl group, HPLC/MS was used to analyze trypsin-digested α1,2-mannosidase. Trypsin digestion was carried out at pH 6.5 in order to minimize disulfide exchange (Robertson *et al.*, 1994). Both reduced and non-reduced tryptic digests were analyzed by HPLC/MS. Some peaks could not be identified as tryptic peptides. Also, peaks after scan 850 contained peptides above 4000 Da and contained mixtures of peptides which could not be resolved. However, 77% of the amino acid sequence was confirmed. Longer incubation

FIGURE 4: Effect of reduction on endoprotease Asp-N-digested peptides. Peptides obtained following CNBr cleavage (250 μg) were digested with endoprotease Asp-N as indicated under "Experimental Procedures". Aliquots (about 5% of total) of the endoprotease Asp-N treated peptides were incubated in the absence (A) or presence (B) of DTT and analyzed by HPLC as indicated under "Experimental Procedures". Peptides which disappear upon reduction are named a_1 and a_2. Peaks that were present when the buffer alone was chromatographed are marked with an *asterisk* (*).

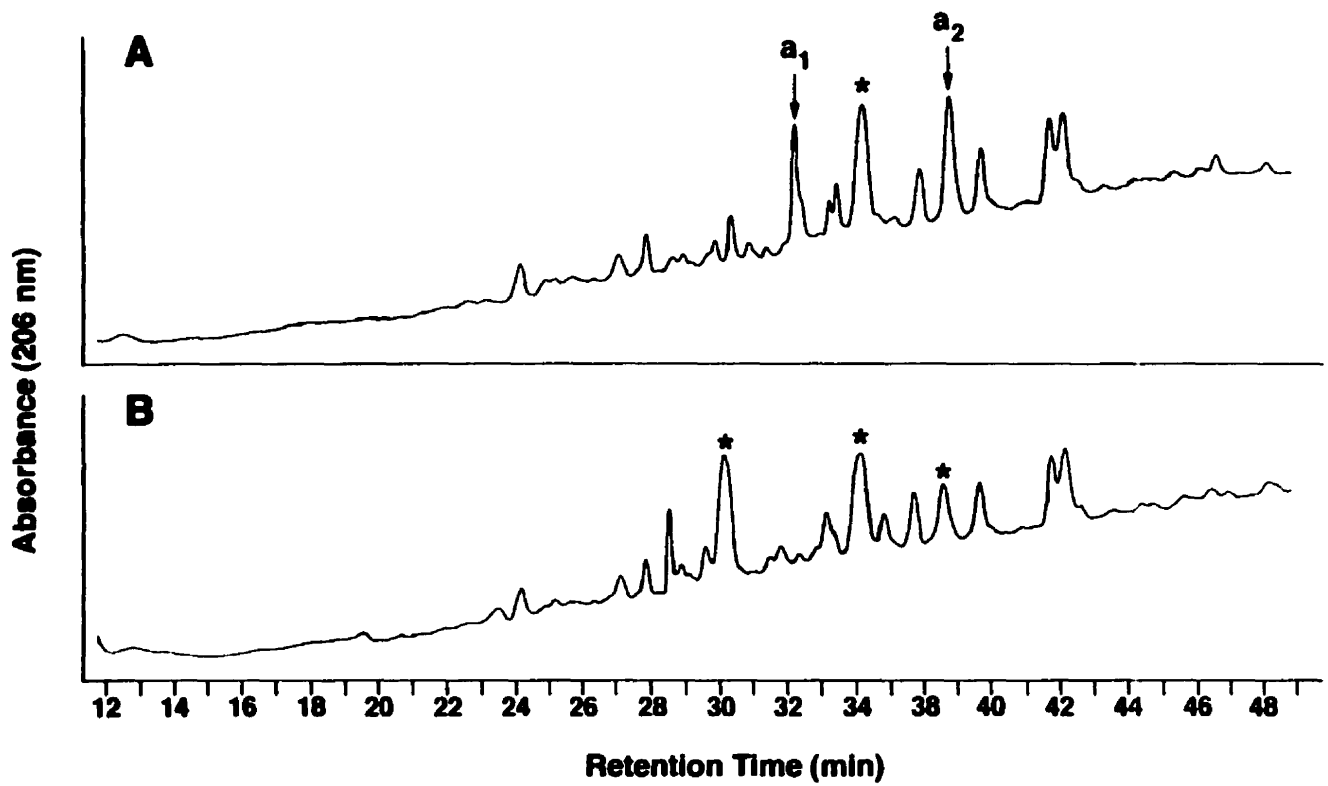

A
B
a1
a2
*
Absorbance (206 nm)
12
14
16
18
20
22
24
26
28
30
32
34
38
40
42
44
46
48
Retention Time (min)

TABLE II

Characterization of peptide a_2

Fraction a_2 from HPLC of endoprotease Asp-N-digested peptides (see Fig. 4) was analyzed for amino acid composition and N-terminal sequence.

Amino acid composition			N-terminal sequence	
Amino acid	Expected ratio	Obtained ratio	Cycle no.	Amino acids
				pmol
Asx	2	2.2	1	Asp (83)
Thr	1	0.9		
Ser	0	0.3	2	Thr (16), His (20)
Hse [a]	2			
Glx	1	1.4	3	Leu (29)
Pro	0	0		
Gly	0	0.2	4	Tyr (33), Val (34)
Ala	0	0.2		
Cys	2	0.7	5	Gln (16), Glu (9) [b]
Val	1	1.1		
Ile	0	0	6	Phe (3), Hse [c]
Leu	1	0.9		
Tyr	1	0.9	7	Hse [c]
Phe	1	1.0		
His	1	0.8		
Lys	0	0.5		
Arg	0	0		

[a] Methionine is converted to homoserine by CNBr cleavage. Homoserine was present, but was not quantitated.

[b] Glutamic acid is obtained due to the deamidation of glutamine.

[c] There was no standard for homoserine, but there was a peak where the homoserine is expected to elute.

with trypsin was not useful, since there were too many peptides which could not be assigned to expected tryptic peptides.

A peptide with mass 4053.3, corresponding to the peptide in which Cys^{340} is bound to Cys^{385} (t_{16}+t_{19}), 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, t_{16} and 1322.9, t_{19}) were only observed in the reduced sample (Fig. 5B and Table III).

The tryptic peptide containing Cys^{485} (t_{23}) 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 (t_{22}) 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 Cys^{468} and Cys^{471}; therefore, peptide t_{22} 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 sulfhydryl residue in the yeast α1,2-mannosidase, each cysteine was individually mutated to serine. The plasmids were transformed into *P. pastoris* and clones were screened for α1,2-mannosidase expression using Western blotting and α1,2-mannosidase assay. Although a significant number (25) of transformants were screened for each mutant, recombinant α1,2-mannosidases containing C340S or C385S mutations were never detected in the medium. Western blotting of cellular extracts showed minimal or no α1,2-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 α1,2-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 α1,2-mannosidase activity were subjected to Western blotting (Fig. 6). According to densitometric scanning, it was determined that the C485S mutation did not affect α1,2-mannosidase activity, whereas the C468S and C471S mutation reduced the specific

FIGURE 5: Effect of reduction on trypsin-treated peptides analyzed by HPLC/MS. A region from the HPLC/MS of the non-reduced sample containing peptides t_{23} and t_{16}+t_{19} is illustrated in (A). The region from the HPLC/MS of the reduced sample containing peptides t_{16}, t_{19}, and t_{23} is shown in (B). Tryptic peptides are named according to their order in the amino acid sequence from the N- to C-terminus. Identified tryptic peptides that do not contain cysteine are also indicated. The unnamed peaks contain mixtures of peptides that could not be identified or contain peptides which do not correspond to the mass of any expected tryptic peptides.

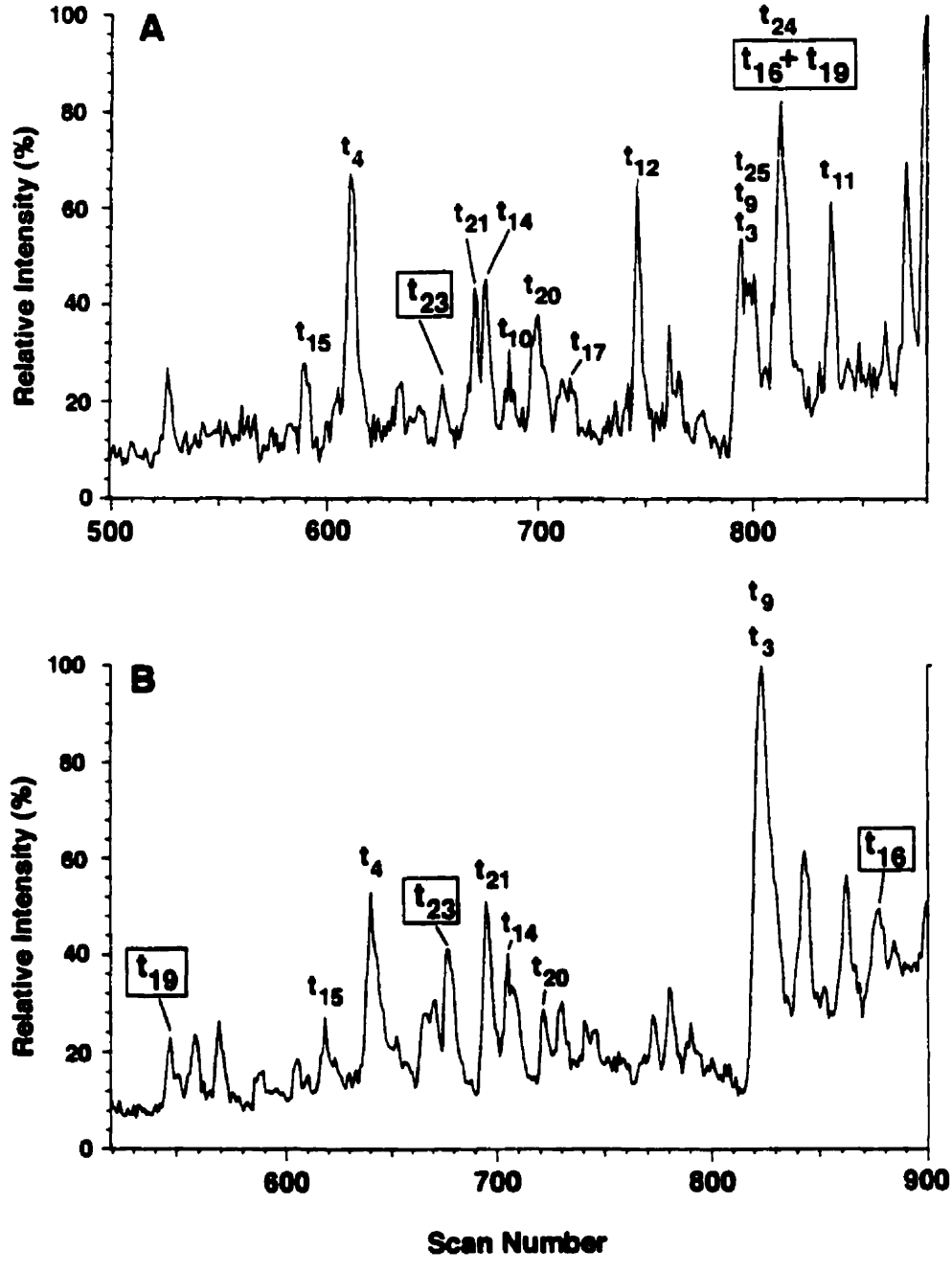

A
B
Relative Intensity (%)
Scan Number
t24
t16+ t19
t4
t12
t25
t9
t3
t11
t21
t14
t23
t20
t10
t17
t15
t19
t16

TABLE III

Cysteine-containing peptides identified by HPLC/MS of reduced and non-reduced trypsin digest of yeast α1,2-mannosidase

Treatment [a]	Tryptic peptides		MH^+		Cysteine [d]
	Peptide no. [b]	Amino acid nos.	Observed	Calculated [c]	
NR	t_{16} + t_{19}	335-360 + 380-390	4053.3	4052.5	C340 -> C385
R	t_{16}	335-360	2731.8	2732.8	C340
R	t_{19}	380-390	1322.9	1322.7	C385
NR	t_{22}	429-475	5625.1	5626.9	C468 -> C471
R	t_{22}	429-475	5626.5	5628.9	C468, C471
NR	t_{23}	479-492	1554.3	1554.1	C485
R	t_{23}	479-492	1554.2	1554.1	C485

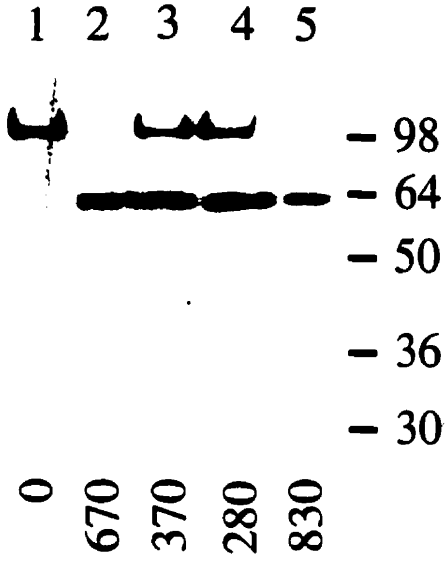
[a] Non-reduced (NR) or reduced (R).

[b] Peptides are numbered according to the amino acid sequence from the N- to C-terminus.

[c] Expected mass calculated from average molecular weights.

[d] 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.

FIGURE 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 two days, then induced in BMMY medium for two days. The medium was diluted 1/8 to 1/150 and assayed for α1,2-mannosidase activity. Different aliquots (0.2 µl for YpHMNS1 in *lane 2*, 2.7 µl for YpHC468S in *lane 3*, 3.4 µl for YpHC471S in *lane 4*, and 0.2 µl for YpHC485S in *lane 5*) containing equal amounts of enzyme activity were loaded on 10% SDS-PAGE and visualized by Western blotting as indicated under "Experimental Procedures". 3.1 µl of medium from pHIL-S1 transformed yeast were loaded as a control in *lane 1*. The molecular size markers (kDa) are shown in the *right margin* and relative α1,2-mannosidase activity (dpm per densitometry unit) is shown at the *bottom*.


