Ktr1p is an α-1,2-mannosyltransferase of *Saccharomyces cerevisiae*

Comparison of the enzymic properties of soluble recombinant Ktr1p and Kre2p/Mnt1p produced in *Pichia pastoris*

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The yeast genome contains a *KRE2/MNT1* family of nine related genes with amino acid similarity to the α1,2-mannosyltransferase Kre2p/Mnt1p, the only member of this family whose enzymic properties have been studied. In this study, the enzymic properties of Ktr1p, another member of this family, were studied and compared to those of Kre2p/Mnt1p. Recombinant soluble forms of Kre2p/Mnt1p and Ktr1p lacking their N-terminal regions were expressed as secreted proteins from the methylotrophic yeast *Pichia pastoris*. After induction with methanol, the medium contained approx. 40 and 400 mg/l of soluble recombinant Kre2p/Mnt1p and Ktr1p respectively. Both recombinant proteins were shown to exhibit α1,2-mannosyltransferase activity. The enzymes have an absolute requirement for Mn^{2+} and a similar K_m for mannose (280–350 mM), methyl-α-mannoside (60–90 mM) and GDP-mannose (50–90 μM), but the V_{max} was approx. 10 times higher for Kre2p/Mnt1p than for Ktr1p. The enzymes have similar substrate specificities and utilize mannose, methyl-α-mannoside, α-1,2-mannobiose and methyl-α-1,2-mannobiose, as well as $Man_{15-30}GlcNAc$, derived from *mnn*2 mutant glycoproteins, as substrates. The enzymes do not utilize α-1,6-mannobiose, α-1,6-mannotriose, α-1,6-mannotetraose, mammalian $Man_9GlcNAc$ or yeast $Man_{9-10}GlcNAc$. These results indicate that Kre2p/Mnt1p and Ktr1p are capable of participating in both N-glycan and O-glycan biosynthesis.

INTRODUCTION

The glycoproteins of *Saccharomyces cerevisiae* contain N-linked oligosaccharides that are greatly enriched in mannose, and O-linked oligosaccharides with up to five mannose residues (see Scheme 1). The mannose residues occur in several different linkages, suggesting that many mannosyltransferases are required for yeast glycoprotein biosynthesis. The *N*-glycan core is formed in the endoplasmic reticulum from the dolichol-linked $Glc_3Man_9GlcNAc_2$ precursor common to most eukaryotes. The glucose residues and one mannose are rapidly removed in the endoplasmic reticulum, and the core is subsequently modified in the Golgi through the addition of α-1,3-linked mannose residues. In some cases, an outer chain of variable size is also attached to the core oligosaccharide. This outer chain consists of a backbone of α-1,6-linked mannose residues substituted with side chains of α-1,2-linked mannose that can, in turn, be terminated with α-1,3-linked mannose residues. The *O*-glycans are linear oligosaccharides containing up to five mannose residues in α-1,2 and α-1,3 linkages. The mannose linked to serine/threonine is transferred from dolichyl mannosyl phosphate in the endoplasmic reticulum, and the other mannose residues are formed from GDP-mannose in the Golgi (reviewed in [1–4]). Only a few of the mannosyltransferases involved in yeast glycoprotein biosynthesis have been characterized biochemically and purified to different extents. These include several α-1,2-mannosyltransferases [5–7], the α-1,6-mannosyltransferase that initiates the outer chains [7–9] and the protein *O*-mannosyltransferase that adds the first mannose to the *O*-glycans [10–13].

Genes involved in the biosynthesis of the dolichol-linked oligosaccharide precursor have been extensively characterized (reviewed in [4]). More recently, yeast genes encoding mannosyltransferases required for the biosynthesis of the O-linked oligosaccharides and for the formation of the N-linked outer chains have been identified, and their role in protein glycosylation is being elucidated. A gene family (*PMT1* to *PMT6*) involved in the initiation of the O-linked glycans [12,14–16] has been identified. The *OCH1* gene has been shown to be responsible for the initiation of the outer chain [17,18] and the *MNN1* gene encodes the α-1,3-mannosyltransferase that adds the terminal α-1,3-linked mannose to both O-linked and N-linked glycans [3,19].

With respect to the genes that encode α-1,2-mannosyltransferases, the only one that has been characterized enzymically is Kre2p/Mnt1p. It was isolated independently by two different approaches. Hill et al. [20] cloned this gene by complementation of a K1 killer-resistant mutant, whereas Häusler and Robbins [6] first purified an α-1,2-mannosyltransferase activity with methyl-α-mannoside as substrate, and then used partial amino acid sequence information to obtain the corresponding gene. Disruption of the *KRE2/MNT1* gene greatly decreased α-1,2-mannosyltransferase activity towards methyl-α-mannoside [21]. Mutants containing the disrupted gene synthesized O-linked oligosaccharides containing only two mannose residues [21], thereby demonstrating *in vivo* that *KRE2/MNT1* encodes the α-1,2-mannosyltransferase responsible for the addition of the third mannose residue of O-linked oligosaccharides (see Scheme 1). The *N*-glycans synthesized by the *kre2/mnt1* mutant were shorter than those obtained from the wild-type strain, but it was not established that Kre2p/Mnt1p is an enzyme directly involved in N-linked glycan biosynthesis [20,22].

