Yeast invertase polymorphism is correlated with variable states of oligosaccharide chain phosphorylation

(Saccharomyces cerevisiae/mannoprotein mutants/endomannanase/endoglucosaminidase)

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ABSTRACT Saccharomyces cerevisiae invertase (EC 3.2.1.26) isolated from wild-type strain X2180 can be resolved by isoelectric focusing into at least seven bands revealed by an activity stain. Most of this polymorphism is eliminated in mutants that are defective in phosphorylation of the mannoprotein carbohydrate chains (mnn4 and mnn6). In contrast to strain X2180, invertase from the mnn9 mutant, which makes mannoprotein lacking the outer portion of the polymannose chains, shows only two major bands on isoelectric focusing. Although mnn2 mannoprotein is thought not to have any branches in its outer chain, the invertase of this mutant shows at least six bands on isoelectric focusing, and digestion of this invertase with an endo-α1→6-mannanase that removes the unbranched outer chain produces an invertase with two bands that are similar to those from the mnn9 mutant. The invertase from mnn2 cells, grown with [32P]orthophosphate and precipitated with specific antiserum, gives at least five radioactive bands on isoelectric focusing, and after digestion with the endomannanase the radioactivity no longer migrates with the residual invertase. Mutants with shortened and unbranched outer chains (mnn2 mnn7, mnn2 mnn8, and mnn2 mnn10) give invertase patterns similar to mnn2. The results suggest that multiple states of outer chain phosphorylation lead to isoelectric polymorphism of S. cerevisiae external invertase and, because invertase has nine carbohydrate chains, no more than one phosphate group per chain would be required to account for this property.

Saccharomyces cerevisiae external invertase (EC 3.2.1.26) is a dimeric mannoprotein with a subunit polypeptide molecular weight of 60,000 (1). To each peptide chain are attached nine oligosaccharide chains (2), six of which have the same core of units whereas three have attached mannoplycosyl outer chains (3). The resultant glycoprotein chain has a molecular weight of about 120,000. The outer chain of S. cerevisiae X2180 mannoproteins is known to contain mannooligosaccharide groups (4), but the core oligosaccharide of this extracellular mannoprotein does not appear to be phosphorylated even though the core of the intracellular mannoprotein carboxypeptidase Y is (5).

We now report that external invertase preparations that give single bands on sodium dodecyl sulfate/polyacrylamide gel electrophoresis show extreme polymorphism by isoelectric focusing. Our study reveals that this phenomenon is due to multiple states of phosphorylation of the polymannose outer chains. Even after eliminating this cause of polymorphism, however, we find that the invertase still shows two differently charged forms. The basis of the latter heterogeneity is unknown.

MATERIALS AND METHODS

Materials. S. cerevisiae X2180 was from the Yeast Genetic Stock Center (University of California, Berkeley), and the various mnn mutants were isolated in this laboratory (6–8). Endoglucosaminidase H was isolated according to Tarentino et al. (9), endo-α1→6-mannanase (10) was prepared by R. Cohen in this laboratory, Zymolase was obtained from Seikagaku (Tokyo, Japan), and alkaline phosphatase was from Sigma. Lyticase (11) and anti-invertase serum were provided by R. Schechman of this department. IgGor (fixed Staphylococcus aureus Cowan I cells) was from the Enzyme Center (Boston, MA), Bio-Lyte ampholyte solutions were from Bio-Rad, H32PO4 was from ICN, H235SO4 was from Amersham, and EN3HANCE was from New England Nuclear.