1 2 3 4 5
98
64
50
36
30
0
670
370
280
830

activity of the enzyme. Several clones expressing the C468S and C471S mutant enzymes had about 50% of the specific activity present in the wild type enzyme. The K_m values were 0.7 mM, 0.7 mM, 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 $Man_9GlcNAc$ and the products were analyzed by HPLC as previously described (Lipari and Herscovics, 1994). The results indicate that the mutant α1,2-mannosidases retained the same specificity as the wild type α1,2-mannosidase (data not shown).

DISCUSSION

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^{485} 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^{485} 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 tryptic 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, support 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 observed 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^{485} are essential for enzyme activity and that removal of the Cys^{340}-Cys^{385} disulfide bond by mutagenesis of Cys^{340} or Cys^{385} results in no secreted α1,2-mannosidase and little or no intracellular recombinant protein. The Cys^{340}-Cys^{385} disulfide bond is therefore essential for the protein to acquire its proper conformation. Although mutagenesis of Cys^{468} or Cys^{471} caused some decrease in specific activity and a small change in K_m, these mutant enzymes were still catalytically active, consistent with the conclusion that the second disulfide bond is not essential for enzyme activity.

From alignment of the amino acid sequences of the known members of the α1,2-mannosidase family (Fig. 7), it is observed that Cys^{340} and Cys^{385} have been conserved through evolution. This observation indicates that this disulfide bond may also play an

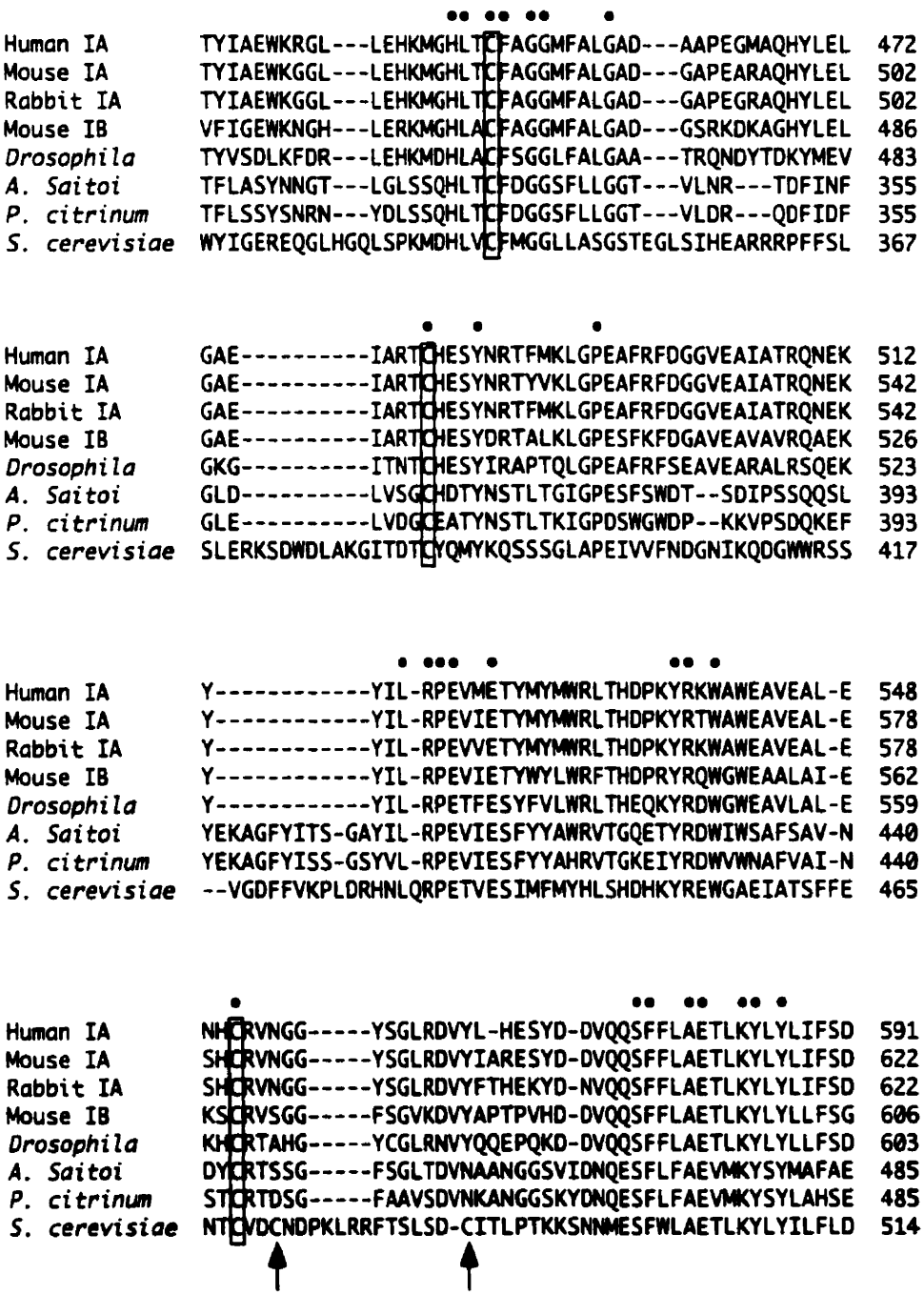
essential role for these enzymes and it supports the conclusion that the yeast enzyme requires formation of the Cys^{340}-Cys^{385} disulfide bond to fold properly.

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 two 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^{468} and Cys^{471} in the yeast enzyme is likely to be important for proper structure and catalytic activity in other members of the family.

The present results demonstrate that mutation of Cys^{485} does not affect activity, showing that the free sulfhydryl 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 ρ-chloromercuribenzoate (Forsee *et al.*, 1989). This inactivation was only observed in the presence of very low Ca^{2+} concentration (0.01 mM) and normal enzyme activity was observed following ρ-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 (Wang *et al.*, 1994).

This study combines results from protein chemistry and mutagenesis in order to elucidate the role of the cysteine residues in the yeast α1,2-mannosidase. It is the only member of the family for which this type of study is possible, since it is the only Family 47 α1,2-mannosidase which can be produced in sufficient quantity as a recombinant enzyme.

FIGURE 7: Alignment of the α1,2-mannosidases of Family 47. The sequences were aligned by the Clustal Method using the Gene Jockey II software. The amino acid sequences containing the conserved cysteine residues are shown from the human IA (Bause *et al.*, 1993), mouse IA (Lal *et al.*, 1994), rabbit IA (Lal *et al.*, 1994), mouse IB (Herscovics *et al.*, 1994), *Drosophila* (Kerscher *et al.*, 1995), *A. saitoi* (Inoue *et al.*, 1995), *P. citrinum* (Yoshida and Ichishima, 1995), and *S. cerevisiae* (Camirand *et al.*, 1991) α1,2-mannosidases. The amino acid numbers are given in the *right margin* and the conserved amino acids are indicated with a *black dot*. The three conserved cysteine residues are highlighted with a *box* and the two additional cysteine residues in the yeast α1,2-mannosidase are marked with an *arrow*.


Human IA TYIAEWKRGL---LEHKMGHLTCFAGGMFALGAD---AAPEGMAQHYLEL 472
Mouse IA TYIAEWKGGL---LEHKMGHLTCFAGGMFALGAD---GAPEARAQHYLEL 502
Rabbit IA TYIAEWKGGL---LEHKMGHLTCFAGGMFALGAD---GAPEGRAQHYLEL 502
Mouse IB VFIGEWKNGH---LERKMGHLACFAGGMFALGAD---GSRKDKAGHYLEL 486
Drosophila TYVSDLKFDR---LEHKMDHLACFSGGLFALGAA---TRQNDYTDKYMEV 483
A. Saitoi TFLASYNNGT---LGLSSQHLTCFDGGSFLLGGT---VLNR---TDFINF 355
P. citrinum TFLSSYSNRN---YDLSSQHLTCFDGGSFLLGGT---VLDR---QDFIDF 355
S. cerevisiae WYIGEREQGLHGQLSPKMDHLVCFMGGLLASGSTEGLSIHEARRRPFFSL 367
Human IA GAE----------IARTCHESYNRTFMKLGPEAFRFDGGVEAIATRQNEK 512
Mouse IA GAE----------IARTCHESYNRTYVKLGPEAFRFDGGVEAIATRQNEK 542
Rabbit IA GAE----------IARTCHESYNRTFMKLGPEAFRFDGGVEAIATRQNEK 542
Mouse IB GAE----------IARTCHESYDRTALKLGPESFKFDGAVEAVAVRQAEK 526
Drosophila GKG----------ITNTCHESYIRAPTQLGPEAFRFSEAVEARALRSQEK 523
A. Saitoi GLD----------LVSGCHDTYNSTLTGIGPESFSWDT--SDIPSSQQSL 393
P. citrinum GLE----------LVDGCEATYNSTLTKIGPDSWGWDP--KKVPSDQKEF 393
S. cerevisiae SLERKSDWDLAKGITDTCYQMYKQSSSGLAPEIVVFNDGNIKQDGWWRSS 417
Human IA Y----------YIL-RPEVMETYMYMWRLTHDPKYRKWAWEAVEAL-E 548
Mouse IA Y----------YIL-RPEVIETYMYMWRLTHDPKYRTWAWEAVEAL-E 578
Rabbit IA Y----------YIL-RPEVVETYMYMWRLTHDPKYRKWAWEAVEAL-E 578
Mouse IB Y----------YIL-RPEVIETYWYLWRFTHDPRYRQWGWEAALAI-E 562
Drosophila Y----------YIL-RPETFESYFVLWRLTHEQKYRDWGWEAVLAL-E 559
A. Saitoi YEKAGFYITS-GAYIL-RPEVIESFYYAWRVTGQETYRDWIWSAFSAV-N 440
P. citrinum YEKAGFYISS-GSYVL-RPEVIESFYYAHRVTGKEIYRDWVWNAFVAI-N 440
S. cerevisiae --VGDFFVKPLDRHNLQRPETVESIMFMYHLSHDHKYREWGAEIATSFFE 465
Human IA NHCRVNGG-----YSGLRDVYL-HESYD-DVQQSFFLAETLKYLYLIFSD 591
Mouse IA SHCRVNGG-----YSGLRDVYIARESYD-DVQQSFFLAETLKYLYLIFSD 622
Rabbit IA SHCRVNGG-----YSGLRDVYFTHEKYD-NVQQSFFLAETLKYLYLIFSD 622
Mouse IB KSCRVSGG-----FSGVKDVYAPTPVHD-DVQQSFFLAETLKYLYLLFSG 606
Drosophila KHCRTAHG-----YCGLRNVYQQEPQKD-DVQQSFFLAETLKYLYLLFSD 603
A. Saitoi DYCRTSSG-----FSGLTDVNAANGGSVIDNQESFLFAEVMKYSYMAFAE 485
P. citrinum STCRTDSG-----FAAVSDVNKANGGSKYDNQESFLFAEVMKYSYLAHSE 485
S. cerevisiae NTCVDCNDPKLRRFTSLSD-CITLPTKKSNNMESFWLAETLKYLYILFLD 514

TRANSITION: CHAPTER 4 TO 5

In Chapter 4, it was demonstrated that the yeast α1,2-mannosidase contains two disulfide bonds and one free sulfhydryl residue. Cys^{340}-Cys^{385} and Cys^{468}- Cys^{471} form the disulfide bonds, and a free thiol group is present on Cys^{485}. Cys^{485} is not at all required for enzymatic activity, whereas the Cys^{468}-Cys^{471} disulfide bond is necessary for optimal enzyme activity. The Cys^{340}-Cys^{385} disulfide bond formed by two invariant cysteine residues is required for proper folding or conformation, since the mutants in either of these residues are not secreted by *P. pastoris*. Since the enzyme was sensitive to reducing agent, it indicated that at least one disulfide bond is essential. This study identified the essential disulfide bond, which is most likely conserved in the other members of the family.

The yeast α1,2-mannosidase catalytic domain is produced as a secreted protein in the methylotrophic yeast, *P. pastoris*, and is quickly purified to obtain up to 30 mg/L of purified protein. The strong alcohol oxidase promoter and the ability of this yeast to sustain a very high cell density allowed for significantly more protein expressed compared to the *S. cerevisiae* expression system.

In Chapter 5, the structure and mechanism of action of the α1,2-mannosidase is further analyzed. The Ca^{2+}-binding properties of the enzyme are characterized and the role of the putative EF hand motif and the nine invariant acidic residues is also investigated.

CHAPTER 5

Effect of mutagenesis of the invariant acidic residues in the yeast α1,2-mannosidase on enzyme activity and calcium binding

SUMMARY

Class 1 α1,2-mannosidases are a family of Ca^{2+}-dependent enzymes that have been conserved through eukaryotic evolution. They are essential for the formation of complex and hybrid N-glycans in mammalian cells and are therefore potential targets for the development of antimetastatic and antiviral agents. There are nine invariant acidic residues and a conserved putative EF hand Ca^{2+}-binding motif found in this enzyme family. Mutagenesis of these conserved residues was done on the processing α1,2-mannosidase from *Saccharomyces cerevisiae* that transforms $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ to identify amino acids that may be involved in catalysis and in Ca^{2+} binding. The effect of mutagenesis was first examined by complementation of a mannosidase-deficient *Saccharomyces cerevisiae* strain. With five of the mutants (E214Q, D275N, E279Q, E435Q, E503Q) there was no detectable α1,2-mannosidase activity whereas with the others (D86N , E132Q, E438Q, E526Q) a very low but significant level of activity (0.07-2%) was observed compared to wild type enzyme. The catalytic domain of the α1,2-mannosidase was then expressed in *Pichia pastoris* as a secreted protein and 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+} was found to protect the enzyme from thermal denaturation. $^{45}Ca^{2+}$ binding of acidic residue mutants (except for E214Q and E435Q that were poorly secreted by *Pichia pastoris*) was determined by autoradiography following non-denaturing polyacrylamide gel electrophoresis or by equilibrium dialysis. The first and twelfth residues of the putative EF hand Ca^{2+}-binding loop sequence, which are the most essential residues in functional EF hands, were mutated (D121N, D121A, E132Q, E132V, and D121A/E132V). These mutations had no effect on Ca^{2+} binding, demonstrating that the EF hand-related sequence is not the site of Ca^{2+} binding in the yeast enzyme. The D275N, E279Q, and E438Q mutants had no detectable or little Ca^{2+} binding, whereas D86N, E503Q, and E526Q mutants exhibited similar binding of Ca^{2+} as the wild type enzyme. The wild type enzyme had a K_m and k_{cat} of 0.5 mM and 700 min^{-1}, respectively. The K_m of E526Q was greatly increased to 4 mM with a small reduction in k_{cat} to 300 min^{-1} whereas the k_{cat} of D86N and E132Q(V) were greatly reduced (0.3-0.4 min^{-1}) with a decrease in K_m (0.07-0.3 mM). These results show that Asp^{275}, Glu^{279}, and Glu^{438} are required for Ca^{2+} binding, whereas Asp^{86}, Glu^{132}, Glu^{503}, and Glu^{526} are important for catalysis.