Abbreviations used: srKtr1p, soluble recombinant Ktr1p; srKre2p, soluble recombinant Kre2p.

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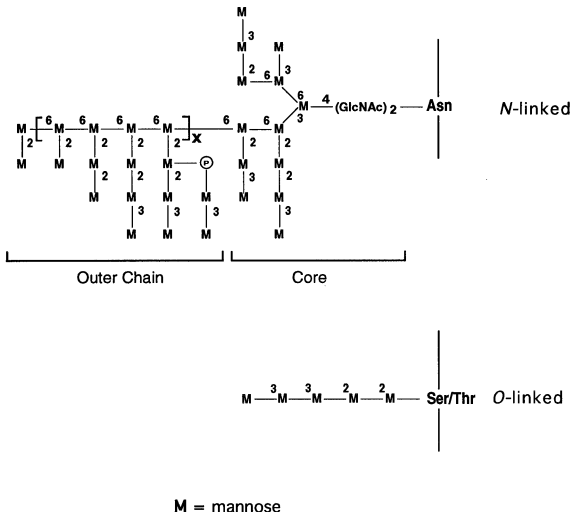
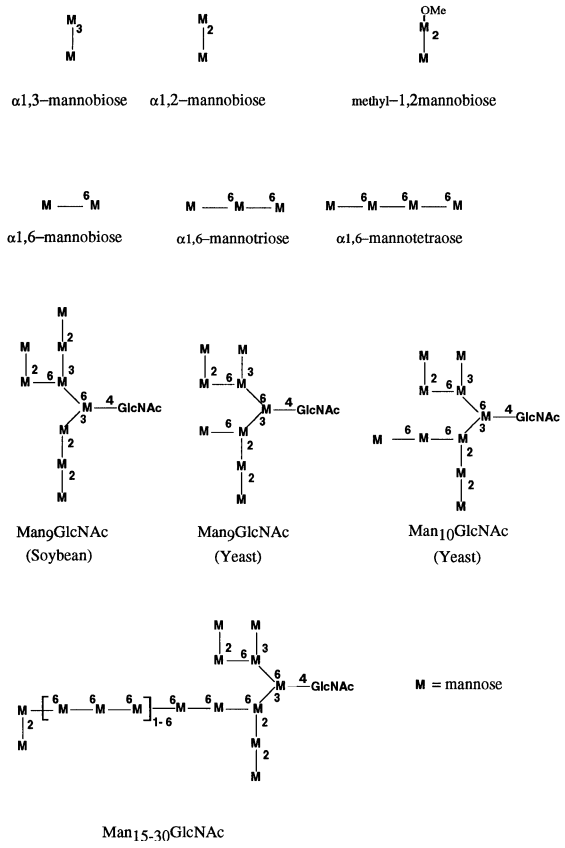


Scheme 1 Structure of *N*-glycans and *O*-glycans in *Saccharomyces cerevisiae*



Scheme 2 Structure of oligosaccharides used as potential acceptors in the present study

The complete yeast genome is now known to contain a *KRE2/MNT1* family of nine related genes. These genes include *KTR1* [20,23], *YUR1* [24], *KTR2* [25] and *KTR3* to *KTR7* ([26], and M. Lussier, A.-M. Scidu, E. Winnett, D. H. Vo, J. Sheraton, A. Düsterhöft, R. K. Storms and H. Bussey, unpublished work). Of all the members of this family, Ktr1p is the one most similar to Kre2p/Mnt1p, with 52% amino acid identity. Because Kre2p/Mnt1p is the only member of this family that has been shown to be an α-1,2-mannosyltransferase [21], it was important to determine whether other members also have α-1,2-mannosyltransferase activity. Therefore the aim of the present study was to produce recombinant Ktr1p to determine its specificity and to compare its enzymic properties with those of recombinant Kre2p/Mnt1. The results demonstrate that, like Kre2p/Mnt1p, Ktr1p is an α-1,2-mannosyltransferase, and that both enzymes can utilize N-type glycans and α-1,2-mannobiose as substrates.

EXPERIMENTAL

Materials

The *Pichia pastoris* expression kit was obtained from Invitrogen (San Diego, CA, U.S.A.); pBluescript KS$^+$II was from Stratagene (La Jolla, CA, U.S.A.); α-1,2-mannosidase (*Aspergillus saitoi*) was from Oxford Glycosystems (Abingdon, Oxon., U.K.); endo-β-*N*-acetylglucosaminidase H was from New England Biolabs (Beverly, MA, U.S.A.); GDP-[^{3}H]mannose (15.7 Ci/mmol) was from New England Nuclear (Du Pont Canada Inc., Mississauga, Ontario, Canada); GDP-mannose, mannose, methyl-α-mannoside and methyl-α-1,2-mannobiose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-1,2-Mannobiose, α-1,3-mannobiose and α-1,6-mannobiose were from Dextra Laboratories (Reading, Berks., U.K.); $Man_9GlcNAc$ was isolated from soybean agglutinin as described previously [7]. All other oligosaccharides used in this work (see Scheme 2) were kindly supplied by Dr. C. E. Ballou (University of California, Berkeley, CA, U.S.A.). Reagents for SDS/PAGE and AG1-X8 anion-exchange resin were from Bio-Rad Laboratories (Mississauga, Ontario, Canada); oligonucleotides were prepared at the Sheldon Biotechnology Center (McGill University, Montréal, PQ, Canada). All other chemicals were of reagent grade.

