The Processing of N-Linked Glycans in Yeast

MUTUALLY EXCLUSIVE STEPS IN THE PROCESSING OF A Man₆ DERIVATIVE BY YEAST MEMBRANE PREPARATIONS

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When a derivatized oligosaccharide isolated from ovalbumin and containing 6 mannose residues was incubated with yeast membranes and GDP-mannose, two sets of products were obtained, a high molecular weight one containing about 25 mannose residues and a low molecular weight one consisting of compounds with 7, 8, and 9 mannose residues, respectively. When the low molecular weight products were reincubated with the yeast membranes and GDP-mannose, no further mannose incorporation was observed, showing that these compounds must be of the wrong structure as substrates for yeast glycan processing enzymes. The structures were investigated by 1H NMR spectroscopy. The high molecular weight products contained an outer chain of an average length of 18 1-6-linked mannose residues attached to a core structure made up of the original 6 mannose residues with one additional 1-2-linked mannose added. The low molecular weight product with 8 mannose residues was deduced to contain a terminal 1-6-linked mannose (on the 1-6 arm) substituted by mannose at the 2-position, and the ones with 7 and 9 mannose residues were identified as having an additional 1-3-linked mannose on the starting Man₆ substrate and on the Man₉ product, respectively. The results lend further support to the picture that the processing steps must occur in proper sequence for specific products to form.

Recent studies on the early steps of the processing of N-linked glycans in Saccharomyces cerevisiae have had a significant impact on our understanding of the conversion of the precursor Glc₃Man₃GlcNAc₂ to oligomannose and polymannose structures in yeast glycoproteins. Based on the observation of a Man₆GlcNAc₂ structure as the smallest intermediate in the process (1), the early steps were deduced to consist of the glucosidase-catalyzed removal of the 3 glucose residues and a mannosidase-catalyzed removal of 1 mannose residue (Man-B in Fig. 1) followed by the mannosyltransferase-catalyzed assembly of the chain of 1-6-linked mannose residues presumably starting on the terminal 1-6-linked residue of the precursor (Man-8 in Fig. 1) (2), and finally by the introduction of 1-2 and 1-3 mannosyltransferase-catalyzed side chain mannose residues on the 1-6-linked chain. The finding of a specific mannosidase capable of removing only the Man-B residue (Fig. 1) (3) lent support for this general model. Finding that the addition of an α1-2-linked mannose to the terminal 1-6-linked mannose would block the elongation of the 1-6 chain (4) suggested that at least one additional 1-6-transferase must be required as the first step in the process (4, 5); and an enzyme capable of introducing one 1-6-linked mannose into the original Man₆ precursor has recently been isolated and characterized (6). The most recent work (5) established that the 1-6-backbone of the outer chain starts at Man-4 instead of at Man-3, leading to the overall model presented in Fig. 1. In our studies of regulatory effectors involved in glycan processing in yeast, we have used an ovalbumin-derived Man₆GlcNAc₂ derivative as substrate. When incubated with yeast membrane preparations this unnatural precursor yielded two kinds of products, one containing polymannose structures with an average of about 25 mannose residues, and another one containing oligomannose structures with 7, 8, or 9 mannose residues; the latter compounds were inactive as acceptor glycans for further addition of mannose. It was felt that to the extent that the structures of the various products could be established these observations might shed some further light on the early steps of yeast glycan processing. In this paper we document the processing steps observed for the Man₆GlcNAc₂ substrates and report the investigation of the structural features of the various products.