INTRODUCTION

The *Saccharomyces cerevisiae* Ca^{2+}-dependent α1,2-mannosidase which removes one mannose residue from $Man_9GlcNAc_2$ to form one isomer of $Man_8GlcNAc_2$ (Jelinek-Kelly *et al.*, 1985; Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991) is a member of Class 1 α1,2-mannosidases involved in the processing of asparagine-linked oligosaccharides in the ER and Golgi apparatus (Moremen *et al.*, 1994). The Class 1 α1,2-mannosidases have been conserved through eukaryotic evolution and have also been classified as Family 47 glycosidases (Henrissat and Bairoch, 1996). In addition to the yeast processing enzyme (Camirand *et al.*, 1991), this family includes fungal (Inoue *et al.*, 1995; Yoshida and Ichishima, 1995), insect (Kerscher *et al.*, 1995; Kawar *et al.*, 1997), and mammalian (Bause *et al.*, 1993; Herscovics *et al.*, 1994; Lal *et al.*, 1994; Bieberich *et al.*, 1997; Tremblay *et al.*, 1998) α1,2-mannosidases. Besides their sequence similarity, the Class 1 α1,2-mannosidases have similar biochemical properties. They specifically cleave α1,2-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, with the possible exception of the fungal enzymes (for reviews see Daniel *et al.*, 1994; Moremen *et al.*, 1994; Herscovics, 1998). The α1,2-mannosidases are distinct from the Class 2 (Family 38) α-mannosidases, which include Golgi α-mannosidase II and lysosomal α-mannosidases. The Class 2 α-mannosidases have different biochemical properties and catalytic mechanism. They are capable of cleaving α1,2- α1,3- α1,6-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. The Class 2 α-mannosidases are Ca^{2+}-independent retaining glycosidases (Howard *et al.*, 1997).

Although several Class 1 α1,2-mannosidases have been cloned and their enzymatic properties have been studied (Bieberich *et al.*, 1997; Lal *et al.*, 1998; Tremblay *et al.*, 1998), 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 is important since Class 1 α1,2-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 (Brockhausen, 1993; Varki, 1993). In particular, there has been considerable interest in the development of inhibitors of processing glycosidases that have potential as antimetastatic and antiviral agents (Winchester and Fleet, 1992; Jacob, 1995).

Since the development of expression systems that produce milligram quantities of the yeast α1,2-mannosidase catalytic domain (Lipari and Herscovics, 1994; Lipari and Herscovics, 1996), the yeast enzyme has served as a model to determine the structure and mechanism of action of the Class 1 α1,2-mannosidases. It was first shown that the yeast enzyme catalyzes removal of mannose with inversion of the anomeric configuration (Lipari *et al.*, 1995), an observation that was recently substantiated for a mammalian member of this enzyme family (Lal *et al.*, 1998). Detailed chemical and mutagenesis studies of the role of cysteine residues (Lipari and Herscovics, 1996) demonstrated that there are two disulfide bonds in the yeast α1,2-mannosidase and that one of these disulfide bonds, between cysteine residues that are conserved in all Class 1 α1,2-mannosidases, is essential for enzyme activity.

The Class 1 α1,2-mannosidases contain a region that is similar to the EF hand Ca^{2+}-binding motifs found in many different Ca^{2+}-dependent proteins (Marsden *et al.*, 1990). An EF hand motif consists of a twelve 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 α1,2-mannosidase. The Ca^{2+} dependence of the Class 1 α1,2-mannosidases has been attributed to this putative Ca^{2+}-binding EF hand motif, but there has been 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, except for studies on the stereochemical course of hydrolysis showing that the Class 1 α1,2-mannosidases are inverting glycosidases (Lipari *et al.*, 1995; Lal *et al.*, 1998) whereas the Class 2 α-mannosidases are retaining enzymes (Howard *et al.*, 1997). Crystal structures and studies on the catalytic mechanisms of both retaining and inverting glycosidases have shown that two acidic residues are usually involved in catalysis (for reviews see Sinnott, 1990; Legler, 1993; Svensson and Søgaard, 1993; McCarter and Withers, 1994; Davies and Henrissat, 1995). For inverting glycosidases, such as the Class 1 α1,2-mannosidases, 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. In either type, the two catalytic residues are usually conserved within each family of glycosidases, but these two catalytic residues have not yet been identified in Class 1 α1,2-mannosidases.

In the current work the yeast enzyme was used to study Ca^{2+} binding properties and the role of the nine invariant acidic residues in the activity of the Class 1 α1,2-mannosidases. It is demonstrated that the yeast α1,2-mannosidase binds one Ca^{2+} ion per mole 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, while four others (Asp^{86}, Glu^{132}, Glu^{503}, and Glu^{526}) are required for catalysis and include potential active site 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, were from Pharmacia Biotech Inc. [^{3}H] labeled and unlabeled $Man_9GlcNAc$ oligosaccharide substrate was prepared as previously described (Lipari and Herscovics, 1994). $^{45}CaCl_2$ (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 Pre-Stained 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

E. 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 (Camirand *et al.*, 1991). 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,

E526Q: GTTGTTTTCAACACACAAGCTCATCCTTTTCC. The open reading frames of the mutants were sequenced by the dideoxy method (Sanger *et al.*, 1977) using either the T7 Sequencing Kit from Pharmacia Biotech Inc. or at the Sheldon Biotechnology Centre (McGill University, Montréal, Canada) by 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 α1,2-mannosidase, beginning at amino acid 23, in the pHIL-S1 vector (Invitrogen)(Lipari and Herscovics, 1996). 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 U.S.E. mutagenesis with the following oligonucleotides:
D121N: GGATAAACGATGTTTTAAATTTTGATATTGATGCCG,
D121A: GGATAAACGATGTGCTAGCTTTTGATATTGATGCCG,
E132V: GATGCCGAAGTTAACGTTTTTGTAACTACTATTAGAATGC,
D121A/E132V:CGATGTTTTGGCTTTTGATATTGATGCCGAAGTTAACGTTTTTG TAACTACTATTAG; oligonucleotides for D275N, E279Q, and E503Q were the same as above. The mutated regions were sequenced as above.

Expression of mutant α1,2-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*))(Camirand *et al.*, 1991) was used to select *ura3* yeast cells by plating on medium containing 5-fluoro-orotic acid and uracil, as described by Boeke *et al.* (1984). The resulting YFL9.26 strain was transformed either with the pYH4 plasmid, the YEp352 vector, or the YEp352 vector containing mutant *MNS1* genes by the lithium acetate method (Ito *et al.*, 1983). Freshly transformed cells were grown overnight at 30 °C in 5 ml supplemented minimal medium without uracil, then 15 ml of YPD were 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 α1,2-mannosidase expressed in P. pastoris

The α1,2-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 (Lipari and Herscovics, 1996). 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 α1,2-mannosidases were purified essentially as described previously (Lipari and Herscovics, 1994; Lipari *et al.*, 1995). 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 was then 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 α1,2-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 α1,2-mannosidase was eluted with a NaCl gradient (0 - 0.4 M) between 0.1 and 0.2 M NaCl. All the purification procedures were performed at 4 °C with buffers containing 1 mM NaN_3.

Atomic absorption spectrophotometry

1.1 ml of purified recombinant α1,2-mannosidase (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 identical conditions to 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 α1,2-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 one degree per 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.* (1983). All plasticware was washed with 10 mM EDTA and rinsed with deionized water. Samples (60-100 µl) of purified mutant and wild type α1,2-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+}. 10 µl aliquots 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. 10 µl aliquots were used to determine the concentration of α1,2-mannosidase by A_{280} using an extinction coefficient of 115,200 $M^{-1}cm^{-1}$ as calculated according to Mach *et al.* (1992). Duplicate samples were used for all measurements. The concentration of free Ca^{2+} in the buffers containing EGTA was determined using the MaxChelator program (Bers *et al.*, 1994).

$^{45}Ca^{2+}$ binding in native polyacrylamide gels

$^{45}Ca^{2+}$ binding to α1,2-mannosidase following electrophoresis in native PAGE gels was assayed essentially as previously described by Schibeci *et al.* (1980). 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 $^{45}Ca^{2+}$. Following 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 seven 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

α1,2-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 (Markwell *et al.*, 1981) with bovine serum albumin as standard. The concentration of purified recombinant α1,2-mannosidase was determined by absorbance at 280 nm using an extinction coefficient of 115,200 $M^{-1}cm^{-1}$ (Mach *et al.*, 1992). The amount of protein on gels was determined by densitometry following staining with Coomassie, using purified recombinant α1,2-mannosidase as standard. SDS-PAGE under reducing conditions was carried out according to Laemmli (1970) using the Bio-Rad Mini-Protean II apparatus. Native PAGE gels (0.75 mm thickness) were prepared as for 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 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 α1,2-mannosidase (Burke *et al.*, 1996). The antibodies were also pretreated with a cell extract from the *S. cerevisiae* strain YFL9.26 in order to reduce background.

α1,2-Mannosidase assays

[^{3}H]mannose released from [^{3}H]$Man_9GlcNAc$ was quantitated using the Concanavalin A method (Herscovics and Jelinek-Kelly, 1987). Depending on the experiment, one of the three following α1,2-mannosidase assay protocols was used: (A) In order to assay α1,2-mannosidase activity in cellular extracts, the assay mixtures contained 25 to 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. Incubation times ranging from 15 min to 15 h at 37 °C are indicated in the legend to Fig. 2. For the mutants with no detectable activity the extracts were incubated for 7 h under the conditions indicated above, another 200 to 550 µg of protein extract (40 µl aliquot) was added and incubated another 15 h at 37 °C. Enzyme activity was quantitated as dpm/h per µg of protein. The assays were done under conditions in which the enzyme activity was linear with time of incubation. (B) In order to measure the specific activity or the kinetic parameters of the purified α1,2-

mannosidase, the assays were perfomed as described previously (Lipari and Herscovics, 1994). The enzyme was assayed for 45 min or 2 h at 37 °C with 0.07 - 0.7 mM unlabelled $Man_9GlcNAc$, 17,400 dpm [^{3}H]$Man_9GlcNAc$, 0.1 M PIPES (pH 6.5), 1 mM NaN_3, and 20 µg BSA in a final volume of 20 µL. Kinetic parameters were determined by Lineweaver-Burke analysis. (C) In order to assay for Ca^{2+}-dependence, purified α1,2-mannosidase was treated for 10 min at 4 °C with Chelex (10 mg/100 µl) in 0.1 M PIPES (pH 6.8). The enzyme sample (7 µM for E132Q or 0.1 µM for wild type and D121N) was 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. $Man_9GlcNAc$ substrate was then added to a final concentration of 0.7 mM (wild type and D121N) or 0.1 mM (E132Q) with 17,400 dpm of [^{3}H]$Man_9GlcNAc$ 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

Effects of mutation of the invariant acidic residues in vivo

The alignment of the Class 1 α1,2-mannosidase catalytic domains indicates that there are some highly conserved regions including nine invariant acidic residues and a putative EF hand consensus sequence in this enzyme family (Fig. 1). Since 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 (Svensson and Søgaard, 1993; McCarter and Withers, 1994), the role of these residues in α1,2-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 (Camirand *et al.*, 1991). 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 the mutants were expressed at appreciable levels as seen by Western blotting of cellular extracts (Fig. 2). With the E214Q, D275N, E279Q, E435Q and E503Q mutants there was no detectable α1,2-mannosidase activity. Measurable α1,2-mannosidase activity was detected in cells transformed with the D86N, E132Q, E438Q, and E526Q mutants, but the enzyme activity was greatly reduced to levels between 0.07 and 2 % of that observed in cells transformed with the wild type enzyme.