Plasmid construction

To obtain the DNA sequence encoding the soluble domains of Kre2p/Mnt1p and Ktr1p, an *Xho*I restriction site was inserted directly after the codon corresponding to the last amino acid of the transmembrane domain of both genes by oligonucleotide-mediated mutagenesis performed by the method of Kunkel et al. [27]. A 1.9 kb *Sst*I–*Xba*I DNA fragment containing the 1329 bp Kre2p/Mnt1p open reading frame was subcloned in pBluescript KS$^+$II and transformed into strain CJ236 from which single-stranded DNA was produced. For *KTR1* a 2.0 kb *Kpn*I–*Hin*dIII fragment containing the 1183 bp Ktr1p open reading frame was subcloned in pFL64$^+$ [28] and transformed into strain CJ236 as indicated above to obtain single-stranded DNA. The *Xho*I site was inserted after mutagenesis with oligonucleotides 5′-TGAATTCCAACAGTAGACTCGAGCTCAGCAATATATTCCG-3′ for *KRE2/MNT1* and 5′-GCTCAGTATGCGAGACTCGAGGCTCTGCCCCAAG-3′ for *KTR*1. Clones containing an *Xho*I restriction site were verified by DNA sequencing of the region around the mutation by using the dideoxy chain-termination procedure [29]. Positive clones were digested with *Xho*I and *Bam*HI and ligated into the *Xho*I/*Bam*HI sites of the pHIL-S1 vector to generate the plasmids pHKRE2 and pHKTR1 respectively.

Transformation of *P. pastoris*

Transformation of *P. pastoris* (GS115 *his4* strain) and isolation of clones was done as indicated by the manufacturers. To obtain His^+Mut^s transformants synthesizing the soluble domain of Kre2p/Mnt1p, plasmid pHKRE2 was digested with *Bgl*II. Similarly, to obtain His^+Mut^s transformants synthesizing the soluble domain of Ktr1p, plasmid pHKTR1 was digested with *Dra*I. The linearized fragments were then used to transform the *P. pastoris* GS115 (*his*4) strain by the spheroplast method. His^+Mut^s mutants were obtained by selecting His^+ transformants that grew well in minimal medium containing glucose but poorly in minimal medium containing methanol. Positive clones were grown at 30 °C (250–300 rev./min), until a D_{600} of 4–6 (approx. 30 h) in medium containing 1 % (w/v) yeast extract, 2 % (w/v) peptone, 1.34 % (w/v) yeast nitrogen base, 4×10^{-5} % biotin and 1 % (v/v) glycerol buffered with 100 mM potassium phosphate (BMGY medium). The medium was removed by centrifugation and the cells were resuspended at five times the original density in medium similar to BMGY, except that it contained 0.5 % (v/v) methanol instead of glycerol (BMMY medium), to induce the expression of the desired proteins.

Mannosyltransferase assays

Mannosyltransferase activity was assayed essentially as described [7]. Standard incubation mixtures contained 50 mM Tris/maleate buffer [pH 7.0 for soluble recombinant Ktr1p (srKtr1p) or pH 7.5 for soluble recombinant Kre2p (srKre2p)], 10 mM $MnCl_2$, 50 mM methyl-α-mannoside, 10 μM GDP-[^{3}H]mannose (0.0135μCi) and 5 mg/ml BSA in a total volume of 50 μl. Unless stated otherwise, incubation was for 15 min at 30 °C. The amount of protein used is indicated in the legends. The reaction was stopped by adding 100 μl of 50 mM EDTA. The reaction mixtures were passed through small columns (approx. 0.4 ml) of AG1-X8 (formate form, 200–400 mesh). The neutral products were eluted with 0.75 ml of water, which was assayed for radioactivity. Samples without methyl-α-mannoside were incubated in parallel and the radioactivity in these controls was subtracted from the experimental values.

Determination of K_m values and substrate specificity

To determine the apparent K_m values of the enzymes the concentration of methyl-α-mannoside was varied from 5 to 100 mM and that of mannose from 25 to 500 mM while the concentration of GDP-[^{3}H]mannose was kept constant at 10 μM (0.0675 μCi). To determine the apparent K_m for GDP-mannose, incubations were performed with a constant amount of GDP-[^{3}H]mannose (10 μM, 0.0675 μCi) and variable amounts of unlabelled GDP-mannose (10–200 μM), at a methyl-α-mannoside concentration of 50 mM. The reactions were performed in a total volume of 250 μl containing either 4.3 ng of srKre2p or 41 ng of srKtr1p. Aliquots of 50 μl were removed at 0, 7.5, 15 and 30 min, added directly to 100 μl of 50 mM EDTA and assayed for mannosyltransferase activity as indicated above.

To study the specificity of the enzymes, incubations were performed in a total volume of 30 μl containing 0.054 μCi of GDP-[^{3}H]mannose (50 μM for srKre2p; 100 μM for srKtr1p), and 4 mM monosaccharide or oligosaccharide under test as acceptors. The reactions were started by the addition of either 8.6 ng of srKre2p or 82 ng of srKtr1p, 8 μl aliquots were removed at 1 and 2 h and processed as indicated above. After 5 h the samples were placed at −20 °C. Next day the incubation mixtures were defrosted on ice, more enzyme was added (4.3 ng of srKre2p or 41 ng of srKtr1p), and an aliquot equivalent to 8 μl of the original incubation mixture was removed immediately and processed as above. The rest of the samples were incubated for an additional 6 h, at which time an aliquot equivalent to approx. 6 μl of the original mixture was assayed for mannosyltransferase activity. The data were normalized to 6 μl of the original incubation.