EXPERIMENTAL PROCEDURES

Preparation of Yeast Membranes—S. cerevisiae was maintained on solid (2% agar) YPD medium (0.3% yeast extract, 1% peptone, and 2% d-glucose). The yeast was grown aerobically on the YPG liquid medium. Fernbach flasks containing 800 ml of medium were inoculated with overnight yeast cultures and incubated at 30 °C on an orbital shaker operating at 150 rpm. The cells were harvested in mid-logarithmic phase by centrifugation and were washed twice with cold 5 mM Tris-HCl buffer, pH 7.2, containing 10 mM MnCl₂, 13-15 g (wet weight) of cells was suspended in 70-80 ml of the Tris-MnCl₂ buffer containing 5% glycerol (homogenizing buffer), and 1 volume of the suspension was mixed with 2 volumes of glass beads. The mechanical disruption of the cells was carried out in an ice-cooled "Bead Beater" homogenizer for 6 min at intervals of 1 min each. After removing the beads, the unbroken cells and cell wall fragments were sedimented by centrifugation at 5000 rpm for 10 min, and the
membrane fraction was next obtained from the supernatant liquid by centrifugation at 48,000×g for 45 min. The isolated membrane pellet was resuspended in the homogenizing buffer to a protein concentration of 13-15 mg/ml; the suspension was stored in 1-ml aliquots at -70°C. The various enzyme activities appeared to be stable for at least 2 months; new membrane preparations were generally prepared every 2 months.

Glycan Processing Reaction—A typical reaction mixture contained 0.1 μmol (0.19 mg) of the acceptor substrate Man6GlcNAC2-(N-biotinamidohexanoyl)Asn (prepared from ovalbumin and derivatized as described (7)), 2 μmol of GDP-[^14]C[Man, 60-70 μl of mucinbase suspension (0.7-0.9 mg of protein), and homogenization buffer to a total volume of 100 μl in capped, conical tubes. The solution was mixed well and incubated at 37°C in a water bath. To terminate the reaction, it was heated at 95°C for 10 min and centrifuged. The supernatant solution was fractionated by gel filtration on 0.3-120-cm columns of Bio-Gel P-6 as described (8). In the cases where larger quantities of products were needed for NMR experiments, the reaction was scaled up to contain up to 4.5 μmol (8.3 mg) of acceptor glycan, 90 μmol of GDP-Man, and 2.5-3.0 ml of membrane suspension in a total of 4.5 ml of buffer. The incubation and work-up was the same as above, but the sample was lyophilized and redissolved in about 1 ml of H2O and was fractionated on a 0.8×120-cm P-6 column.

Characterization of Glycan Substrates and Products—The various radioactive peaks from the gel filtration columns were pooled and lyophilized and desalted by additional gel filtration as previously described (8). The final lyophilized product was then subjected to various analyses to be described below.

RESULTS AND DISCUSSION

A typical time course of the incorporation of mannose from GDP-[^14]C[Man to the acceptor substrate Man6GlcNAC2-[R] is illustrated in Fig. 24. Three distinct product peaks are evident from the gel filtration on Bio-Gel P-6; of these only peaks II and III are products of the added acceptor substrate. Peaks I, IV, and V were observed both in the absence and presence of acceptor substrate; they consequently must be derived from an endogenous acceptor and/or from GDP-Man and have not been included in any of the subsequent analyses. The mass spectrometer results for peak III show that it consists of the three early products Man6GlcNAC2-[R, Man6GlcNAC2-[R, and Man6GlcNAC2-[R at a ratio of 4:5:1 (Fig. 2B). Peak II did not yield any product that could be detected by mass spectrometry. The chemical analysis of monosaccharide composition and estimates based on [14]C incorporation (Table I) suggested that the product on the average contained from 28 to 32 mannose residues and GlcNAc.

The abbreviation used is: R, (6-biotinamidohexanoyl)Asn.
FIG. 2. A, gel filtration patterns for the yeast-membrane-catalyzed reaction of Man$_6$GlcNAc$_2$-(6-biotinamidohexanoyl)Asn with GDP-$[^{14}C]$Man. Peak V, the only peak observed in zero time controls, is GDP-Man; peaks I and IV, and possibly also a product in the same position as peak V after long incubations, are unknown products formed in control experiments in which the Man$_6$GlcNAc$_2$-R substrate was omitted. B, fast atom bombardment-mass spectrum of the pooled peak III (18-h incubation). The calculated molecular ion (M + H$^+$) masses are M$_r$ 2013.6, M$_s$ 2175.8, M$_g$ 2337.