Ca^{2+} binding properties of recombinant α1,2-mannosidase

Since all the invariant acidic residues were shown to be essential for α1,2-mannosidase activity, including Glu^{132} that is part of the 12 residue consensus sequence found in the putative EF hand Ca^{2+}-binding motif observed in Class 1 α1,2-mannosidases (Fig. 1, Fig. 3), the Ca^{2+} binding properties of the enzyme and the role of the EF hand in Ca^{2+} binding were examined. It was previously shown that the yeast α1,2-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 (Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991), but the stoichiometry and affinity of the enzyme for Ca^{2+} were never determined. For this purpose the catalytic domain of the yeast α1,2-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

FIGURE 1: Alignment of the Class 1 α1,2-mannosidases. The sequences were aligned by the Clustal Method using the GeneJockey II software. The catalytic domains of the mouse IA (Lal *et al.*, 1994), mouse IB (Herscovics *et al.*, 1994), and *S. cerevisiae* (Camirand *et al.*, 1991) α1,2-mannosidases are shown. The putative EF hand loop sequence of the yeast α1,2-mannosidase is *underlined.* Although only three sequences are indicated in the figure, the nine invariant acidic residues, indicated with a *box*, are observed in all Class 1 α1,2-mannosidases cloned so far and those in the *S. cerevisiae* protein are *numbered.*

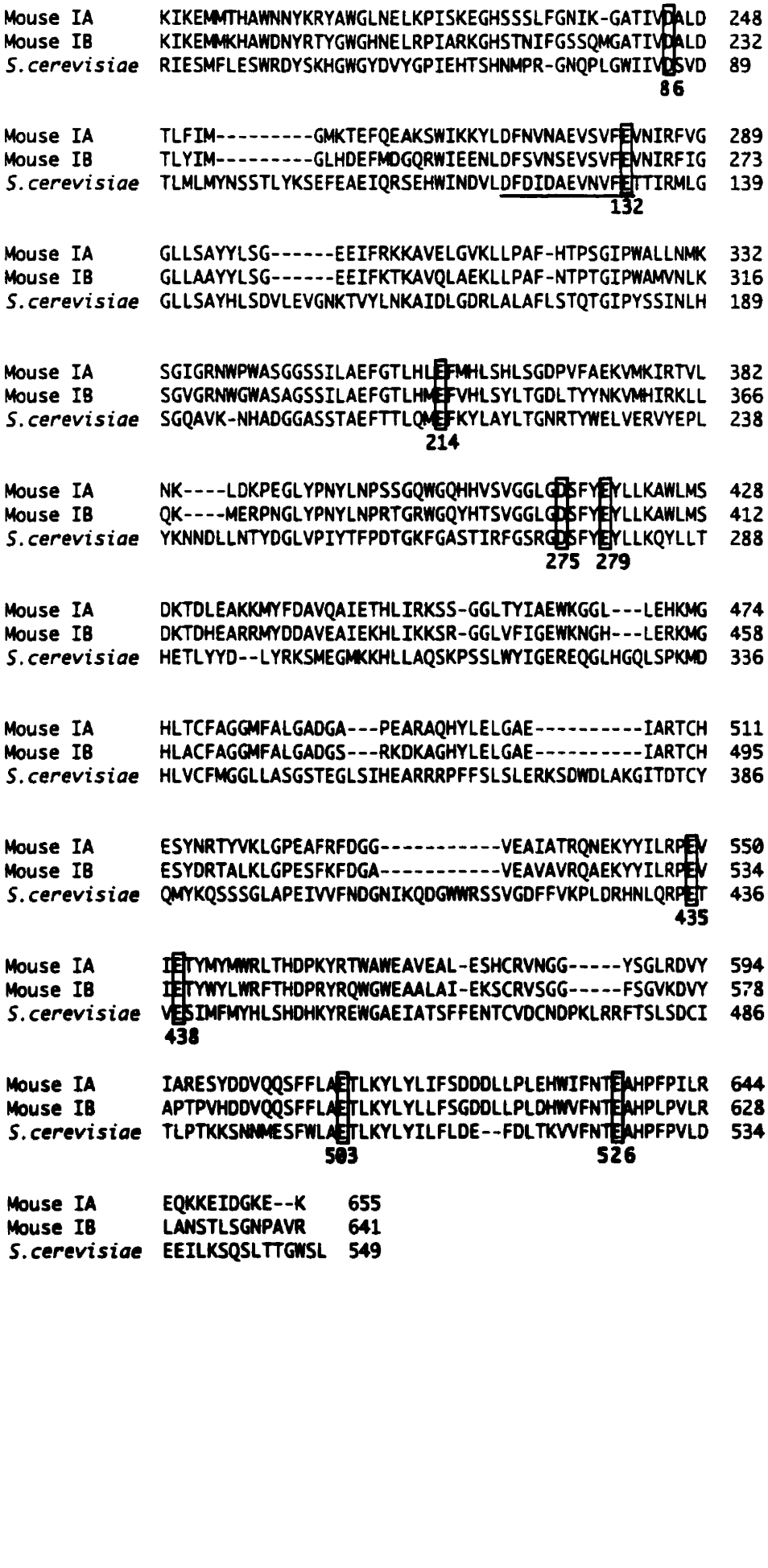

Mouse IA KIKEMMTHAWNNYKRYAWGLNELKPISKEGHSSSLFGNIK-GATIVDALD 248
Mouse IB KIKEMMKHAWDNYRTYGWGHNELRPIARKGHSTNIFGSSQMGATIVDALD 232
S.cerevisiae RIESMFLESWRDYSKHGWGYDVYGPIEHTSHNMPR-GNQPLGWIIVDSVD 89
86
Mouse IA TLFIM---------GMKTEFQEAKSWIKKYLDFNVNAEVSVFEVNIRFVG 289
Mouse IB TLYIM---------GLHDEFMDGQRWIEENLDFSVNSEVSVFEVNIRFIG 273
S.cerevisiae TLMLMYNSSTLYKSEFEAEIQRSEHWINDVLDFDIDAEVNVFETTIRMLG 139
132
Mouse IA GLLSAYYLSG------EEIFRKKAVELGVKLLPAF-HTPSGIPWALLNMK 332
Mouse IB GLLAAYYLSG------EEIFKTKAVQLAEKLLPAF-NTPTGIPWAMVNLK 316
S.cerevisiae GLLSAYHLSDVLEVGNKTVYLNKAIDLGDRLALAFLSTQTGIPYSSINLH 189
Mouse IA SGIGRNWPWASGGSSILAEFGTLHLEFMHLSHLSGDPVFAEKVMKIRTVL 382
Mouse IB SGVGRNWGWASAGSSILAEFGTLHMEFVHLSYLTGDLTYYNKVMHIRKLL 366
S.cerevisiae SGQAVK-NHADGGASSTAEFTTLQMEFKYLAYLTGNRTYWELVERVYEPL 238
214
Mouse IA NK----LDKPEGLYPNYLNPSSGQWGQHHVSVGGLGDSFYEYLLKAWLMS 428
Mouse IB QK----MERPNGLYPNYLNPRTGRWGQYHTSVGGLGDSFYEYLLKAWLMS 412
S.cerevisiae YKNNDLLNTYDGLVPIYTFPDTGKFGASTIRFGSRGDSFYEYLLKQYLLT 288
275 279
Mouse IA DKTDLEAKKMYFDAVQAIETHLIRKSS-GGLTYIAEWKGGL---LEHKMG 474
Mouse IB DKTDHEARRMYDDAVEAIEKHLIKKSR-GGLVFIGEWKNGH---LERKMG 458
S.cerevisiae HETLYYD--LYRKSMEGMKKHLLAQSKPSSLWYIGEREQGLHGQLSPKMD 336
Mouse IA HLTCFAGGMFALGADGA---PEARAQHYLELGAE----------IARTCH 511
Mouse IB HLACFAGGMFALGADGS---RKDKAGHYLELGAE----------IARTCH 495
S.cerevisiae HLVCFMGGLLASGSTEGLSIHEARRRPFFSLSLERKSDWDLAKGITDTCY 386
Mouse IA ESYNRTYVKLGPEAFRFDGG----------VEAIATRQNEKYYILRPEV 550
Mouse IB ESYDRTALKLGPESFKFDGA----------VEAVAVRQAEKYYILRPEV 534
S.cerevisiae QMYKQSSSGLAPEIVVFNDGNIKQDGWWRSSVGDFFVKPLDRHNLQRPET 436
435
Mouse IA IETYMYMWRLTHDPKYRTWAWEAVEAL-ESHCRVNGG-----YSGLRDVY 594
Mouse IB IETYWYLWRFTHDPRYRQWGWEAALAI-EKSCRVSGG-----FSGVKDVY 578
S.cerevisiae VESIMFMYHLSHDHKYREWGAEIATSFFENTCVDCNDPKLRRFTSLSDCI 486
438
Mouse IA IARESYDDVQQSFFLAETLKYLYLIFSDDDLLPLEHWIFNTEAHPFPILR 644
Mouse IB APTPVHDDVQQSFFLAETLKYLYLLFSGDDLLPLDHWVFNTEAHPLPVLR 628
S.cerevisiae TLPTKKSNNMESFWLAETLKYLYILFLDE--FDLTKVVFNTEAHPFPVLD 534
503 526
Mouse IA EQKKEIDGKE--K 655
Mouse IB LANSTLSGNPAVR 641
S.cerevisiae EEILKSQSLTTGWSL 549

FIGURE 2: Activity of the invariant acidic residue mutants expressed in *S. cerevisiae*. Extracts of *S. cerevisiae* strain YFL9.26 expressing the wild type (pYH4) or mutant α1,2-mannosidases were prepared and the α1,2-mannosidase activity in the extracts was determined using assay protocol A according to "Experimental Procedures". The incubation times for the α1,2-mannosidase assays were as follows: pYH4 (15 to 30 min), D86N (4 to 15 h), E132Q (3 to 7 h), E438Q (15 h), E526Q (30 min to 3 h). No detectable activity was observed in the other mutants even though more extended incubation times were used as explained in "Experimental Procedures". Cell extracts containing 25 μg of protein were subjected to 8 % SDS-PAGE and Western blotting and the α1,2-mannosidase bands were quantitated by densitometry as explained under "Experimental Procedures". The α1,2-mannosidase activity in cells transformed with pYH4 was about 17 dpm/h per μg of protein. The specific activity (α1,2-mannosidase activity / relative densitometry units) as % of wild type is shown at the *bottom* of the Western blot. The molecular size markers (kDa) are indicated on the *left*.

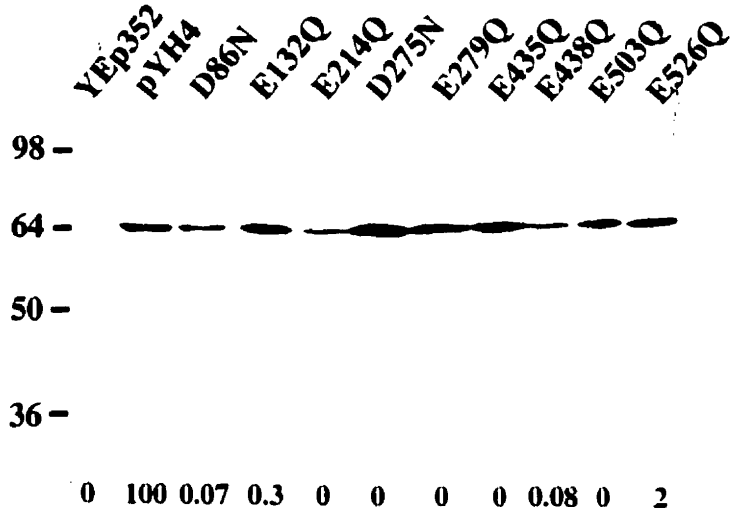

YEp352
pYH4
D86N
E132Q
E214Q
D275N
E279Q
E435Q
E438Q
E503Q
E526Q
98 –
64 –
50 –
36 –
0 100 0.07 0.3 0 0 0 0 0.08 0 2

FIGURE 3: The conserved EF hand motif in the Class I α1,2-mannosidases. The putative EF hand consensus sequence of the human IA (Bause *et al.*, 1993), human IB (Tremblay *et al.*, 1998), pig IA (Bieberich *et al.*, 1997), rabbit IA (Lal *et al.*, 1994), mouseIA (Lal *et al.*, 1994), mouse IB (Herscovics *et al.*, 1994), and the *S. cerevisiae* (Camirand *et al.*, 1991) α1,2-mannosidases are aligned and compared to the established EF hand consensus sequence. The consensus includes the 12-residue Ca^{2+}-binding loop sequence and the subsequent residue based on sequence alignment of known EF hand proteins (Bairoch, 1993). The amino acids in the α1,2-mannosidase sequences that do not agree with the EF hand consensus are in normal type, while the others are in bold. The positions in the EF hand sequence are *numbered* and Ca^{2+} coordinating positions are indicated as X, Y, Z, -X, -Y, and -Z *above* the alignment. In the consensus EF hand sequence, a *capital* letter denotes that only these residue(s) were found at that position, while a *lower case* letter(s) indicates that these residues were not found at that position. An "x" signifies that any amino acid is possible.

	1	2	3	4	5	6	7	8	9	10	11	12	13
	X		Y		Z		-Y		-X			-Z	
Human IA	D	F	N	V	N	A	E	I	S	V	F	E	V
Human IB	D	F	S	V	N	S	E	V	S	V	F	E	V
Pig IA	N	F	N	V	N	A	E	V	S	V	F	E	V
Rabbit IA	D	F	N	V	N	A	E	I	S	V	F	E	V
Mouse IA	D	F	N	V	N	A	E	V	S	V	F	E	V
Mouse IB	D	F	S	V	N	S	E	V	S	V	F	E	V
S. cerevisiae	D	F	D	I	D	A	E	V	N	V	F	E	T
Consensus for EF hand	D	x	D N S	i l v f y w	D E N S T G	D N Q G H R K	g p	L I V M C	D E N Q S T A G C	x	x	D E	L I V M F Y W

bound per 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 per molecule, respectively. In order 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 to 5 μM free Ca^{2+} in the buffer. Scatchard analysis of the binding data (Fig. 4) demonstrated that about 0.8 equivalent of Ca^{2+} was bound per mole of enzyme with a K_D value of 4×10^{-7} M.

Role of Ca^{2+} on thermostability

The possibility that the bound Ca^{2+} found associated with the yeast α1,2-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+} (Fig. 5). In the presence of 5 mM Ca^{2+} and 5 mM EGTA the α1,2-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 α1,2-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+}.

Circular dichroism was used as a probe of α1,2-mannosidase structure upon exposure of the enzyme to increased temperature. When the α1,2-mannosidase was incubated with 10 mM EGTA, there was a dramatic decrease in the signal beween 200 and 250 nm after exposure to a temperature gradient (25-80 °C), indicating unfolding of the α1,2-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 indicate that Ca^{2+} binding to the α1,2-mannosidase greatly reduces its susceptibility to thermal denaturation, indicating that Ca^{2+} plays a structural role.

Characterization of the EF hand mutants

One of the conserved motifs in the Class 1 α1,2-mannosidases is similar to EF hand regions that act as Ca^{2+} binding sites in many proteins. An EF hand consists of a 12 residue loop consensus sequence flanked by two α-helices. A comparison of the putative EF hand loop sequences of the Class 1 α1,2-mannosidases and the established consensus EF hand loop sequence (Bairoch, 1993) is illustrated in Fig. 3. It has been shown that the first and twelfth 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 (Marsden *et al.*, 1990; Bairoch, 1993). In order to determine whether this motif is

FIGURE 4: Scatchard analysis of Ca^{2+} binding to wild type α1,2-mannosidase. Equilibrium dialysis with purified recombinant α1,2-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.