Methods. Except for the radioactive labeling experiment, protoplast digestes were prepared according to Schwenke and Nagy (12). After mercaptoethanol treatment, cells were incubated with Zymolase (1.5 mg/ml) for 1 hr, the digest was centrifuged at 2,000 × g for 10 min, and the supernatant solution was dialyzed for 20 hr against distilled water and then concentrated 10-fold by lyophilization. Cultures of mnn2, mnn2 mnn9, and wild-type cells were grown on Wickerham’s minimal medium (13) to mid-logarithmic phase. A suitable portion of each culture was centrifuged and resuspended in 2 ml of a minimal medium, in which sulfate and phosphate salts were replaced by chloride salts, such that OD600 = 2. After growth for 4 hr at 30°C, the cultures were centrifuged and the cells were suspended in 2 ml of a medium for induction and labeling of invertase that contained 50 mM ammonium sulfate, 0.1% D-glucose, 0.1 mM potassium phosphate, 3 mM H32PO4, and 1 mM H235SO4 (1 Ci = 3.7 × 1010 becquerels). During a 1-hr incubation, invertase was induced, after which the cells were lysed by addition of 50 units of lyticase in 200 μl of 1.4 M sorbitol that was 5 mM in Na2SO4, 2.5 mM in MgCl2, and 40 mM in mercaptoethanol. After incubation for 30 min at 30°C, the protoplasts and insoluble material were removed by centrifugation, and 15 μl of anti-invertase serum was added. After a 2-hr incubation, 100 μl of Staph. aureus cells in 0.5% Triton X-100 was added, and 30 min later the cells were washed three times with 0.1 M Tris-HCl buffer, pH 7.5, containing 2 M urea, 0.2 M NaCl, and 0.1% Triton X-100, and three times with 1% mercaptoethanol. The immune complex bound to the cells was solubilized by boiling them in 75 μl of 1% mercaptoethanol. For electrophoresis and enzyme digestions, 10-μl samples were used.

Invertase activity was determined according to Goldstein and Lampen (14). To remove the mannoprotein outer chain, radioactive invertases were incubated for 12 hr at 45°C with 0.02 unit of endomannanase in 5 mM sodium citrate buffer, pH 5.5, containing 10 μM CaCl2 (15). Alkaline phosphatase digestions were done with 0.06 unit in 1 M Tris-HCl buffer, pH 9.0, for 12 hr at 35°C.

Isoelectric focusing was carried out on 0.2-unit samples of invertase in 2 μl of dialyzed and concentrated supernatant extract applied to a polyacrylamide gel containing 1.4% Bio-Lyte.
RESULTS

Isoelectric Focusing Patterns of External Invertases Show Multiple Bands. External invertase is a major inducible glycoprotein of the periplasm that can be solubilized by digestion of the yeast cells with Zymolyase. Fig. 1 shows the gel electrophoresis patterns of native invertases obtained from different mutants that possess truncated oligosaccharide chains (7). The fastest-migrating invertase was from the mnn2 mnn9 mutant, the mannoproteins of which are known to lack all of the outer chain. Although the enzymes exhibited a single band on electrophoresis, they showed a high degree of heterogeneity when analyzed by isoelectric focusing in a pH 3–5 gradient. Invertases from the mnn2, mnn2 mnn7, mnn2 mnn8, mnn2 mnn10, and wild-type strains gave similar patterns with at least seven different forms (Fig. 2, lanes 1 and 2). In contrast, the mnn2 mnn9 invertase shows only two major bands by the activity stain (Fig. 2, lane 3).

![Fig. 1. Mutant invertases show altered migration rates on gel electrophoresis. Cells induced for invertase secretion were treated with Zymolyase to form protoplasts and samples of each supernatant with equivalent amounts of invertase activity were electrophoresed on a nondenaturing gel. The invertase bands were detected with an activity stain. The mnn9 invertase, with the lowest carbohydrate content, shows the highest rate of migration. The mnn7–mnn10 strains contain also the mnn2 defect.](image)

![Fig. 2. Yeast invertases show polymorphism by isoelectric focusing. The invertase extracts were subjected to isoelectric focusing and then the isoenzymes were detected by an activity stain. Lanes 1–3 are untreated invertases; lane 1 is X2180 wild type, lane 2 is mnn2, and lane 3 is mnn2 mnn9. Lanes 4 and 5 are invertases treated with endomannanase; lane 4 is strain mnn2 and lane 5 is X2180. Lanes 6–8 are invertases treated with endoglucosaminidase; lane 6 is mnn2, lane 7 is X2180, and lane 8 is mnn2 mnn9. Endoglucosaminidase H digestion reduces the polymorphism of all three invertases, whereas endomannanase digestion affects only the mnn2 invertase.](image)

