# Isolation and Characterization of a Phosphomannose Isomerase Mutant in Yeast

by

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ABSTRACT

A mutant of Saccharomyces cerevisiae lacking 92 percent of its phosphomannose isomerase (E.C.5.3.1.8.) activity was isolated after mutation with E.M.S.. The mutant was found to have the same auxotrophic requirements as the parent strain A364a. Thermostability studies indicated the mutation was in the structural genome and *in vivo* incorporation studies with $C^{14}$(C-1)mannose indicated a similar rate of decarboxylation as the parent strain.

This paper follows *The Journal of Biological Chemistry* in its style.

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# Isolation and Characterization of a Phosphomannose Isomerase Mutant in Yeast

Phosphomannose Isomerase (E.C.5.3.1.8.) deficient mutants of Saccharomyces cerevisiae have been isolated and characterized for use in studies of hexose metabolism (Alvarez, Matria and Lobo). Phosphomannose isomerase catalyzes the conversion of mannose-6-phosphate to fructose-6-phosphate with a $K_m$ of 1.35 mM and a specific activity of 800 moles substrate converted/min/mg protein at 30°C. The enzyme has a molecular weight of 45,000 $\pm$ 1000 in Saccharomyces (Noltman and Gracy I,III).

Mannose is used as a major component of yeast glycoproteins. It enters glycolysis through a pathway separate and distinct from all other six-carbon sugars. If a mutant of Saccharomyces is obtained with little of no residual activity in phosphomannose isomerase, a sensitive assay for glycoprotein synthesis and turnover can be easily developed. Alternative methods of labeling, either by supplying the cells with labeled dolichol-mannose or with labeled GDP-mannose are neither as sensitive nor prevent incorporation of the label into other compounds once turnover occurs. This paper describes isolation and characterization of a mutant in Saccharomyces cerevisiae, which is defective in a phosphomannose isomerase.

## Experimental Procedure

### Materials

A364a (a,adel,ade2,ural,his7,lys2,argl,gal) was used as the parent strain in the mutating procedure. Yeast extract casamino acid media (YCG) consisted of (per liter) yeast extract, 10g; casamino acids, 5g; and glucose, 10g. Yeast nitrogen base (Difco) without amino acids or ammonium sulfate was used as the defined media with 1% glucose (or mannose)

and growth factors as required (adenine, 20 g; uracil, 20mg; histidine, 10mg; lys, 20mg; arg, 20 mg). Solid media was prepared by adding 10g/l Difco "Noble Agar." In critical mutating steps and differential plating mannose and glucose were filter sterilized and added to the autoclaved media to avoid isomerization of the sugars. $C^{14}$ (C-1) Mannose (Amersham) at .19 ci/$\lambda$ was used in the radioactive assay procedure. Glucose-6-phosphate dehydrogenase (Sigma), mannose-6-phosphate (Sigma) and $NADP^+$ (Sigma) were used in enzymatic analysis of the phosphomannose isomerase (PMI) mutant.

## Methods

A364a was mutated according to a modification of Lindegren et. al., with 5% EMS. Mutated cells were outgrown and enriched 2-3 logs with Nystatin (Methods in Yeast Genetics). The cells were then plated on YNB glucose supplemented with casamino acids and colonies were replicated onto similar media with mannose as a carbon source. Colonies were selected for no growth on mannose, tested further on solid media and finally by enzyme analysis.

For enzyme analysis cells were prepared by harvesting log phase cells, grown in YCG broth, through centrifugation in a Sorvall centrifuge. Pellets were resuspended in a phosphate buffer (pH=7.15) and cells broken in a French press at 15-17,000 lbs/$in^2$. Cellular lysates were centrifuged at 9,000 rpm in a Sorvall SS-34 fixed angle rotor.

Phosphomannose isomerase was assayed spectrophotometrically at 340 nm, as described by Noltman and Gracy, scaled down to give a total volume of 1 ml and substituting a phosphate buffer in the assay mixture.

In vivo determinations of the breakdown of mannose was carried out by a modification of a procedure described in The Genera of Bacteria for determination of $CO_2$ production. Log phase cells were harvested and put into 5 ml YNB broth with growth factors at a cell concentration of 1-2 x $10^6$ cells/ml. Mannose and glucose were added to a concentration of 1mM

as a carbon source and 10λ $C^{14}$ (C-1) Mannose (.19 ci/λ) was added to start the assay. Air, passed through an Ascarite $CO_2$ trap, was bubbled through the broth and displaced air/$CO_2$ was bubbled through 5 ml .25N NaOH. After 30 minutes, trapped $CO_3^{2-}$ was precipitated by addition of 5 ml .25N $BaCl_2$ to form $BaCO_3$. $BaCO_3$ and cells were centrifuged, washed, and resuspended in 1 ml $BaCl_2$ or media respectively. 50λ samples were then added to 10 ml scintillation cocktail and assayed in a Beckman Liquid Scintillation Spectrometer.

## Results

Of the mutants isolated for no growth on mannose, 137c had the smallest specific activity (2μg/min/mg protein) and contained about 8% the residual activity of the parent strain (Table I). Genetic analysis of the auxotrophic requirements of the mutant 137c indicated no new growth requirements (except for the inability to grow on mannose).

The effect of mannose on the generation time of the mutant can be observed in Table II. As can be seen, mannose concentrations tested did not affect generation time. Composition ratios in this study only went to 1 while Herra et. al. found increasing inhibition of growth at higher mannose to glucose ratios, obtaining 50% inhibition at a ratio of approximately 2. Growing 137c in 1% mannose resulted in no appreciable growth.

Testing the thermostability of the mutant enzyme showed (figure 1) that the mutation was in the structural gene coding for PMI rather than in a regulator for the gene.