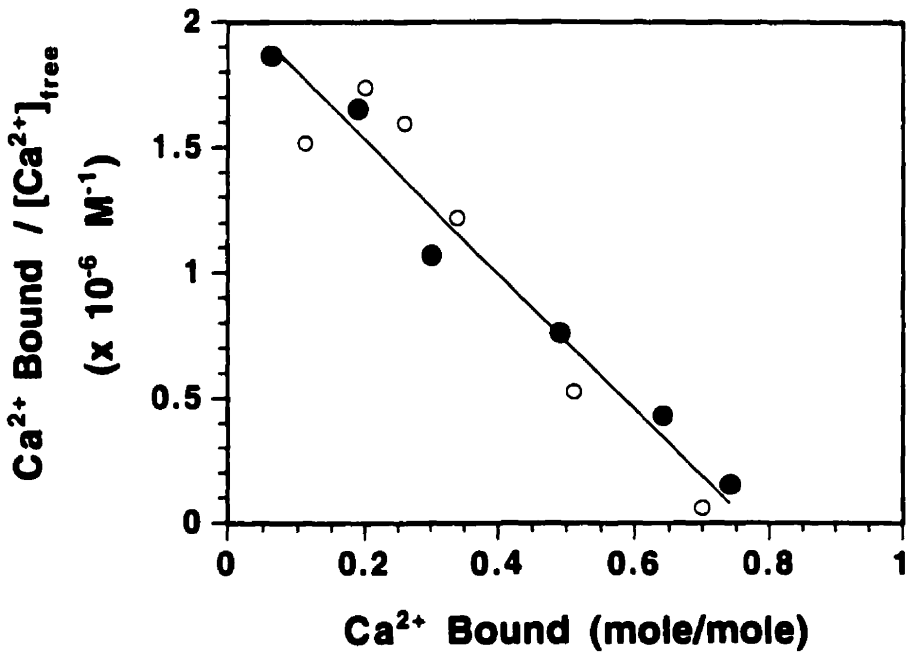
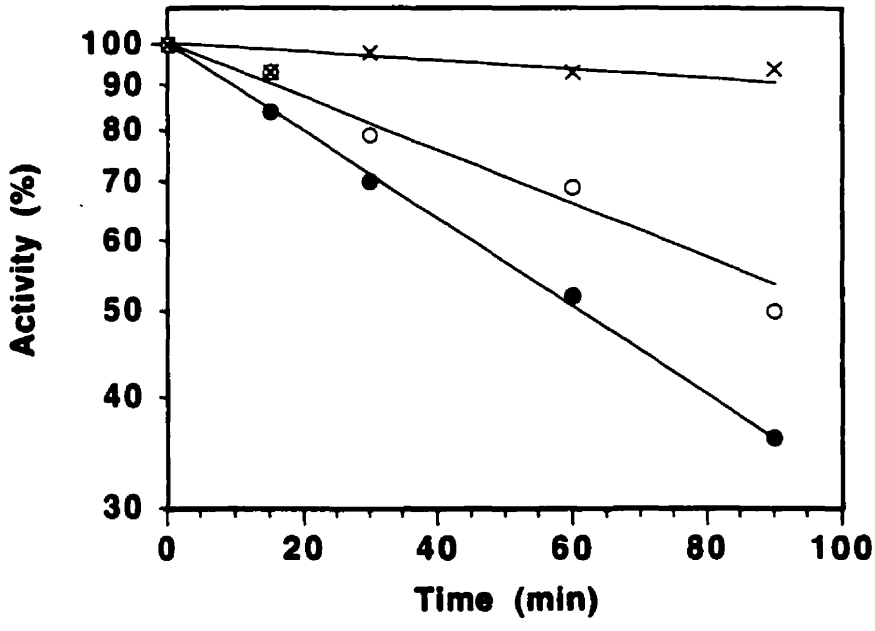

2
1.5
1
0.5
0
0
0.2
0.4
0.6
0.8
1
Ca2+ Bound / [Ca2+]free
(x 10-6 M-1)
Ca2+ Bound (mole/mole)

FIGURE 5: Role of Ca^{2+} on α1,2-mannosidase thermostability. Purified recombinant α1,2-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. α1,2-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 % of the activity before incubation at 50 °C.


Activity (%)
100
90
80
70
60
50
40
30
0
20
40
60
80
100
Time (min)

involved in Ca^{2+} binding in the yeast α1,2-mannosidase, the two most conserved residues (Asp^{121} and Glu^{132}) were mutated and Ca^{2+} binding and enzyme activity of the mutants expressed in *P. pastoris* were studied. The first residue, which is almost always Asp, was mutated to Asn or Ala. The twelfth residue, which is either Glu or Asp, was mutated to Gln or Val and a double mutant (D121A/E132V) with changes in both the first and twelfth residues of the EF hand was constructed. The results shown in Table I demonstrate that the D121N and D121A mutants retain complete enzyme activity, while the E132Q, E132V and D121A/E132V mutants have only 0.06 to 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 α1,2-mannosidase (Fig. 6A and Table I). 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 one Ca^{2+} ion per protein molecule as determined by equilibrium dialysis and had very similar dissociation constants as the wild type enzyme (0.3-0.4 μM)(Fig. 6B and Table I). These results clearly demonstrate that mutation of the most essential residues of the EF hand-related sequence, including the invariant Glu^{132}, does not change the Ca^{2+} binding characteristics of the enzyme.

Ca^{2+} binding to invariant acidic residue mutants

Since the putative EF hand is not the site of Ca^{2+} binding and acidic residues are often involved in binding this divalent cation (McPhalen *et al.*, 1991), the possibility that some of the invariant acidic residues that were shown to be required for enzyme activity *in vivo* (Fig. 1) 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 gels (Fig. 7). Since the level of expression of the mutants varied, the amounts of medium were adjusted to obtain comparable amounts of α1,2-mannosidase on the gels and two different amounts of medium obtained from cells transformed with the pHIL-S1 vector were also loaded (vec.1 and vec.2) for comparison. The α1,2-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 Fig. 7 that D86N, E132Q, E503Q, and E526Q mutants retained a similar affinity for Ca^{2+} as the

TABLE I

Characteristics of EF hand mutants

Mutants	Specific activity (%)[a]	$[Ca]_{50}$ (mM)[b]	Ca^{2+} / protein (mole/mole) [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". 100 % activity is 30,000 dpm/h per μg α1,2-mannosidase.

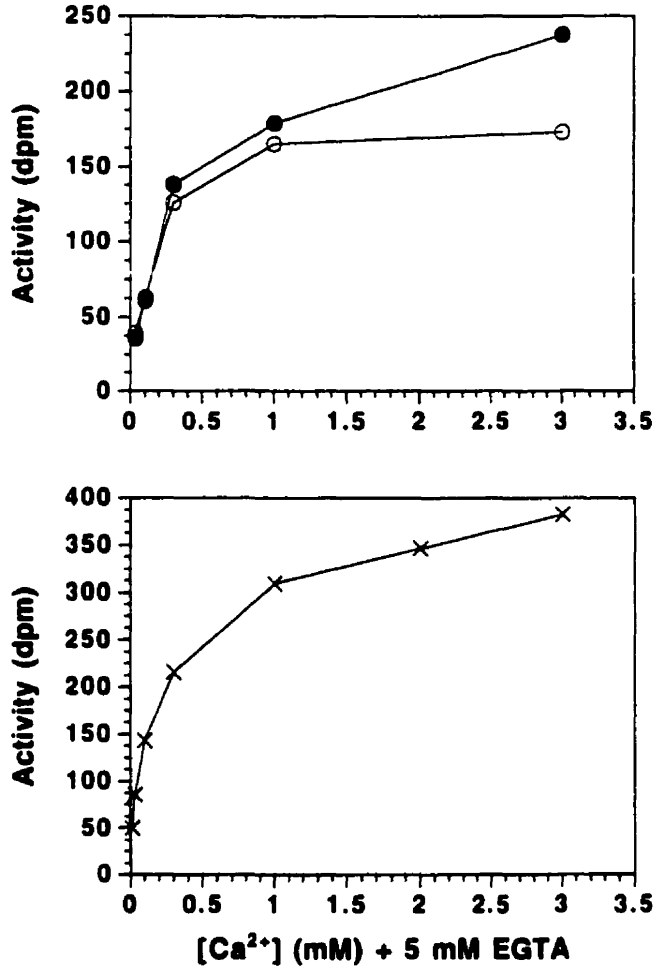
[b] The concentration of Ca^{2+} needed in the presence of 5 mM EGTA to obtain 50% of maximum activity (see Fig. 6A).

[c] Number of Ca^{2+} ions per α1,2-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 Fig. 6B.

[d] The dissociation constants (K_D) were determined by equilibrium dialysis (Fig. 6B) and Scatchard plots (not shown).

FIGURE 6: Effect of EF hand mutations on α1,2-mannosidase activity and on Ca^{2+} binding. (A) Purified wild type (●), D121N (○), and E132Q (×) α1,2-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".

A


Activity (dpm)
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
3
3.5
Activity (dpm)
400
350
300
250
200
150
100
50
0
[Ca2+] (mM) + 5 mM EGTA

B

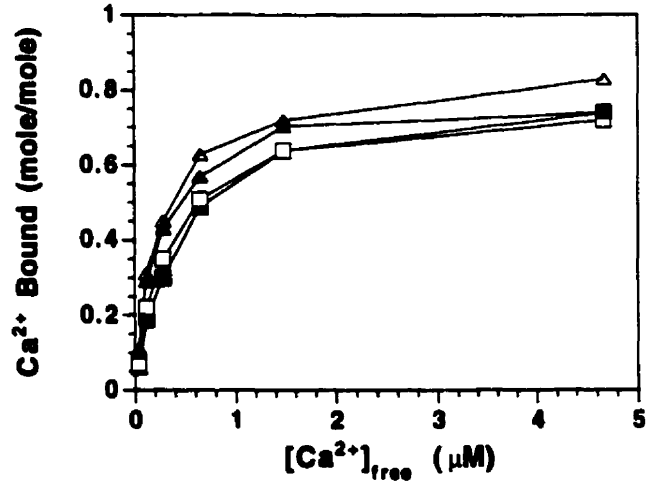
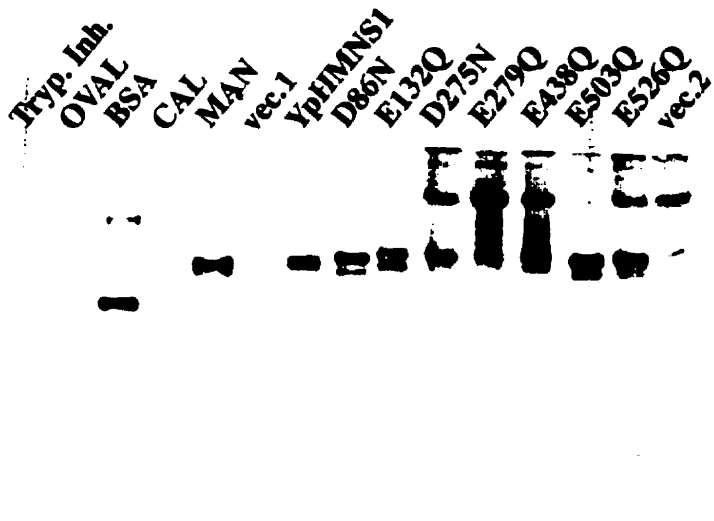
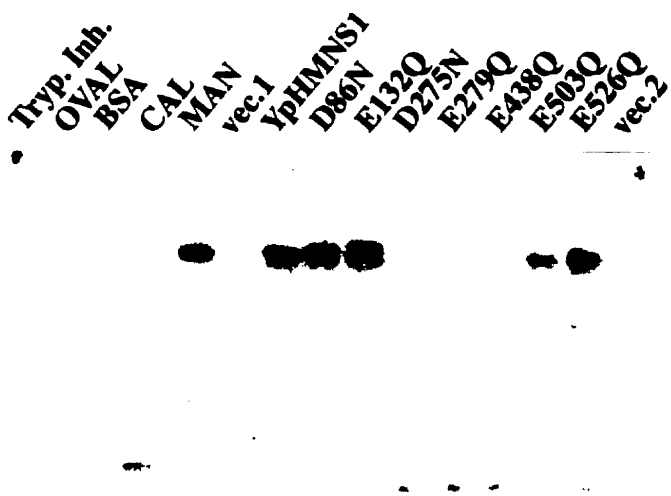

Ca2+ Bound (mole/mole)
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
[Ca2+]free (μM)

FIGURE 7: Role of the invariant acidic residues in Ca^{2+} binding to recombinant α1,2-mannosidase. *P. pastoris* clones transformed with wild type (YpHMNS1) or mutant α1,2-mannosidases were induced with methanol in BMMY medium for four days as previously described (Lipari and Herscovics, 1996). The medium was concentrated 20 to 60-fold and an aliquot containing 2-3 μg of α1,2-mannosidase per lane was subjected to PAGE on native gels. For cells transformed with the vector (pHIL-S1), similar aliquots 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 either comparable (D86N, E132Q, E503Q, E526Q) to the wild type, < 0.1 % (D275N, E279Q), or < 10 % (E438Q). The $^{45}Ca^{2+}$ blot was performed at least twice for all the mutants with similar results. 2 μg trypsin inhibitor (Tryp. Inh.), 2 μg ovalbumin (OVA), 2 μg bovine albumin (BSA), 0.2 μg calmodulin (CAL), and 2 μg purified recombinant α1,2-mannosidase (MAN) were used as controls.

A


Tryp. Inh.
OVAL
BSA
CAL
MAN
vec.1
YpHMNS1
D86N
E132Q
D275N
E279Q
E438Q
E503Q
E526Q
vec.2

B


Tryp. Inh.
OVAL
BSA
CAL
MAN
vec.1
YpHMNS1
D86N
E132Q
D275N
E279Q
E438Q
E503Q
E526Q
vec.2

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 α1,2-mannosidase (data not shown). The specific activity of the mutants in the medium was evaluated (data not shown) and found to be similar to the specific activity of the mutants expressed intracellularly in *S. cerevisiae* (Fig. 2). 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 α1,2-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

Since 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 II). Mutation of Asp^{86} resulted in a dramatic decrease in k_{cat} of 1750-fold and a 2.5-fold decrease in K_m. Mutation of Glu^{132} also greatly decreased the k_{cat} (2300-fold) and decreased the K_m (2 to 7-fold). In contrast, mutation of Glu^{526} resulted in an increase in K_m of 8-fold, with only a 2-fold decrease in k_{cat}, suggesting that this mutation mostly affected substrate binding. The E503Q mutant that was inactive in *S. cerevisiae in vivo* was also inactive *in vitro* as a protein secreted from *P. pastoris*.