A. saitoi α-1,2-mannosidase treatment

The product(s) of the incubation of srKtr1p with 100 μM GDP-[^{3}H]mannose and 50 mM methyl-α-mannoside were desalted through coupled columns of AG 50W-X8 (H^+ form, 200–400 mesh) and AG1-X8 (formate form, 200–400 mesh), and incubated at 37 °C in 15 μl of 0.1 M sodium acetate, pH 5.0, containing 15 μ-units of *A. saitoi* α-1,2-mannosidase as described [7]. An incubation without enzyme was done in parallel as control. At 40 h a 3 μl aliquot was removed, added to 100 μl of water and boiled for 5 min. The mixture was centrifuged and the supernatant was combined with 100 μl of acetonitrile and [^{14}C]mannose as internal standard, followed by HPLC on an Aminospherisorb column (Phase Separations, packed by Chromatography Sciences Co., Ville Mont Royal, Québec, Canada) with isocratic elution at 0.5 ml/min for 60 min in acetonitrile/water (4:1, v/v). The water contained 15 mM potassium phosphate and 5 mM NaN_3, pH 5 [30]. Aliquots of 0.5 ml were assayed for radioactivity.

Endo-β-*N*-acetylglucosaminidase H treatment

The recombinant proteins present in 100 μl of medium for srKre2p and in 50 μl of medium for srKtr1p were denatured by boiling for 10 min in 0.5 % SDS/1 % (v/v) 2-mercaptoethanol and incubated with or without 100 m-units of endo-β-*N*-acetylglucosaminidase H for 30 h at 37 °C in 0.5 % SDS/1 % (v/v) 2-mercaptoethanol/50 mM sodium citrate (pH 5.5).

Analytical methods

SDS/PAGE was performed by the method of Laemmli [31] with the Bio-Rad Mini-Protean II apparatus. Slab gels were 0.75 mm thick. Protein bands were revealed by staining with Coomassie Blue. For Western blotting, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and detected by the enhanced chemiluminescence Western blotting detection system (Amersham, Little Chalfont, Bucks., U.K.) with rabbit polyclonal antisera raised against synthetic peptides containing 10 amino acid residues of the C-terminus of Kre2p/Mnt1p and 14 amino acid residues of the C-terminus of Ktr1p respectively. Protein concentration was determined on SDS/PAGE gels with BSA as standard for srKre2p and ovalbumin as standard for srKtr1p. The recombinant proteins were quantified by densitometry with a Bio Imager (Bio Image, Millipore Ann Arbor, MI, U.S.A.).

RESULTS

Production of recombinant proteins

KRE2/MNT1 and *KTR1* encode type II membrane proteins of 51.5 and 46 kDa respectively, with a relatively short cytoplasmic N-terminus (11 and 16 residues) followed by a membrane-spanning domain (19 and 21 residues) and a large C-terminal catalytic domain. To characterize and compare their enzymic activities, soluble forms of Kre2p/Mnt1p and Ktr1p were produced as proteins secreted from *P. pastoris*. For this purpose, the DNA sequence encoding the soluble domain of each protein was subcloned into the pHIL-S1 expression vector. In these

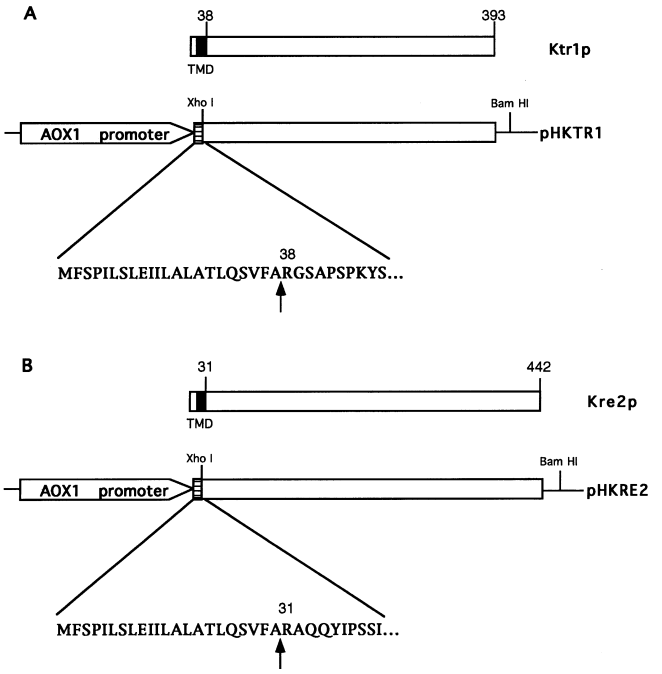


Figure 1 Cloning of the soluble domains of Kre2p and Ktr1p into the expression vector pHIL-S1