TABLE I
Characterization of fractions II and III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Compositional analysis*</th>
<th>$[^{14}C]$Man incorporation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>GlcNH$_2$ 56.3 Man 32/2 Man/GlcNAc 22/2</td>
<td>Total Man/GlcNAc 28/2</td>
</tr>
<tr>
<td>III</td>
<td>GlcNH$_2$ 66.0 Man 10/2 Man/GlcNAc 2.2/2</td>
<td>Total Man/GlcNAc 8.2/2</td>
</tr>
</tbody>
</table>

* Obtained by ion exchange chromatography of trifluoroacetic acid hydrolysates of the fractions (10).

*Calculated from the specific activity of $[^{14}C]$Man and the dpm per GlcNH$_2$ for the aliquots analyzed.

ion peaks by fast atom bombardment-mass spectroscopy with oligosaccharides containing up to 20 sugars (12), it appears that this represents a practical upper limit at this time, and especially in view of the likelihood that peak II contains a mixture of different molecular ions, the failure to detect the peak II product(s) in the mass spectrometer is not surprising.

The ion exchange chromatography analysis of the reaction mixtures in Fig. 2 shed some additional light on the course of the reaction. As shown in Fig. 3, different components can be discerned by the further resolution provided by the ion exchange system, and even in the absence of authentic reference compounds it is possible to make reasonable interpretations of the data. The time course suggests that each of the early intermediates containing 7, 8, and perhaps also 9 mannose residues may appear as two or more resolved isomers; some of these isomers (peaks 4, 6, and 8) increase and then decrease in the fashion of a typical reaction intermediate, while others (peaks 2, 3, 5, and 7) increase to a constant value in the way a reaction product accumulates. All the intermediates beyond the putative Man$_6$GlcNAc$_2$-R intermediates (peak 7) appear to be actively converted to the final product(s) corresponding to peak II in Fig. 2. The data in both Figs. 2 and 3 thus are consistent in showing that the starting acceptor substrate is converted to two sets of products, one low molecular weight set containing primarily Man$_6$GlcNAc$_2$-R and Man$_7$GlcNAc$_2$-R with some additional Man$_6$GlcNAc$_2$-R and a second set containing higher molecular weight polymannose structures of unknown size. The possibility that certain enzymes might be entrapped in the membranes was considered, and the above experiments were rerun in the presence of Triton X-100 (8). The results (not shown) revealed no significant detergent-induced changes in the yield of peaks II and III or in the composition of peak III as established by fast atom bombardment-mass spectroscopy and ion exchange chromatography. To establish unequivocally that the low molecular weight components are indeed inactive as acceptors in the reaction and at the same time explore whether the high $M_r$ fraction could be processed further, the components of peaks II and III in Fig. 2, collected separately from the P 6 column after 18 h incubation were reincubated with the enzyme preparation.
and GDP-Man under the same conditions as those used for the original incubation, and the products were monitored for a movement of the radioactivity of the starting materials toward the higher molecular weight range. The results are given in Fig. 4 and show clearly that the intermediates in peak III are inactive as substrates for the mannosyltransferases that produce the polymer products in peak II. Although a slight shift toward higher molecular weight is observed, this shift should only signify a lower proportion of Man₉ structures and a higher proportion of Man₈ structures in the peak III mixture. A similar shift toward the higher M₉ range was also observed in the case of peak II. Again, the most reasonable explanation is that some of the lower M₉ components are converted to higher M₉ component yielding what appears to be a more homogeneous mixture of slightly higher M₉ than the starting mixture in peak II. The results in Fig. 4 also show that mannosidases cannot be significant components of the system.