TABLE II

Kinetic parameters of acidic residue mutants

Mutants	Specific activity (%)[a]	K_m (mM)[b]	k_{cat} (min^{-1})
wild type	100	0.5	700
D86N	0.1	0.2	0.4
E132Q	0.1	0.07	0.3
E132V	0.06	0.3	0.3
E526Q	7	4	300

[a] Specific activity of the α1,2-mannosidases was assayed using purified recombinant enzymes and 0.2 mM $Man_9GlcNAc$ as substrate (see Table I).

[b] The kinetic parameters were determined according to assay protocol B in "Experimental Procedures".

DISCUSSION

In the present work the Ca^{2+} binding characteristics of the yeast processing α1,2-mannosidase were determined, and the role of conserved acidic residues in Ca^{2+} binding and in enzyme activity were studied by mutagenesis. It was demonstrated by atomic absorption analysis and equilibrium dialysis that the yeast α 1,2-mannosidase binds one Ca^{2+} ion per mole with high affinity ($K_D \sim 10^{-7}$ M) and that the 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 1 α1,2-mannosidases are necessary for enzyme activity *in vivo*. Mutagenesis of five of these residues (Glu^{214}, Asp^{275}, Glu^{279}, Glu^{435}, Glu^{503}) completely prevents complementation of an α1,2-mannosidase-deficient *S. cerevisiae* strain and mutagenesis of the other four residues (Asp^{86}, Glu^{132}, Glu^{438}, Glu^{526}) results in very low levels of α1,2-mannosidase activity compared to that observed following complementation with the wild type enzyme. *In vitro* studies on recombinant yeast α1,2-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 following native PAGE and that mutations of three other invariant acidic amino acids (Asp^{86}, Glu^{132}, Glu^{526}) results in the production of recombinant enzymes that exhibit very low, but significant, activity with greatly altered kinetic properties.

The yeast α1,2-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 (Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991; Lipari and Herscovics, 1994), 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 α1,2-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 to 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 experiments 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 α1,2-mannosidase also has a high affinity for Ca^{2+} and that Ca^{2+} is required for substrate binding (Schutzbach and Forsee, 1990).

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 (Cotton *et al.*, 1979; Scott *et al.*, 1990). In other enzymes, such as xylanase (Spurway *et al.*, 1997), β-glucanase (Keitel *et al.*, 1993), and proteinase K (Bajorath *et al.*, 1989), 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 (Chauvaux *et al.*, 1995), α-amylase (Boel *et al.*, 1990), and gelatinase B (Bu and Pourmotabbed, 1996), 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.

The present results clearly establish that the EF hand motif is not the site of Ca^{2+} binding in the yeast processing α1,2-mannosidase even though the sequence between amino acid residues 121 and 132 is very similar to the consensus loop region, including all the crucial amino acids at positions X, Y, Z, -Y, -X, -Z that coordinate Ca^{2+} in functional EF hands of many proteins (Fig. 3). It is evident that sequence homology is not sufficient to identify this type of Ca^{2+} binding site and that mutagenesis studies are essential to determine functionality of EF hand sequences, as shown for other proteins that contain non-functional EF hands (Drayer *et al.*, 1995; Bertauche *et al.*, 1996).

Analyses of crystal structures of Ca^{2+}-binding proteins show that Ca^{2+} ions can be coordinated by 5 to 8 oxygen atoms and that oxygen ligands often come from the carboxylate group of acidic amino acids (McPhalen *et al.*, 1991). In the present work it is shown that at least three of the invariant acidic residues (Asp^{275}, Glu^{279}, and Glu^{438}) are important for $^{45}Ca^{2+}$ binding to recombinant yeast α1,2-mannosidase. Mutation of Asp^{275} and Glu^{279} completely eliminated both $^{45}Ca^{2+}$ binding and enzyme activity whereas the E438Q mutant exhibited minimal Ca^{2+} binding equivalent to < 10% of the wild type binding and some residual enzyme activity. Therefore, the invariant acidic residues, Asp^{275},Glu^{279}, and Glu^{438} 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. It is possible that Glu^{214} and Glu^{435} may also be important for Ca^{2+} binding since mutations of these residues completely inactivated the enzyme, but the binding of Ca^{2+} to these mutants could not be examined since their secretion was impaired and insufficient protein was produced for the $^{45}Ca^{2+}$ binding assay.

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 to 7-fold (Table II). Two conserved acidic amino acids are usually involved in catalysis by glycosidases (for reviews see Sinnott, 1990; Legler, 1993; Svensson and Søgaard, 1993; McCarter and Withers, 1994; Davies and Henrissat, 1995) and the changes observed in the behavior of D86N and E132Q are typical for mutants of catalytic residues in inverting glycosidases (Chauvaux *et al.*, 1992; Frandsen *et al.*, 1994; Totsuka *et al.*, 1994; Damude *et al.*, 1995; Davies *et al.*, 1995). 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 α1,2-mannosidase from *A. saitoi* that five conserved acidic residues corresponding to Asp^{275}, Glu^{279}, Glu^{435}, Glu^{438}, and Glu^{503} in the yeast α1,2-mannosidase are crucial for enzyme activity (Fujita *et al.*, 1997). While the residues essential for activity in the *A. saitoi* enzyme are consistent with the results obtained for the yeast α1,2-mannosidase, Ca^{2+} binding to the *A. saitoi* mutants was not examined and kinetic parameters of the mutants were not studied. However, this α1,2-mannosidase may differ from other Class 1 enzymes since it has a lower pH optimum and has been reported not to require Ca^{2+} for activity.

Considering the amino acid similarity between the yeast and mammalian Class 1 α1,2-mannosidases, it is likely that the EF hand motif is not the site of Ca^{2+} binding in the mammalian enzymes and that the residues corresponding to Asp^{275}, Glu^{279}, and Glu^{438} in those proteins may also be required for Ca^{2+} binding. In addition, the four potential active site residues (Asp^{86}, Glu^{132}, Glu^{503}, Glu^{526}) identified in the yeast α1,2-mannosidase may also be important for activity in the mammalian enzymes. The yeast α1,2-mannosidase has recently been crystallized and preliminary X-ray crystallographic data have been obtained (Dole *et al.*, 1997). Determination of the three-dimensional structure of the enzyme 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.

CHAPTER 6

General Discussion

The Class 1 α1,2-mannosidases play a very important role in N-glycan biosynthesis and have been conserved through eukaryotic evolution (Chapter 1). They have been implicated in the degradation of misfolded ER glycoproteins and also as potential therapeutic targets to treat viral infection and cancer. In order to understand the function of the α1,2-mannosidases in N-glycan biosynthesis, it is necessary to determine their mechanism of action. In addition, a knowledge of the catalytic mechanism and active site topology of these enzymes may be useful to design novel α1,2-mannosidase inhibitors as potential drugs. Previous to the work presented in this thesis, there was only minimal information available on the structure and mechanism of action of these α1,2-mannosidases. One major obstacle was to obtain enough protein for such studies since only microgram levels could be purified from cell extracts. An expression system was developed to produce the yeast α1,2-mannosidase, the first Class 1 α1,2-mannosidase to be available in milligram quantities (Chapter 2).

Glycosidases are classified into families according to amino acid sequence similarity. Within a glycosidase family, the catalytic mechanism and overall protein topology of its members are very similar. Therefore, the structural and mechanistic properties described for the yeast α1,2-mannosidase in this thesis provide a model for other Class 1 α1,2-mannosidases.

ADVANTAGES OF *PICHIA PASTORIS* FOR EXPRESSION OF α1,2-MANNOSIDASE

A yeast expression system was selected over a prokaryotic expression system to produce recombinant α1,2-mannosidase, because it had been previously established that the enzyme contained at least one disulfide bond. The possibility that N-glycans may be important was also considered. A system whereby the recombinant protein is secreted was preferred due to the low amount of protein naturally present in the medium of yeast cultures, which significantly simplifies purification of the recombinant protein. Furthermore, since the enzyme is present in the secretory pathway, it would acquire its proper structure and post-translational modifications.

P. pastoris was chosen as the expression system to produce the recombinant yeast α1,2-mannosidase because up to 50 times more protein can be obtained than with the *S. cerevisiae* system. Furthermore, the protein purified from *P. pastoris* shows only one band on SDS-PAGE (Appendix I), whereas a doublet (60 and 63 kDa) is purified from *S. cerevisiae* (Chapter 2).

More recently, human α1,2-mannosidase IB (Tremblay *et al.*, 1998) and mouse α1,2-mannosidases IA and IB (Lal *et al.*, 1998) catalytic domains have also been expressed in *P. pastoris* as secreted proteins; however, the yields are considerably lower for these mammalian enzymes (≤ 1 mg/l). Recombinant *P. citrinum* α1,2-mannosidase (Yoshida *et al.*, 1998) was also produced as a secreted protein using *Aspergillus oryzae* as the host. The availability of other Class 1 α1,2-mannosidases will be useful for comparison of the structure and mechanism of action of these enzymes.

ROLE OF THE CYSTEINE RESIDUES

The yeast α1,2-mannosidase contains five cysteine residues, three of which are invariant in the Class 1 α1,2-mannosidases. It was demonstrated by peptide analysis that the invariant Cys^{340} and Cys^{385} residues form a disulfide bond. The C340S and C385S mutants were not secreted in *P. pastoris* and they did not accumulate intracellularly, suggesting that the mutants were not stable and that this disulfide bond plays an important role in protein conformation (Chapter 4). Therefore, reduction of the Cys^{340}-Cys^{385} disulfide bond is most likely responsible for the inactivation of the α1,2-mannosidase upon incubation in DTT. The DTT-reduced protein tended to precipitate and did not retain activity upon lyophilization, an observation consistent with a role for this disulfide bond in conformation. This disulfide bond is most likely conserved in the other members of the α1,2-mannosidase family; however, the function of the cysteine residues has not been studied for any of the other enzymes.

The second disulfide bond (Cys^{468}-Cys^{471}) is unique to the yeast enzyme, since the other α1,2-mannosidases of the family contain only three cysteine residues in the catalytic domain. The Cys^{468}-Cys^{471} disulfide bond is not essential for enzymatic activity, but the C468S and C471S mutants have a 50 % decrease in substrate affinity. This disulfide bond is required for stability since the C471S mutant was easily degraded during purification. The non-conserved Cys^{485} in the yeast α1,2-mannosidase does not play a role in enzyme activity; however, the C485S mutant was significantly more susceptible to proteases during purification compared to the wild type enzyme (unpublished results). This result indicates that the Cys^{485} residue is not part of the active site, but stabilizes the protein. Quantitative modification of the free thiol group with alkylating agents (iodoacetate and iodoacetamide) was not possible under non-denaturing conditions (unpublished results), suggesting that the residue is not exposed to the surface.

ROLE OF CALCIUM

The yeast α1,2-mannosidase requires one tightly bound Ca^{2+} ion for activity, as was demonstrated by atomic absorption analysis of the purified enzyme (Chapter 5). This result was the first direct evidence of Ca^{2+} binding to any Class 1 α1,2-mannosidase. A sequence similar to the EF hand Ca^{2+}-binding loop has been conserved in the Class 1 α1,2-mannosidases and the motif was previously considered as a possible site for Ca^{2+} binding (Camirand *et al.*, 1991; Bause *et al.*, 1993; Herscovics *et al.*, 1994; Lal *et al.*, 1994; Bieberich *et al.*, 1997; Kawar *et al.*, 1997). However, it was clearly shown by mutagenesis and characterization of the Ca^{2+} binding properties of the mutants that the EF hand motif is not involved in Ca^{2+} binding (Chapter 5) and that Ca^{2+} binds at other sites in the Class 1 enzymes.

Three invariant residues (Asp^{275}, Glu^{279}, and Glu^{438}) were found to be required for Ca^{2+} binding in the yeast α1,2-mannosidase (Chapter 5). These three residues are within two highly conserved areas of the Class 1 α1,2-mannosidases (Chapter 1, Fig. 4), suggesting that their function has been conserved in all the enzymes of the family. This conclusion is supported by a recent report showing that the corresponding three residues are also crucial for activity in the *A. phoenicis* enzyme (Fujita *et al.*, 1997). Since these three invariant amino acids are located in distant regions of the polypeptide, a complex tertiary structure is most likely required to form the Ca^{2+} binding site. The fact that Ca^{2+} binding is lost after denaturation in 8 M urea (Chapter 5) is consistent with this hypothesis.

The Ca^{2+} ion in the yeast α1,2-mannosidase is important for catalysis without greatly altering the conformation, as demonstrated by CD and fluorescence spectra, and V8 protease sensitivity experiments (Chapter 5). It was previously shown that Ca^{2+} is required for substrate binding of the rabbit Class 1 α1,2-mannosidase. The K_m increased upon decreasing the Ca^{2+} concentration, whereas the V_{max} was unchanged (Schutzbach and Forsee, 1990). Determination of the three-dimensional structure of the yeast α1,2-mannosidase, which is currently under way, is required to locate the exact Ca^{2+} coordinating residues and to establish the role of Ca^{2+}.

There is some apparent discrepancy in the results obtained with the recombinant enzyme in this work (Chapter 2) and previous results (Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991) regarding the role of other cations on yeast α1,2-mannosidase activity. Although Ca^{2+} was the most efficient in restoring enzyme activity after treatment with EDTA or EGTA, other divalent cations had different effects on enzyme activity. The soluble endogenous enzyme was inhibited by zinc, whereas zinc restored the activity of the CHAPS-solubilized enzyme and of the recombinant *S. cerevisiae* produced enzyme in the

presence of chelator. In addition, cobalt and manganese were inhibitory for the endogenous enzymes, but not for the recombinant enzyme. Since Ca^{2+} is bound to the yeast α1,2-mannosidase with high affinity (Chapter 5), the ability of other cations to restore activity in the presence of chelators may be due to the presence of contaminating Ca^{2+} that is released from the Ca^{2+}-chelator complex upon addition of excess cation such as zinc.