The diagrams represent the complete type II membrane proteins Ktr1p (**A**) and Kre2p (**B**). Both have a short cytoplasmic tail, a transmembrane domain (TMD) and a large soluble domain. A partial sequence of each construct, pHKTR1 (**A**) and pHKRE2 (**B**), is shown, including the amino acid sequence of the *PHO1* signal peptide (hatched box) and the following first 10 residues of the soluble domain. The numbers indicate the first and last residue of the soluble domain and the arrows indicate the sites of the expected signal peptidase cleavage.

constructs the N-terminal cytoplasmic and transmembrane domains of the proteins are replaced with the cleavable signal sequence of *PHO1* and the fusion proteins are placed under the control of the *AOX1* promoter. The resulting constructs pHKTR1 (Figure 1A) and pHKRE2 (Figure 1B) contain DNA sequences encoding Ktr1p and Kre2p/Mnt1p, beginning at residues 38 and 31 respectively.

The *P. pastoris* (GS115 strain) transformed with pHKRE2 or pHKTR1 was grown in BMGY medium and then transferred to BMMY medium to induce the production of the recombinant proteins. Aliquots of culture supernatants (10 μl) obtained at different times of induction were subjected directly to SDS/PAGE followed by staining with Coomassie Blue (Figure 2). When cells were transformed with vector pHKTR1, a major specific protein band of about 40 kDa (srKtr1p) was produced after induction (Figure 2A, lanes 2–6). This protein was absent from medium obtained from cells transformed with the vector alone (Figure 2A, lane 1). The amount of 40 kDa protein increased with time of induction up to approx. 5 days. Two minor products of approx. 43 and 27 kDa were also produced. The size of the major protein band was close to the predicted molecular mass of 41.9 kDa for unglycosylated srKtr1p.

When cells were transformed with pHKRE2, a specific protein band of approx. 60 kDa (srKre2p), absent from cultures transformed with pHIL-S1 alone, was detected beginning after 2 days of induction (Figure 2B, lane 3). The 60 kDa band (srKre2p) was most intense after approx. 4 days of induction. After longer induction, a protein band of approx. 43 kDa appeared while the 60 kDa band decreased in intensity, most probably due to partial

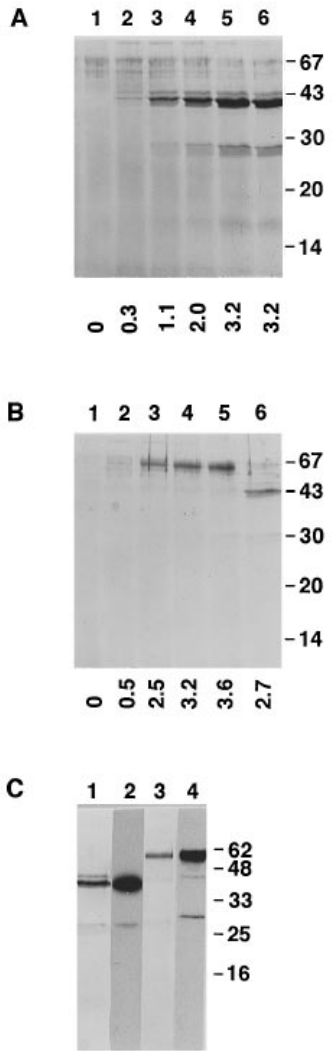


Figure 2 SDS/PAGE of medium from *P. pastoris* transformed with either pHKTR1 or pHKRE2

(**A**) Coomassie Blue staining of medium (10 μl) from cells containing pHIL-S1 vector (lane 1) after 6 days of induction with methanol, or pHKTR1 (lanes 2–6) after 1, 2, 3, 5 and 6 days of induction with methanol respectively. (**B**) Coomassie Blue staining of medium from cells containing pHIL-S1 vector (lane 1) after 6 days of induction with methanol, or pHKRE2 (lanes 2–6) after 1, 2, 3, 4 and 6 days of induction with methanol respectively. In (**A**) and (**B**) the mannosyltransferase activity in the medium (0.05 μl) was determined with methyl-α-mannoside as substrate, as indicated in the Experimental section. (**C**) Cells containing either pHKTR1 (lanes 1 and 2) or pHKRE2 (lanes 3 and 4) were induced with methanol for 3 days. In lanes 1 and 3 the medium (10 μl) was subjected to SDS/PAGE and revealed by staining with Coomassie Blue. In lane 2, 0.2 μl of medium was applied to detect srKtr1p by Western blotting; in lane 4, 1 μl of medium was used to detect srKre2p by Western blotting, as described in the Experimental section. Medium from cells containing vector alone did not react with the antibodies on Western blots (results not shown). Molecular masses (kDa) of protein standards are indicated at the right; 10^{-3} × mannosyltransferase activity (in c.p.m.) is shown under the lanes.

proteolysis. The predicted molecular mass of the unglycosylated srKre2p is 48.2 kDa. The reasons for the apparent discrepancy between the predicted molecular mass of srKre2p and the value found experimentally are not known.

To further identify the proteins detected by staining with Coomassie Blue, the constituents of the medium were also detected by Western blotting with antibodies prepared against synthetic peptides derived from either Ktr1p or Kre2p/Mnt1p.