The components of peaks II and III in Fig. 2 were subjected to NMR spectroscopy in an attempt to establish their structure. No attempt was made to fractionate the high molecular weight components of peak II, and the spectrum in Fig. 5 can therefore only be interpreted in terms of a possible mixture of compounds. From the integration of the various peaks it can be concluded that the components of peak II have an average of 18 mannose residues linked 1→6 in an outer chain which contains no branches (the signal at 4.90 ppm). The compound must also contain one 1→2-linked mannose (5.04 ppm) attached to a 1→6-linked mannose (5.11 ppm) in addition to the 6 mannoses of the starting substrates, as indicated in Fig. 5. The total of 26 mannose residues per GlcNAc₂ unit is in reasonable agreement with the analytical data in Table I. Unfortunately, as the figure indicates, more than one possible structure is consistent with the data. The mixture of compounds in peak III gave a more complicated spectrum, reflecting the contributions of multiple components. In order to facilitate the interpretation of the NMR spectra, an attempt was made to fractionate the peak III components by a preparative gel filtration system. A large sample of acceptor substrate (4.5 μmol) was incubated with yeast membrane preparations and GDP-Man for 24 h, and the products were fractionated on a 0.8 × 114-cm column of Bio-Gel P-6 to yield three separate fractions, a (leading edge), b (central region), and c (trailing edge) from the single, broad peak III. Mass spectrometry showed that fraction c contained about 90% of Man₉ derivatives, fraction b virtually pure Man₈ derivatives, and fraction a a 40:60 mixture of Man₇ and Man₈ derivatives. Ion exchange chromatography of the various fractions was further applied to permit an assessment of the possible positional isomers that might be present (Fig. 6). The NMR spectra for the various fractions are given in Fig. 6, along with the possible structures involved. The Man₉ derivative (Fig. 6C) was the purest and consequently the easiest to characterize. The key issue is the location of the 1→2 substituent (position 10) on the 1→6 chain, and the basis for its assignment to the terminal mannose (position 9) is based entirely on the chemical shift of the unsubstituted 1→6 Man (position 8). As pointed out by Tsai et al. (13), the resonance of the mannose at position 8 at 4.90 ppm is characteristic of a mannose linked 1→6 to a 1→3-substituted mannose. If it was linked to a 1→3-substituted mannose, the resonance would be at 4.91–4.925 ppm (5, 6, 13). This lower field resonance is observed for the high molecular weight product and, in fact, provides the basis for the proposed structures for this derivative. It is interesting to note that the Man₉ precursor of this Man₉ structure must also be the precursor for structure.
Processing of a Man₆ Derivative by Yeast Enzymes

FIG. 5. Expanded ¹H NMR spectrum for the anomeric proton region for peak II. The integration of the resonance at 4.89-4.92 ppm gave a value of 18 relative to those at 4.76, 4.87, 5.12, and 5.34 ppm, all at about 1.0. (Only part of the peak at 4.90 ppm could be included in this representation.) The assignment of the resonances according to the literature (4-6, 12-14) is indicated in the figure. Since the resonance of DM has been found to be at 4.91 ppm (5, 6) instead of the 4.911 we have assigned to it in B and C, structure A is considered the most likely one.

In Fig. 5, if the 1→6-transferase acts on the Man₇ precursor, polymannose structures result; if the 1→2-transferase acts, it yields the deduced Man₆ structure as the final product. The assignment of the Man₇ (Fig. 6B) structure also appears to be unambiguous in spite of the fact that the Man₆ structure is the major component in the mixture. The new signal at 5.14 ppm is characteristic of the 1→3-linked mannose (position 11), and the associated slight upfield shift of the 1→3-linked mannose (position 7) suggests that this is the residue to which it is attached (position 7'). The resonance at 5.14 ppm was also observed for the Man₆ derivatives but without any effect on the other 1→3-linked mannose (position 7). Since the two 1→2-linked mannose residues (5 and 10) show an upfield shift along with a slight downfield shift of Man-4, the best interpretation of the spectrum in Fig. 6D is that it represents a mixture of two positional isomers (the ion exchange strongly suggests the presence of a major and a minor (trailing component) both having a new 1→3-linked mannose (position 11) attached to either of the two 1→2-linked residues in the Man₆ derivative (positions 5 or 10); based on the ratio of the 4 and 4' resonances the derivative with the new mannose linked to Man-5 is likely to be the major component.

What is the relevance of this structural information to the specificity of the early processing steps? As outlined in Fig. 1, it seems clear at this stage that at least four mannosyltransferases are needed to produce the various structures found in yeast glycoproteins. In addition, since the 2-linked Man-B is
absent in most, if not all known structures, the known mannosidase, specific for the release of that 1 residue (3), must also be involved.