CATALYTIC MECHANISM

The yeast α1,2-mannosidase is an inverting glycosidase, as demonstrated by NMR analysis of the reaction process (Chapter 3). It was later shown that mouse α1,2-mannosidase IA also acts by an inverting mechanism (Lal *et al.*, 1998), consistent with the concept that glycosidases within a family have the same stereospecificity. According to previous studies described in Chapter 1, inverting glycosidases contain two catalytic carboxylate residues, one acting as an acid catalyst while the other is a base catalyst. In order to identify catalytic residues, several reagents were tested as potential irreversible inactivators of the yeast α1,2-mannosidase, including (2,3)-epoxypropyl α-D-mannoside and α-D-mannopyranosylmethyl-ρ-nitrophenyltriazene, with no success (unpublished results). Site-directed mutagenesis of the nine invariant acidic residues combined with Ca^{2+} binding and kinetic analysis of the mutants was therefore used to identify potential catalytic residues (Chapter 5). Mutation of Asp^{86} and Glu^{132} resulted in a 2000-fold decrease in catalytic efficiency (k_{cat}) and 2-7-fold increase in substrate affinity (K_m), which is consistent with a possible catalytic function for these residues. Mutation of Glu^{503} resulted in complete inactivation of enzyme activity. These three mutants still bound Ca^{2+}; therefore, these three amino acids are potential catalytic residues. The remaining 6 mutants were either not secreted, were required for Ca^{2+} binding, or were necessary for substrate binding.

The yeast α1,2-mannosidase is the only Class 1 α1,2-mannosidase for which putative catalytic residues have been identified by kinetic analysis. In the *A. phoenicis* α1,2-mannosidase, the invariant residues corresponding to Glu^{132} and Glu^{503} in the yeast enzyme were also found to be crucial for enzyme activity, but no kinetic analysis was reported (Fujita *et al.*, 1997). Mutation of Phe^{592} in mouse α1,2-mannosidase IB, which is adjacent to the invariant Glu residue corresponding to Glu^{503} in the yeast enzyme, caused inactivation of the enzyme (Schneikert and Herscovics, 1995). These results are consistent with the mutagenesis studies on the yeast enzyme.

SUBSTRATE BINDING

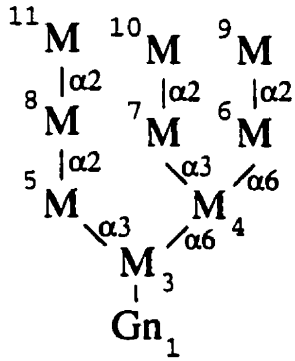
Glu^{526} was demonstrated to be required for substrate binding since its mutation caused an 8-fold increase in K_m with only a 2-fold reduction in k_{cat} (Chapter 5). Glu^{526} is invariant, suggesting that it plays a similar role in the other Class I enzymes.

The structural determinants of the oligosaccharide substrate, $Man_9GlcNAc$ (Fig. 1A), required for binding to the yeast enzyme have been studied by testing different oligosaccharides as substrates. With the CHAPS-solubilized enzyme, different isomers of $Man_7GlcNAc$, $Man_8GlcNAc$, and $Man_9GlcNAc$ were compared (Ziegler and Trimble, 1991). It was concluded that the mannose residues at positions 8 and 11 of $Man_9GlcNAc$ (Fig. 1A) are not essential for enzyme activity, but that mannose residue 9 is required. In another study, the kinetic parameters of recombinant yeast α1,2-mannosidase were determined with different synthetic oligosaccharide substrates (Scaman *et al.*, 1996). The pentasaccharide $Man_5(OCH_2)_8COOCH_3$ (Fig. 1B), a structure containing mannose residues corresponding to positions 4,6,7,9, and 10 of $Man_9GlcNAc$, has a 13-fold increase in K_m and a 2.5-fold reduction in V_{max} compared to $Man_9GlcNAc$ (K_m = 0.3 mM, V_{max} = 15 mU/μg); therefore, residues 1, 3, 5, 8, and 11 together are very important for substrate binding. The trisaccharide α-Man1,2α-Man1,3α-$ManO(CH_2)_8COOCH_3$ (Fig. 1C) is an extremely poor substrate for the yeast α1,2-mannosidase, with a 30-fold increase in K_m and a 20-fold decrease in V_{max} compared to $Man_9GlcNAc$. The initial rates of hydrolysis for the trisaccharides α-Man1,2α-Man1,2α-$ManO(CH_2)_8COOCH_3$ (Fig. 1D) and α-Man1,2α-Man1,6α-$ManO(CH_2)_8COOCH_3$ (Fig. 1E) were 4 and 9 fold decreased, respectively, compared to α-Man1,2α-Man1,3α-$ManO(CH_2)_8COOCH_3$ (Fig. 1C). Therefore, out of the three trisaccharides tested the enzyme prefers α-Man1,2α-Man1,3α-$ManO(CH_2)_8COOCH_3$ (Fig. 1C), which has the α1,3-linkage adjacent to the cleaved mannose.

It is evident from the above studies that various parts of the $Man_9GlcNAc$ substrate may be important for binding to the enzyme. Due to the significance of these interactions in determining substrate specificity, it may be useful to directly analyze substrate binding of the enzyme. Common approaches for examining interactions of carbohydrates with their receptor proteins are high resolution NMR spectroscopy and X-ray crystallography of the protein bound to its ligand (Homans *et al.*, 1998). For glycosidases, these types of studies are only possible with substrate analogues that are not hydrolyzed. Considering the complexity of the $Man_9GlcNAc$ substrate, it would be difficult to synthesize a substrate analogue to mimic all the interactions with the yeast enzyme. However, a completely inactive α1,2-mannosidase obtained by mutagenesis of one or more of the essential

FIGURE 1: Structures of the oligosaccharide substrates tested for recombinant yeast α1,2-mannosidase. The sugar residues in oligosaccharide A are *numbered*.

A



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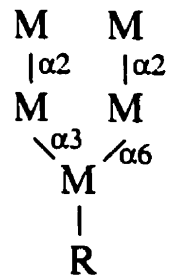

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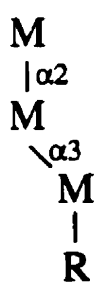

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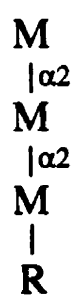
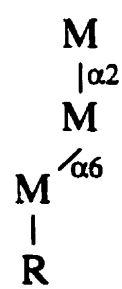


E



Key

M / Gn	Manβ1,4-GlcNAc
R	$O(CH_2)_8COOCH_3$

catalytic residues may be useful for such experiments, since the mutant may bind the oligosaccharide substrate without hydrolyzing it.

TOWARDS A CRYSTAL STRUCTURE OF THE YEAST α1,2-MANNOSIDASE

Elucidation of the three-dimensional structure of the yeast α1,2-mannosidase will be an important step towards understanding its mechanism of substrate binding and catalysis. The structure determination in conjunction with the mutagenesis studies described in Chapters 4 and 5 will lead to the identification of the catalytic residues, to the determination of the Ca^{2+} binding amino acids, and to the identification of residues involved in substrate binding. Structural information will also be useful to design more potent inhibitors of the Class 1 α1,2-mannosidases and to model other members of the family. In addition, the structural determinants responsible for the difference in specificity between the yeast α1,2-mannosidase and the other members of the family may be elucidated. Since NMR techniques are not useful for solving the structure of proteins over 30 kDa, the only technique now available to determine the three-dimensional structure of the α1,2-mannosidase is X-ray crystallography.

Progress towards the elucidation of the three-dimensional structure has been made. The recombinant yeast α1,2-mannosidase has been crystallized and preliminary X-ray diffraction data have been collected (Dole *et al.*, 1997). In Appendix 1, the different approaches that have been taken to improve the reproducibility of the crystals are described. To eliminate the heterogeneity in the recombinant α1,2-mannosidase due to the N-glycans, Endo H was used under native conditions with no significant loss of enzyme activity. To eliminate any variability due to proteolytic degradation of the N-terminal end, constructs expressing shorter α1,2-mannosidases were tested and an α1,2-mannosidase starting at amino acid 34 instead of 22 was found to be more favorable for reproducible crystallization conditions.

CONCLUSION

Significant advances have been made towards understanding the structure and mechanism of catalysis of the yeast α1,2-mannosidase. Important structural components and mechanistic properties have been characterized. In addition, possible active site carboxylates and residues important for Ca^{2+} binding have been identified. These investigations have led to further questions concerning the mechanism of catalysis of the yeast α1,2-mannosidase: What is the precise role of the Ca^{2+} ion? What is the mechanism of substrate binding? Which are the catalytic acid and base residues? The knowledge gained on the yeast α1,2-mannosidase can also be applied to the other members of the α1,2-mannosidase family, for which minimal information is currently available.

APPENDIX I

INTRODUCTION

The recombinant yeast α1,2-mannosidase catalytic domain, which is produced as a secreted glycoprotein from *P. pastoris* (Chapter 4), was successfully crystallized and preliminary X-ray diffraction data were obtained (Dole *et al.*, 1997). To improve the reproducibility of the crystals, it is important to establish a method to obtain a homogeneous set of α1,2-mannosidase molecules within all preparations (McPherson, 1982). The yeast α1,2-mannosidase is N-glycosylated and N-glycans often exhibit heterogeneity in their structure. In addition, it was observed that the N-terminal sequence of the α1,2-mannosidase is susceptible to proteolysis, resulting in a mixture of N-termini. The following report describes the steps taken to eliminate variability caused by the N-glycans and the protease-sensitive N-terminal sequence.

RESULTS AND DISCUSSION

Deglycosylation of recombinant yeast α1,2-mannosidase

Two approaches were taken to obtain deglycosylated yeast α1,2-mannosidase. The first was removal of the N-glycosylation sites by mutating the asparagine (N) residues in the consensus N-X-S/T sequence to glutamine (Q). Each of the three sites was mutated individually and the different recombinant α1,2-mannosidases were expressed in *P. pastoris* as secreted glycoproteins and purified (Fig. 1A). The yield (2 to 7 mg/L) and specific activity of the purified mutants was very similar to that obtained for wild type recombinant α1,2-mannosidase. When all three N-glycosylation sites were mutated, no α1,2-mannosidase was found in the medium as indicated by Western blot analysis and by enzyme assay of 50 clones, but Western blot analysis of cellular extracts showed an α1,2-mannosidase band of about 60 kDa, while the non-mutated α1,2-mannosidase was 64 kDa (Fig. 1B). Therefore, the N-glycans are required for secretion of the α1,2-mannosidase from *P. pastoris.*

Glycosidase treatment was then used to remove the N-glycans from the purified α1,2-mannosidase. Both Endo H and PNGase F were capable of complete deglycosylation under native conditions after extended incubation; however, the enzyme activity was not significantly affected by deglycosylation with Endo H, but it was greatly reduced upon PNGase F treatment (Fig. 2). PNGase F cleaves the N-glycan between the asparagine and GlcNAc residues and transforms the amido group to a carboxylate, while Endo H leaves a GlcNAc residue linked to asparagine upon hydrolysis. Therefore, it is likely that the acidic group formed upon PNGase F treatment destabilizes the protein conformation.

N-terminal deletions

The N-terminal sequences of different preparations of α1,2-mannosidase Δ21 were determined (Table II) and it was found that some proteolytic degradation was occurring to variable extents without affecting the specific activity of the α1,2-mannosidase (data not shown). To eliminate the protease sensitive sites, systematic deletion of the N-terminus was carried out (Table I), and the mutants were expressed in *P. pastoris*. α1,2-Mannosidases Δ28 and Δ33 were secreted at normal levels compared to Δ21, but α1,2-mannosidase Δ38 was barely visible upon Western blot analysis (data not shown).

Since α1,2-mannosidase Δ33 was the smallest α1,2-mannosidase expressed at high levels, it was chosen for further crystallization trials. The protein was purified and

the specific activity of the enzyme was shown to be the same as α1,2-mannosidase Δ21 (data not shown). Upon S-Sepharose chromatography of the culture medium containing α1,2-mannosidase Δ33 (Fig. 3A), only one peak of protein was observed, whereas purification of α1,2-mannosidase Δ21 often resulted in three peaks (Fig. 3B). This observation indicates that α1,2-mannosidase Δ33 is less heterogeneous than α1,2-mannosidase Δ21.

The purified α1,2-mannosidase Δ33 (12.3 mg) was treated with Endo H under native conditions to remove the N-linked oligosaccharides. Approximately 60 % of the α1,2-mannosidase no longer contained N-linked oligosaccharides, while 40 % had one N-glycan remaining (data not shown). This deglycosylated α1,2-mannosidase was then purified by sequential Concanavalin A-Sepharose and S-Sepharose chromatography and 2.7 mg (22 % yield) were obtained. It was demonstrated that deglycosylation has no effect on the specific activity of α1,2-mannosidase Δ33 (data not shown).

Therefore, a method was established to obtain deglycosylated α1,2-mannosidase Δ33, which is more favorable for obtaining reproducible crystals. Indeed, the crystals formed from the first batch of this enzyme are suitable for further crystallographic analysis (Vallee and Howell, personal communication).

FIGURE 1: Expression of N-glycosylation mutants in *P. pastoris*. α1,2-mannosidase Δ21 (see Table I) and N-glycosylation mutant α1,2-mannosidases were purified as described in EXPERIMENTAL PROCEDURES. 0.75 μg of Δ21 (WT), N96Q , N155Q, and N224Q were run on 8 % SDS-PAGE and stained with Coomassie Blue (A). The specific activity of the α1,2-mannosidases was determined using 0.2 mM [^{3}H]$Man_9GlcNAc$ as substrate as previously described (Chapter 5). The activity is expressed as % of the wild type activity (30,000 dpm/h per μg α1,2-mannosidase) and is shown at the *bottom*. Intracellular extracts of cells transformed with pHIL-S1, YpHMNS1 or YpHΔN were analyzed by Western blot (B) as explained in EXPERIMENTAL PROCEDURES. The molecular weight markers are shown in the *right margins*.