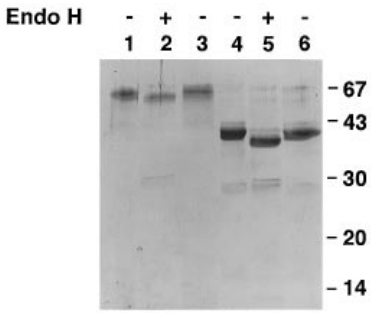


Figure 3 Endo-β-*N*-acetylglucosaminidase H treatment of the recombinant proteins

Medium containing srKre2p or srKtr1p was incubated with (lanes 2 and 5) or without (lanes 3 and 6) endo-β-*N*-acetylglucosaminidase H (Endo H) as described in the Experimental section. The samples were then subjected to SDS/PAGE [15% (w/v) gel] and revealed with Coomassie Blue. Lanes 1–3, srKre2p; lanes 4–6, srKtr1p; lanes 1 and 4, untreated medium.

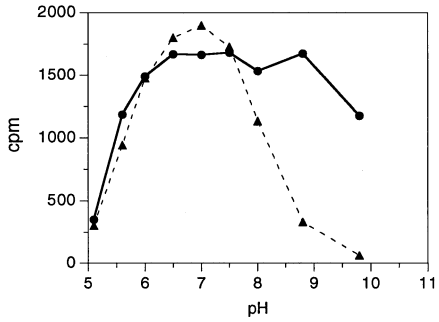


Figure 5 Effect of pH on the activity of the recombinant α-1,2-mannosyltransferases

Incubations were performed for 30 min, as indicated in the Experimental section, with 2.15 ng of srKre2p (●) or 20.5 ng of srKtr1p (▲) in 50 mM Tris/maleate buffers of different pH values.

The results (Figure 2C) indicate that the bands detected by Coomassie Blue are specific for each protein. After induction, the medium (0.05 μl) was also assayed for mannosyltransferase activity with methyl-α-mannoside as acceptor (Figures 2A and 2B). Both recombinant soluble proteins were enzymically active, and the enzyme activity corresponded to the intensity of the specific recombinant proteins observed on the gels. These results clearly demonstrate that srKtr1p, like srKre2p, is a mannosyltransferase capable of using methyl-α-mannoside as substrate *in vitro*.

Both *KRE2/MNT1* and *KTR1* encode proteins that have a single consensus sequence for N-glycosylation. To test whether these sites are used, medium containing the recombinant proteins were treated with endo-β-*N*-acetylglucosaminidase H and analysed by SDS/PAGE. The apparent molecular mass of both proteins decreased by approx. 2.5 kDa, indicating that both enzymes are N-glycosylated by *P. pastoris* (Figure 3). These results show that the discrepancy in size of srKre2p might be due to O-glycosylation or some other post-translational modifications, or to aberrant migration on SDS/PAGE.

Recombinant proteins obtained after 4 days of induction with methanol were used for subsequent studies. The amount of protein produced was determined by quantification of the Coomassie Blue staining on SDS/PAGE with the Bio-Imager. It is estimated that approx. 40 mg of srKre2p per litre of BMMY, and about 400 mg of srKtr1p per litre of BMMY, were produced.

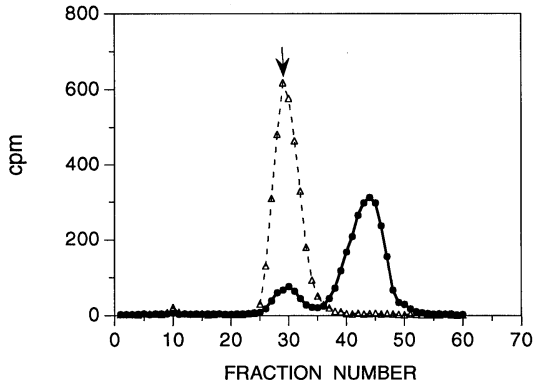


Figure 4 Effect of *A. saitoi* α-1,2-mannosidase on the products synthesized by srKtr1p

Medium containing srKtr1p was incubated with methyl-α-mannoside and GDP-[^{3}H]mannose as described in the Experimental section. The products of the reaction were incubated with (▲) or without (●) α-1,2-mannosidase from *A. saitoi*. The mixtures were then fractionated by HPLC. The arrow indicates the elution position of the [^{14}C]mannose used as an internal standard.

Recombinant Ktr1p is an α-1,2-mannosyltransferase

Previous studies showed that Kre2p/Mnt1p is an α-1,2-mannosyltransferase [6], but the specificity of Ktr1p was unknown. To determine the linkage of the mannose residue added to methyl-α-mannoside by srKtr1p, the medium containing the recombinant protein was incubated with GDP-[^{3}H]mannose and methyl-α-mannoside, and the reaction products were treated with *A. saitoi* α-1,2-mannosidase and fractionated by HPLC. Figure 4 shows that all the radioactivity added to methyl-α-mannoside was released as [^{3}H]mannose after incubation with the specific α-1,2-mannosidase. These results demonstrate that srKtr1p is also an α-1,2-mannosyltransferase.