Mutations That Prevent Phosphorylation Reduce Invertase Polymorphism. One explanation for the heterogeneity observed on isoelectric focusing could be that the chains possess a variable degree of phosphorylation. To test this hypothesis, mutants were analyzed that lack the enzyme activity responsible for phosphorylation of the mannoprotein outer chain (18). Isoelectric focusing patterns for invertases from strains possessing the mnn4 mutation are shown in Fig. 3, and it is apparent that the more acidic invertase bands have largely disappeared. It is notable, however, that the mnn2 mnn9 invertase is not affected by these mutations and that after introduction of the mnn4 or mnn6 mutation all other strains show similar patterns to this invertase.

 Isoenzymes of External Invertase Can Be Labeled with $^{32}$P. Support for this hypothesis was obtained by labeling yeast cells with $^{32}$P and $^{35}$S and then comparing the immunoprecipitated invertases from mnn2, mnn2 mnn9, and wild-type strains. The autoradiogram of the sodium dodecyl sulfate gel electrophogram is shown in Fig. 4. Another portion of each invertase was subjected to isoelectric focusing and, after autoradiography, the radioactive bands were cut out and the ratio of phosphate to sulfate incorporation was determined in each. The results in Fig. 5 demonstrate that the more acidic bands exhibit a higher ratio of $^{32}$P to $^{35}$S, corresponding to a higher degree of phosphorylation of the carbohydrate chains.

 Enzymatic Removal of Carbohydrate Chains Reduces Heterogeneity of Invertase. The outer chain portion of the asparagine-linked oligosaccharide chains in the mnn2 mutant mannoproteins can be removed by endo-α1→6-mannanase digestion,
but the enzyme does not act on wild-type or mnn9 mannoproteins. Table 1 summarizes the results of endomannanase digestion, and it is seen that there is a large decrease in the $^{32}$P-to-$^{35}$S ratio for the mnn2 invertase. Incubation of the invertases with alkaline phosphatase had no effect, which suggests that the phosphate groups are diesterified, as they are known to be in other yeast mannoproteins (8). Endomannanase treatment of the mnn2 invertase also reduced its isoelectric focusing heterogeneity (Fig. 2, lane 4), although the presence of a third band indicates that there was not a complete removal of the phosphorylated sites. As expected, the pattern of the wild-type enzyme was not altered by this treatment.

Endoglucosaminidase H will cleave about 85% of the carbohydrate chains from nondenatured wild-type invertase (2) and, after treatment with this enzyme, all of the invertases show the two bands on isoelectric focusing that are characteristic of the mnn2 mnn9 invertase (Fig. 2, lanes 6–8). Because there was very little radioactive phosphate incorporated into the mnn2 mnn9 invertase, the more acidic of these two bands does not appear to be generated by phosphorylation of the remaining core oligosaccharide chains.

**DISCUSSION**

The carbohydrate chains of *S. cerevisiae* invertase are linked to asparagine through di-N-acetylchitobiose units in a manner typical of eukaryotic glycoproteins (15, 19). Yeasts differ from higher eukaryotes, however, in that the core oligosaccharide, derived from a dolichol-linked precursor, is enlarged after transfer to protein by addition of an outer polymannose chain. Both the core and outer chain are highly branched, but they are distinguished by the types of linkages between mannose units and by the presence or absence of covalently linked phosphate. Previous studies show that the core of extracellular mannoproteins is not phosphorylated but the outer chain is (8), whereas intracellular mannoproteins lack an outer chain but have a phosphorylated core (5).