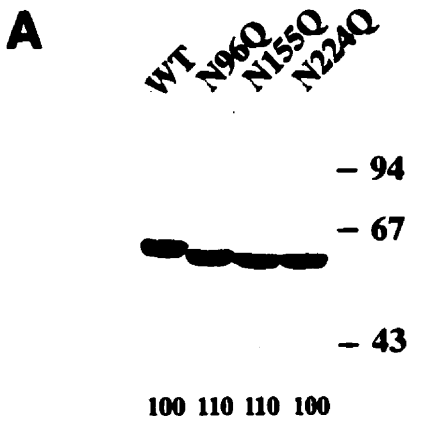

A
WT
N96Q
N155Q
N224Q
– 94
– 67
– 43
100 110 110 100
B
pHIL-S1
YpHMNS1
YpHΔN
– 98
– 64
– 50

TABLE I

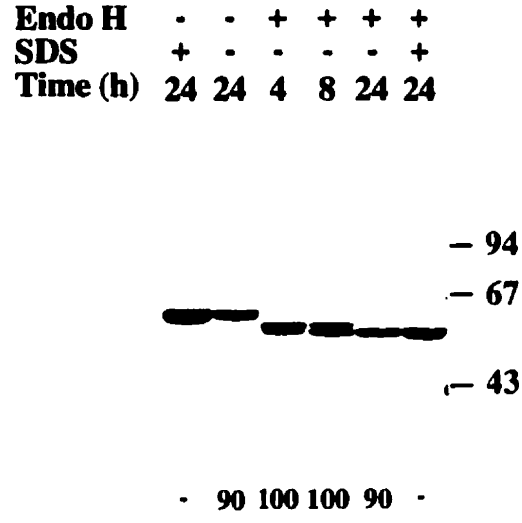
Predicted N-terminal sequences of recombinant α1,2-mannosidases

Plasmid	α1,2-Mannosidase	Predicted N-terminal sequence [a]
YpHMNS1	Δ21	RV^{22}PWYEHFERKSPGAGEMRDRIESMFLE...
YpHΔ28	Δ28	RE^{29}RKSPGAGEMRDRIESMFLE...
YpHΔ33	Δ33	RG^{34}AGEMRDRIESMFLE...
YpHΔ38	Δ38	R^{39}DRIESMFLE...

[a] The recombinant α1,2-mannosidase is expressed containing the *PHO1* signal peptide at the N-terminal end, which is subsequently cleaved by signal peptidase, and the α1,2-mannosidase is secreted into the medium. The predicted N-terminal sequence of the recombinant secreted α1,2-mannosidase is shown. In all cases, an arginine (R) is the first amino acid because it is required for signal peptidase cleavage. The amino acid with a number at the top right is the first amino acid corresponding to the published primary amino acid sequence (Camirand *et al.*, 1991).

FIGURE 2: Deglycosylation of α1,2-mannosidase Δ21 with Endo H and PNGase F. Purified α1,2-mannosidase Δ21 was treated with Endo H (A) or PNGase F (B) under native or denaturing conditions as described in EXPERIMENTAL PROCEDURES. The presence of Endo H, PNGase F, or SDS, and incubation times for each sample are indicated above each figure. 0.75 μg of protein samples were loaded for 8 % SDS-PAGE followed by Coomassie Blue staining. 4 μl of sample were removed and diluted 1/1000 or 1/2000 in 10 mM PIPES, pH 6.5 containing 1 mM NaN_3 and 1 mg/ml BSA to measure α1,2-mannosidase activity as described previously (Chapter 5) with 0.2 mM [^{3}H]$Man_9GlcNAc$ as substrate. The % activity remaining after treatment is illustrated at the *bottom* of each figure. The molecular weight markers are shown in the *right margins*.

A


Endo H - - + + + +
SDS + - - - - +
Time (h) 24 24 4 8 24 24
94
67
43
- 90 100 100 90 -

B

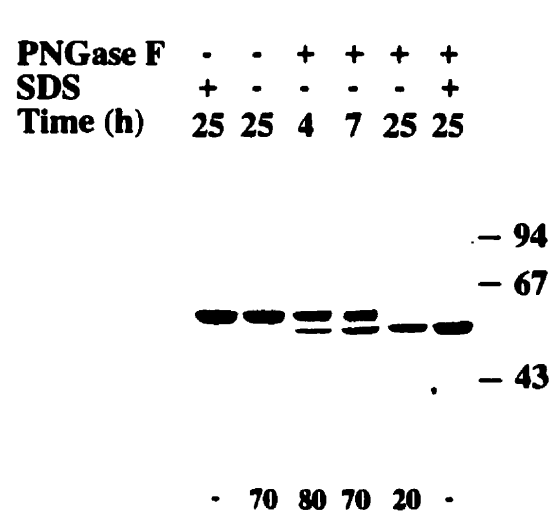

PNGase F - - + + + +
SDS + - - - - +
Time (h) 25 25 4 7 25 25
94
67
43
- 70 80 70 20 -

TABLE II

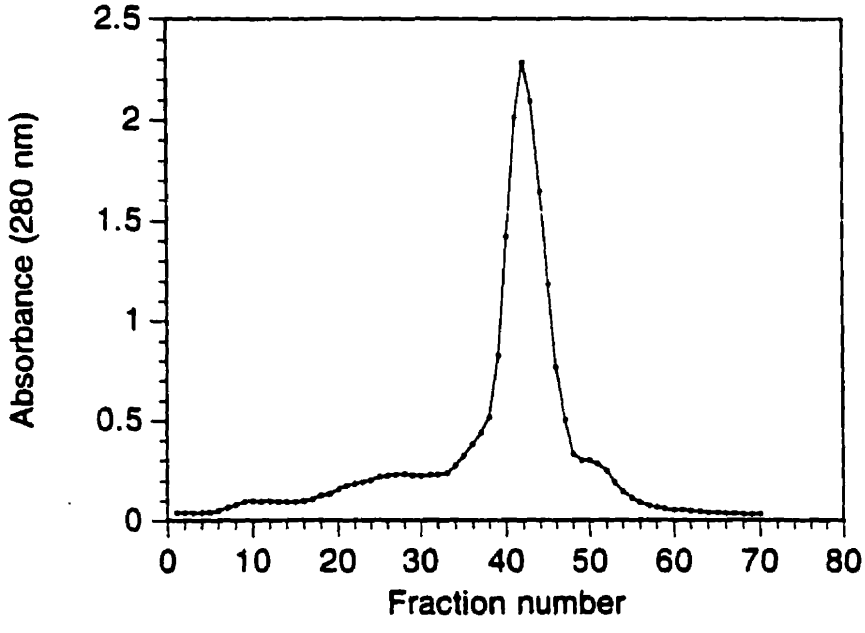
N-terminal sequences observed for α1,2-mannosidase Δ21

α1,2-Mannosidase preparation [a]	N-terminal sequence
#1	RVPWY
#2	RVPWY
#2	ERKSP
#3	RVPWY
#3	VPWYE

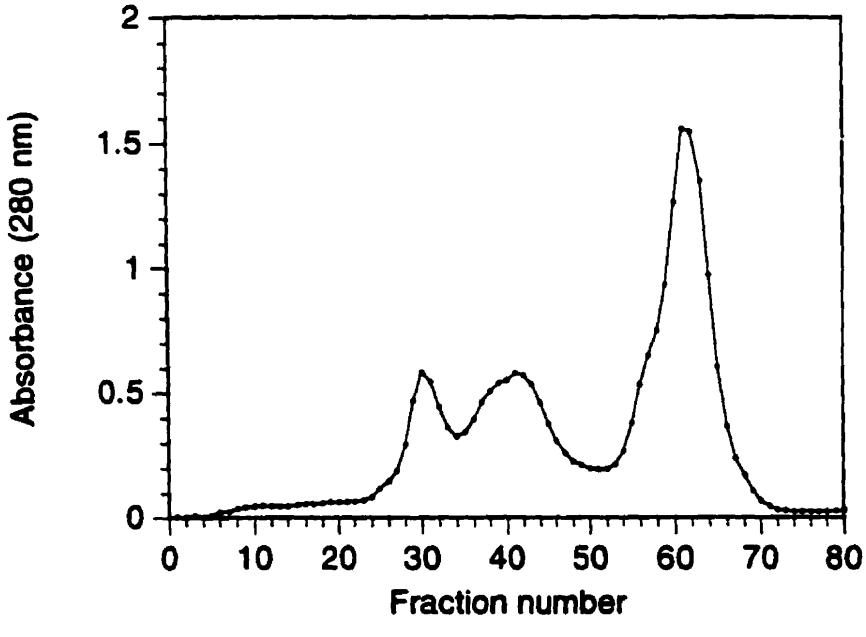
[a] Three different recombinant α1,2-mannosidase Δ21 preparations were analyzed by N-terminal sequencing and the first five amino acids observed in the analysis are shown. For preparations #2 and #3 there were two N-terminal sequences observed.

FIGURE 3: S-Sepharose chromatography of α1,2-mannosidases Δ33 and Δ21. α1,2-Mannosidases Δ33 (A) and Δ21 (B), from 4.4 l or 4.2 l of culture medium, respectively, were chromatographed on S-Sepharose according to EXPERIMENTAL PROCEDURES. Although three peaks containing α1,2-mannosidase were eluted from the column for α1,2-mannosidase Δ21, only fractions 54-67 of the third peak were pooled and further purified for crystallization.

A


Absorbance (280 nm)
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
Fraction number

B


Absorbance (280 nm)
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
Fraction number

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis

Mutation of the N-glycosylation sites in the yeast α1,2-mannosidase was accomplished by the unique site elimination procedure (U.S.E. Mutagenesis Kit (Pharmacia)) as previously described (Chapter 4). The plasmid YpHMNS1 used for mutagenesis contains the nucleotide sequence encoding amino acids 22-549 of the α1,2-mannosidase (Chapter 4)(Table I). The following oligonucleotides were used: GATGTTAATGTATCAGAGCTCCACACTATACAAAAG for YpHN96Q, CTATCT GATGTTCTCGAGGTAGGTCAGAAGACTGTCTACTTG for YpHN155Q, and GGCG TATTTGACCGGTCAGCGTACTTATTGGG for YpHN224Q. To obtain the plasmid containing an α1,2-mannosidase devoid of N-glycosylation sites (YpHΔN), the YpHN224Q plasmid was mutated in one reaction with the two oligonucleotides used to construct YpHN96Q and YpHN155Q. The mutated regions were sequenced by the dideoxy method (Sanger *et al.*, 1977).

N-terminal deletions were constructed by PCR using the plasmid pBS9.5 (Grondin and Herscovics, 1992) that contains the *MNS1* gene, an antisense 3' oligonucleotide GGT*GGATCC*CTACAACGACCAACCTGTG containing a BamHI site, and one of three of the following 5' sense oligonucleotides containing an XhoI site: ATA*CTCGAG*AGAGAAAGTCACCGGGGGCC for YpHΔ28; ATA*CTCGAG*GGGC CGGAGAAATGAGAGATC for YpHΔ33; ATA*CTCGAG*ATCGGATTGAAAGCATG for YpHΔ38. The PCR conditions were as previously described (Chapter 2) and the specific PCR products were digested with XhoI and BamHI, and ligated into the XhoI/BamHI sites of the pHIL-S1 vector. The YpHΔ28, YpHΔ33, and YpHΔ38 plasmids contain α1,2-mannosidase nucleotide sequence encoding amino acids 29-549, 34-549, and 39-549, respectively (Table I).

Expression and purification of recombinant α1,2-mannosidase

Recombinant α1,2-mannosidase was expressed in the *P. pastoris* strain GS115 (*his4*) or KM71 (*his4, aox1*) from Invitrogen as previously described (Chapter 4). For purification of the α1,2-mannosidase, the culture medium was first concentrated using either a Diaflo Hollow Fiber Cartridge (Cartridge type H1P30-43, Amicon) or polyethylene glycol (average molecular weight of 8,000 or 20,000, American Chemicals Ltd.), followed by sequential S-Sepharose and Q-Sepharose chromatography (Chapter 5).

To purify Endo H treated α1,2-mannosidase Δ33, the sample in 50 mM sodium citrate buffer, pH 5.5, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, and 0.02

% (w/v) NaN_3 was applied to a Concanavalin A Sepharose (Pharmacia) column (2 ml) equilibrated in the same buffer. The fractions containing α1,2-mannosidase that did not bind to this column were dialyzed in succinate buffer, pH 5.5, containing 1 mM NaN_3 and then loaded onto an S-Sepharose column (2 ml) equilibrated in the same buffer. The column was eluted with a 0-0.5 M NaCl gradient. SDS-PAGE followed by Coomassie Blue staining was used to identify the fractions that contained α1,2-mannosidase free of Endo H.

Protein analysis

The concentration of purified α1,2-mannosidase was determined using the absorbance at 280 nm (extinction coefficient = 115,200 $M^{-1}cm^{-1}$)(Mach *et al.*, 1992). SDS-PAGE under reducing conditions was carried out according to Laemmli (1970) using the Bio-Rad Mini-PROTEAN II apparatus. N-terminal sequence analysis was performed at Harvard Microchemistry (Cambridge, Massachusetts) or at the Biotechnology Service Centre, University of Toronto (Toronto, Ontario). For Western blot analysis of intracellular proteins, *P. pastoris* transformed with pHIL-S1, YpHMNS1, or YpHΔN were grown overnight in 1.5 ml BMGY medium, then induced in 0.3 ml of BMMY medium for 48 h. 40 µl of the culture was centrifuged and the cells were resuspended in 20 µl of SDS-PAGE sample buffer. After boiling for 2 min, 2 µl of the sample were used for SDS-PAGE and Western blot analysis using polyclonal antibodies to the yeast α1,2-mannosidase (Chapter 4).

Endo H treatment

Purified recombinant α1,2-mannosidase (0.5 mg/ml) was treated with Endo H (New England Biolabs) under native conditions in 50 mM sodium citrate buffer, pH 5.5 using a final concentration of 1250 units/ml added at 0 and 4 h of incubation, and the incubation was carried out for 24 h at 37 °C. For treatment under denaturing conditions, the α1,2-mannosidase (1 mg/ml) was first boiled for 10 min in 0.5 % SDS and 1.0 % mercaptoethanol. An equal volume of 100 mM sodium citrate buffer, pH 5.5 and 6250 units/ml of Endo H were added and the mixture was incubated for 24 h at 37 °C. Control incubations were done under identical conditions without Endo H.

PNGase F treatment

Purified recombinant α1,2-mannosidase (0.5 mg/ml) was treated with PNGase F (New England Biolabs) under native conditions in 50 mM sodium phosphate buffer, pH

7.5. PNGase F (final concentration of 6250 units/ml) was added and the incubation was carried out for 25 h at 37 °C. For treatment under denaturing conditions, the α1,2-mannosidase (1 mg/ml) was first boiled for 10 min in 0.5 % SDS and 1.0 % mercaptoethanol. An equal volume of 100 mM sodium phosphate buffer, pH 7.5 containing 2 % NP-40 and 6250 units/ml of PNGase F were added and the mixture was incubated for 25 h at 37 °C. Control incubations were done under identical conditions without PNGase F.

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