The first 1→6-transferase (the “initiator” 1→6-transferase) catalyzes the introduction of 1 mannose residue; the enzyme has been purified (6), and the position of the “initiation” of the outer chain has been established (5). The next steps appear to be the integrated action of the second 1→6-transferase (the “elongation” 1→6-transferase) and a 1→2-transferase, the former elongating the 1→6-linked backbone, the latter inserting the 1→2 linked sidechains along the backbone. The action of these two enzymes must apparently be coordinated so that the 1→6 addition stays ahead of the 1→2 addition; if the terminal 1→6 residue receives a 1→2 substituent before the next 1→6 addition is made, the entire polymerization process stops (4, 5). The 1→3-transferase is assumed to catalyze the final steps of the process. The mutants that do not express this activity, make N-linked polymannose structures differing from normal ones only by the absence of nonreducing terminal 1→3 mannoses (14). It is as if the 1→3-transferase “caps” each completed chain of 1→2 oligomers, and terminates further reaction at that point. The removal of Man-B from the core structure cannot be assigned to any precise point in this sequence of events. The initial 1→6-transferase step proceeds whether or not the Man-B residue is present in the substrate (6), so the mannosidase is certainly not an obligatory first step in the reaction scheme.

Three of the above enzymes (the 1→6 elongation, the 1→2 and the 1→3 transferases) can definitely be accounted for in the conversion of the unnatural Manα substrate to the various products identified in this work. The specific mannosidase should not be involved, since its substrate is not present; in fact, based on several observations, in particular the recombination of the products in peaks II and III with Golgi membranes in Fig. 4, it is concluded that mannosidases do not play a significant role in the reactions with the unnatural substrate. Based on the absence of the characteristic resonance of the initiator mannose at 4.925 ppm (5, 6) in the products observed in this work, we also consider it unlikely that the initiator 1→6-transferase (structures B and C in Fig. 5) is involved with this substrate.

The reactions required to yield the Manα and Manβ derivatives (Fig. 6, C and D) are consistent with the recent conclusions by Gopal and Ballou (4) and Hernandez et al. (5). If the 1→2 addition takes place at a terminal 1→6-linked mannose, the product is inert for further processing by 1→6-transferases, but can be modified by 1→3-transferases. Of particular interest is the inert Manα and product (Fig. 6B). The introduction of the 1→3-linked mannose in this position is presumably a natural step in yeast glycan biosynthesis but, in the case of the unnatural substrate used here, it apparently renders the product inert to further processing. Perhaps this is consistent with the view that the introduction of the terminal 3-linked glycans in yeast takes place only after the processes catalyzed by the 1→6- and 1→2-transferases are completed.

The phenomenon of two enzymes competing for the same substrate in mutually exclusive fashion has been well established as a key regulating mechanism in glycan processing in animals (15, 16). Perhaps the best example is the competition between GlcNAc-transferase III and mannosidase II for the substrate GlcNAc-Manα-GlcNAc protein (16). If the transferase acts first to insert the bisecting GlcNAc residue, that product (GlcNAc-Manα-GlcNAc protein) is resistant to the action of mannosidase. If, on the other hand, the mannosidase acts first, that product (GlcNAc-Manα-GlcNAc protein) is no longer a substrate for transferase III. In this way the pathways leading to either hybrid (transferase fast) or complex (mannosidase fast) structures are determined by the relative activity of these two enzymes in a given tissue (16) and by the effect of the protein environment on each individual glycan substrate (17). It now appears that the same regulatory mechanism applies to yeast glycan processing with the relative activity of the 1→2, 1→3, and 1→6 (elongation) transferases on a given substrate determining whether a given N-linked glycan will become an oligomannose (1→2- and/or 1→3-transferase fast) or a polymannose (1→6-transferase fast) structure (4, 5).

With this picture we visualize the unnatural substrate used here as a poor substrate for the elongation 1→6-transferase and for the 1→3-transferase, and perhaps not a substrate at all for the initiation 1→6-transferase and the 1→2-transferase. Once a new mannose has been added 1→6 to the 1→6 arm, the 1→2-transferase becomes active and can compete with the 1→6-transferase to give either the precursors for further elongation or the Manα product documented above. Because of the slow initial step, the starting Manα as well as this Manα product can be modified further by the 1→3-transferase. Further experiments will be needed to document this model.

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