The present study demonstrates that invertase from *S. cerevisiae* X2180, a wild-type strain, is highly polymorphic as revealed by isoelectric focusing and that this polymorphism is a result of multiple states of phosphorylation of the outer chain. This conclusion was documented in several ways. First, mutations that are known to alter phosphorylation of the outer chain reduced the multiplicity of bands by eliminating the more acidic ones. Second, invertase labeled with radioactive phosphate had the highest density of label in the more acidic bands. Third, the polymorphism and radioactivity of labeled mnn2 invertase were reduced by endomannanase digestion, which removed the unbranched outer chain of this mannoprotein, whereas endoglucosaminidase digestion reduced the heterogeneity of both the mnn2 and wild-type strains.

The observation that mnn2 invertase is polymorphic and contains phosphate was unexpected because it is known that this mutation makes mannan without the side chains to which phosphate is linked (6). There is evidence, however, that this mutant may be slightly leaky. Raschke et al. (6) observed from methylation studies that mnn2 mannoprotein still contained a small amount of 1→2-linked mannone and Nakajima and Ballou (20) reported that cell extracts still possessed a measurable amount of α1→2-mannosyltransferase activity, although at the time it
Fig. 5. Comparison of $^{32}$P and $^{35}$S in invertase isomers separated by isoelectric focusing. The radiolabeled invertases were precipitated with specific antiserum and separated by isoelectric focusing, and an autoradiogram of the gel was made to detect the bands, which were cut out and assayed for $^{32}$P (hatched columns) and $^{35}$S (open columns). The number in each column gives the ratio of $^{32}$P to $^{35}$S in that band, and the heights of the $^{35}$S columns indicate the amount of invertase in each band. The acidity of the bands increases with the slice number.

It was concluded that this activity might be involved in synthesis of the core rather than the outer chain. Subsequently, it has been shown that membranes prepared from the mnn2 mutant make endogenous mannan with properties of the wild-type polymer (21) and that the same membranes catalyze formation of products with exogenous acceptors that are similar to those obtained with membranes from wild-type strains (22). Thus, it appears that the S. cerevisiae mnn2 mutant, like the mnn2-2 mutant of Kluyveromyces lactis (23), has a defect that prevents expression of an enzyme activity in the intact cell even though the glycosyltransferase activity is demonstrable in cell extracts. Our present conclusion is that S. cerevisiae mnn2 mannosylprotein has a few side chains on the otherwise unbranched outer chain that serve as acceptor sites for addition of mannosylphosphate units, and this leads to the multiplicity of bands we find on isoelectric focusing.

Several S. cerevisiae mannosylprotein mutants have shortened outer chains (mnn7, mnn8, and mnn10) or lack all of the outer chain (mnn9) (7). The first three mutations, superimposed on the mnn2 background, give strains with multiple invertase bands on isoelectric focusing and, like the mnn2 strain, these mutants show less heterogeneity when the mnn4 lesion is introduced. The mnn9 mutant is of interest because it shows only two bands regardless of the presence or absence of other mutations, including mnn4. If the heterogeneity seen in mnn9 mannosylproteins is related to oligosaccharidase phosphorylation, the unit must be inaccessible to phosphomonoesterase or endoglucosaminidase attack because these treatments have no effect, and its presence must not be regulated by the mnn4 or mnn6 focus. The isoelectric focusing pattern given by internal invertase also has two bands (data not shown), one of which migrates with one of the mnn9 invertase bands, even though these two preparations differ dramatically when electrophoresed on polyacrylamide gels because the latter invertase is not glycosylated. Although invertase molecules that differ in the number of carbohydrate chains can be separated by gel electrophoresis (3), the heterogeneity we are dealing with here is observed only on isoelectric focusing. The structural basis of this heterogeneity is unknown.

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Table 1. Ratio of $^{32}$P to $^{35}$S in labeled and treated invertases

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Ratio of $^{32}$P to $^{35}$S</th>
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<tbody>
<tr>
<td>mnn2 mnn9</td>
<td>0.03</td>
</tr>
<tr>
<td>mnn2</td>
<td>0.28</td>
</tr>
<tr>
<td>X2180 wild type</td>
<td>0.42</td>
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</tbody>
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* Because so little $^{32}$P was incorporated, these analyses were not done.