Properties of the recombinant α-1,2-mannosyltransferases

The srKtr1p in Tris/maleate buffer exhibits maximum α-1,2-mannosyltransferase activity between pH 6.5 and 7.5, with a definite peak at pH 7.0, whereas srKre2p exhibits a very broad pH optimum between pH 6.5 and 8.7 (Figure 5). With both enzymes the reaction is linear for approx. 15–30 min. Under standard conditions the mannosyltransferase activity of srKre2p is proportional to protein concentration up to approx. 17 ng/ml and that of srKtr1p is proportional up to approx. 165 ng/ml (results not shown). The enzymes have an absolute requirement for Mn^{2+} with an optimum concentration of approx. 10 mM; Ca^{2+}, Co^{2+}, Mg^{2+} or Zn^{2+} at a similar concentration cannot substitute for Mn^{2+}.

Acceptor affinity

The affinity of the two α-1,2-mannosyltransferases for methyl-α-mannoside and mannose as acceptors, was compared. The data obtained from plots of V against $V/[S]$ indicate that the apparent

Table 1 Apparent K_m and V_{max} values of recombinant α-1,2-mannosyltransferases

Values were obtained from plots of V against $V/[S]$ as described in the Experimental section. The results are the averages for two independent assays.

Enzyme	Substrate	K_m (mM)	V_{max} (nmol/min per mg of protein)
srKre2p	Methyl-α-mannoside	55	3870
srKtr1p	Methyl-α-mannoside	85	460
srKre2p	Mannose	345	1830
srKtr1p	Mannose	280	120

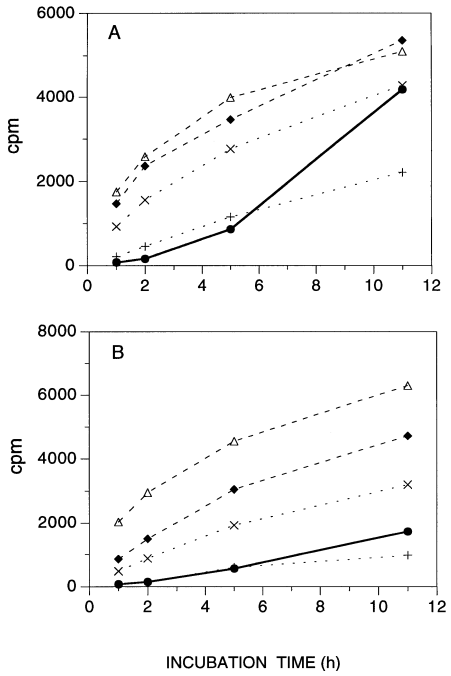


Figure 6 Specificity of the recombinant α-1,2-mannosyltransferases towards different substrates

srKre2p (**A**) and srKtr1p (**B**) were incubated for different periods with the oligosaccharides shown in Scheme 2 and were assayed for mannosyltransferase activity as described in the Experimental section. Symbols: +, mannose; ◆, methyl-α-mannoside; ×, α-1,2-mannobiose; △, methyl-α-1,2-mannobiose; ●, $Man_{15-30}GlcNAc$.

K_m values were similar for both enzymes (Table 1): approx. 60–90 mM for methyl-α-mannoside and 280–350 mM for mannose, but the V_{max} for srKtr1p was approx. one-tenth that of srKre2p with both substrates. Similar K_m values were obtained for a recombinant Kre2p/Mnt1p expressed in *Escherichia coli* [32], showing that glycosylation does not affect its enzymic properties. The affinity of both enzymes for the donor GDP-mannose was similar (50–90 μM) but the V_{max} for srKtr1p was also lower than that observed with srKre2p.

Specificity of srKre2p and srKr1p

The specificity of the enzymes was studied with a series of potential oligosaccharide substrates (shown in Scheme 2). Besides mannose and methyl-α-mannoside, both enzymes utilize α-1,2-mannobiose and methyl-α-1,2-mannobiose as substrates (Figure 6) but not α-1,3- or α-1,6-mannobiose. The α-1,2-disaccharides (α-1,2-mannobiose and methyl-α-1,2-mannobiose) are better acceptors than their respective monosaccharides (mannose and methyl-α-mannoside). Both srKre2p (Figure 6A) and to a smaller extent srKtr1p (Figure 6B) can use $Man_{15-30}GlcNAc$ as substrate, thereby demonstrating that they can both act on *N*-glycans *in vitro*. Whereas the reaction of srKtr1p with $Man_{15-30}GlcNAc$ is linear with time of incubation, there is a significant increase in the rate of mannose incorporation into this substrate during the second part of the incubation with srKre2p. This increase suggests that some of the mannose incorporation is dependent on the formation of products during the first part of the incubation. Of the other oligosaccharides tested as acceptors, α-1,6-mannotriose, α-1,6-mannotetraose, soybean agglutinin $Man_9GlcNAc$, yeast $Man_9GlcNAc$ or yeast $Man_{10}GlcNAc$ oligosaccharides were not substrates for either enzyme.

DISCUSSION

The yeast genome contains a *KRE2/MNT1* family of nine related genes (M. Lussier, A.-M. Scidu, E. Winnett, D. H. Vo, J. Sheraton, A. Düsterhöft, R. K. Storms and H. Bussey, unpublished work). Kre2p/Mnt1p is the only member of this family that has been previously characterized enzymically. In the present study we have established that *KTR1*, another gene in this family, also encodes an α-1,2-mannosyltransferase with properties very similar to those of Kre2p/Mnt1p. Soluble forms of Kre2p/Mnt1p and Ktr1p were efficiently expressed as secreted proteins (40 and 400 mg/l of medium respectively) from the methylotrophic yeast *P. pastoris*. Both recombinant soluble proteins were shown to be enzymically active with different substrates and to have similar specificities.

The substrate specificity studies indicate that Kre2p/Mnt1p and Ktr1p have the capacity for participating in both *N*-glycan and *O*-glycan biosynthesis. They both utilize the *N*-glycan-type oligosaccharide, $Man_{15-30}GlcNAc$, which contains the α-1,6-mannose outer-chain backbone. Because $Man_{15-30}GlcNAc$ lacks all the α-1,2-mannose-containing branches of the outer chain (with the exception of the terminating α-1,2-mannose; see Scheme 2 and below) and in accordance with the structure of the carbohydrates of yeast mannoproteins, the present results indicate that both Kre2p/Mnt1p and Ktr1p have the ability to add the first α-1,2-linked mannose residues to the outer-chain backbone *in vitro*. The observation that in the presence of Kre2p/Mnt1p the incorporation of mannose into $Man_{15-30}GlcNAc$ increased considerably during the second part of the incubation (Figure 6A) suggests that this enzyme might also be capable of adding the second row of α-1,2-linked mannose residues to the outer chain.

The results obtained with $Man_{15-30}GlcNAc$ as substrate demonstrate that Kre2p/Mnt1p and Ktr1p can participate in *N*-glycan biosynthesis. Kre2p/Mnt1p and other members of the protein family have been implicated in N-linked outer-chain elaboration *in vivo* [20,22]. The involvement of Kre2p/Mnt1p was suggested in studies showing that the *N*-glycans of invertase and acid phosphatase were shorter in a *kre2/mnt1* mutant [20,22]. However, in another study there was no effect of a *kre2/mnt*1 null mutation on the structure of the total oligosaccharides found in an *mnn1 mnn10* mutant background even though a decrease in glycosylation of invertase was observed [21]. The present results support the idea that Kre2p/Mnt1p participates directly in *N*-glycan biosynthesis *in vivo*. Invertase was also found to be underglycosylated in a *ktr1 ktr2 yur1* triple null mutant compared with a wild-type strain but not in single or double disruptants. It receives even less glycosylation when

synthesized in the quadruple *ktr1 ktr2 yur1 kre2* null strain, consistent with these four proteins (Kre2p, Ktr1p, Ktr2p and Yur1p) having redundant functions in N-linked glycosylation [22].The substrate specificities of srKtr1p and srKre2p *in vitro* demonstrated in the present study support a role for these enzymes in the synthesis of the outer chain of N-linked glycans.

In previous work [5,33], evidence was presented for the existence of an α-1,2-mannosyltransferase whose role is to add the α-1,2-mannose residue that acts as a signal for termination of the outer chain. This terminating α-1,2-mannosyltransferase was capable of utilizing α-1,6-mannobiose, α-1,6-mannotriose, α-1,6-mannotetraose and yeast $Man_{9-10}GlcNAc$ as substrates. The observation that these compounds are not substrates of srKre2p and of srKtr1p, coupled with the fact that the $Man_{15-30}GlcNAc$ acceptor already has an α-1,2-mannose residue at the non-reducing end (see Scheme 2), rules out a role of Kre2p/Mnt1p and Ktr1p in the addition of the terminating α-1,2-mannose.

Both srKre2p and srKtr1p utilize simple carbohydrates such as mannose, α-1,2-mannobiose and their methyl derivatives as substrates *in vitro*, an observation that suggests that both are capable of participating in O-glycosylation. This role of Kre2p/Mnt1p has been clearly demonstrated *in vivo* because a *kre2/mnt1* null mutant was blocked in the addition of the third α-1,2-linked mannose residue of the *O*-glycans [21]. Whereas srKtr1p has a similar specificity *in vitro* to that of srKre2p, a *ktr1* null mutation showed no defect in O-linked glycosylation *in vivo* [22].

Although Kre2p/Mnt1p and Ktr1p share high sequence similarities and are both α-1,2-mannosyltransferases with similar requirements and specificities *in vitro*, their enzymic properties differ in one major respect. Although they have similar K_m values towards mannose (280–350 mM), methyl-α-mannoside (60–90 mM) and GDP-mannose (50–90 μM), the V_{max} of srKtr1p with each of these substrates is about one-tenth that of srKre2p/Mnt1p. These kinetic properties of srKtr1p might partly explain why it has been difficult to demonstrate its role as a mannosyltransferase *in vivo*. No decrease in mannosyltransferase activity was observed in membrane preparations derived from a *ktr1* null strain, and a modest effect of overexpression of *KTR1* was seen in membranes obtained from a quadruple *kre2 ktr1 ktr2 yur1* null strain [22]. The fact that *KTR1* is one of nine related genes in yeast might also be a salient factor. It is also possible that its subcellular localization precludes demonstrating its activity *in vivo* in the presence of other mannosyltransferases. Ktr1p might participate with other mannosyltransferases in assembling *N*-glycan or *O*-glycan chains, but its fractional involvement might not become apparent unless the other contributing gene products are removed. The enzymic properties of the other members of this family are not known, but it is possible that they have overlapping specificities that allow them to compensate for one another depending on the physiological conditions. In any case it is clear from the present work that both Kre2p/Mnt1p and Ktr1p are capable of modifying *N*-glycans of *S. cerevisiae*.

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