Finally a radioactive incorporation experiment was conducted to determine if the residual activity in the mutant could result in an appreciable in vivo degradation of mannose. Table III shows the incorporation of radioactive mannose and the production of $CO_2$ from that mannose.

## Discussion

The results of the mutant characterization showed the mutant 137c was 92% inactive yet had an in vivo metabolism of mannose

| | Specific Activity | % Specific Activity |
|---|---|---|
| 137a | 3.7 ug/min/mg pro. | 14.4 |
| 137c | 2 ug/min/mg pro. | 7.8 |
| 135 | 18 ug/min/mg pro. | 70 |
| A364a | 25.7 ug/min/mg pro | 100 |

Table I: Specific Activity of Phosphomannose Isomerase in Mutant and Parent Strains

| | 137c | A364a |
|---|---|---|
| cpm/$10^6$ cells (mannose) | 1113 | 2392 |
| cpm/$10^6$ cells ($CO_2$) | 80 | 112 |
| percent incorporation into $CO_2$ | 7.18% | 4.68% |

Table III: Incorporation of Radioactive Mannose into Mutant and Parent Strains

Table II: GROWTH RATES OF 137c IN VARIOUS CONCENTRATIONS OF MANNOSE
( Glucose Held Constant at 1% )

| | 0% mannose | 0.05% | 0.3% | 0.6% | 0.8% | 1.0% |
|---|---|---|---|---|---|---|
| A364a | 4.5 hr. | -----* | ------ | ------ | -------- | ------- |
| 137c+ | 4.8 hr. | 4.6 hr. | 3.6 hr. | 4.3 hr. | 3.8 hr. | 4.6 hr. |

*A364a had similar generation times in different concentrations of mannose.

+137c did not grow in mannose without glucose as an alternate source of carbon

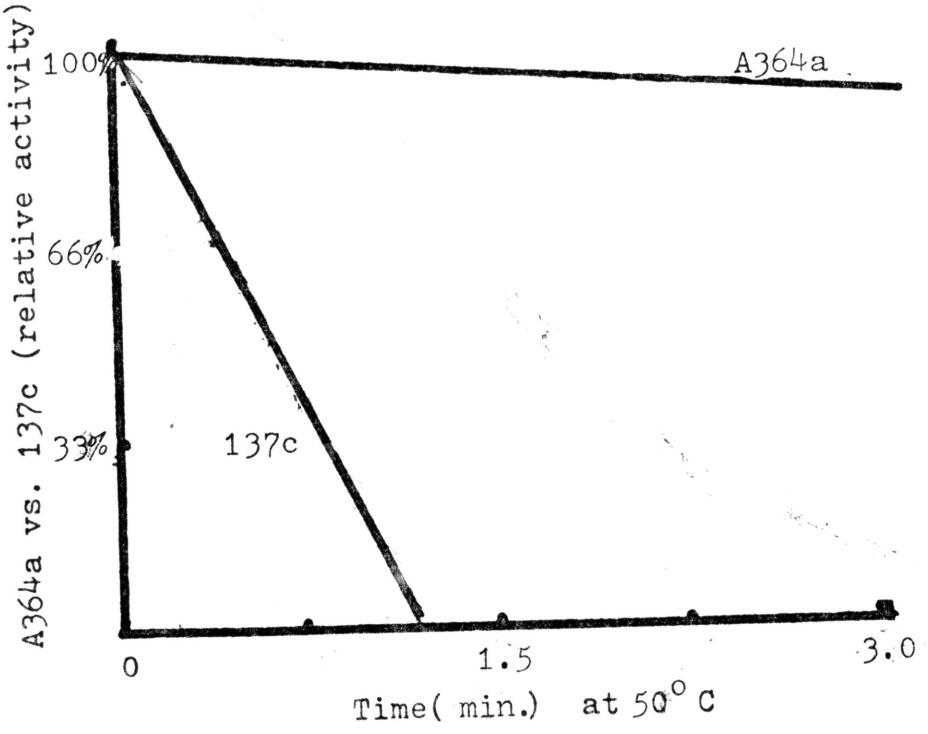


Figure 1: Thermal Stability of Mutant and Parent Enzyme

similar to the parent strain. Since the mutation is in the structural genome rather than the regulator I can take this mutant and re-mutate to isolate a tighter mutation. It is interesting to note previous investigators (Lobo, Herra) were unable to isolate a mutant without residual activity. Lobo suggested, in a paper on a phosphoglucose isomerase mutant inhibition of cell growth was caused through build up of glucose-6-phosphate acting on RNA synthesis. If mannose-6-phosphate acted in a similar manner phosphorylation of mannose in the media could prevent cells completely lacking the enzyme from growing into visible colonies.

Once a tight mutation is obtained and the cell cannot make its own mannose, mannose should be required for glycoprotein synthesis. The problem then becomes one of inhibition of cell growth by over-phosphorylation of mannose versus inhibition through under-mannosylation of glycoproteins.

The growth problem can be overcome by finding a mannose nonrepressor and using this mutant to obtain a PMI mutant or growing the cells out on trace amounts of mannose, with some other sugar as a carbon source. While I have isolated a pyruvate kinase deficient mutant that grows off acetate and succinate in the presence of mannose, and thus seems to be a mannose nonrepressor, I feel re-mutating and isolation of 137c for a further mutation in phosphomannose isomerase is the better choice. A second-step mutation obtained by growing cells out on galactose, a sugar without mannose contaminants, has been carried out resulting in a phenotypic "super-sensitive" yeast. Further enzymatic analysis will indicate if this was the proper procedure to isolate a mutant with no residual activity in phosphomannose isomerase.

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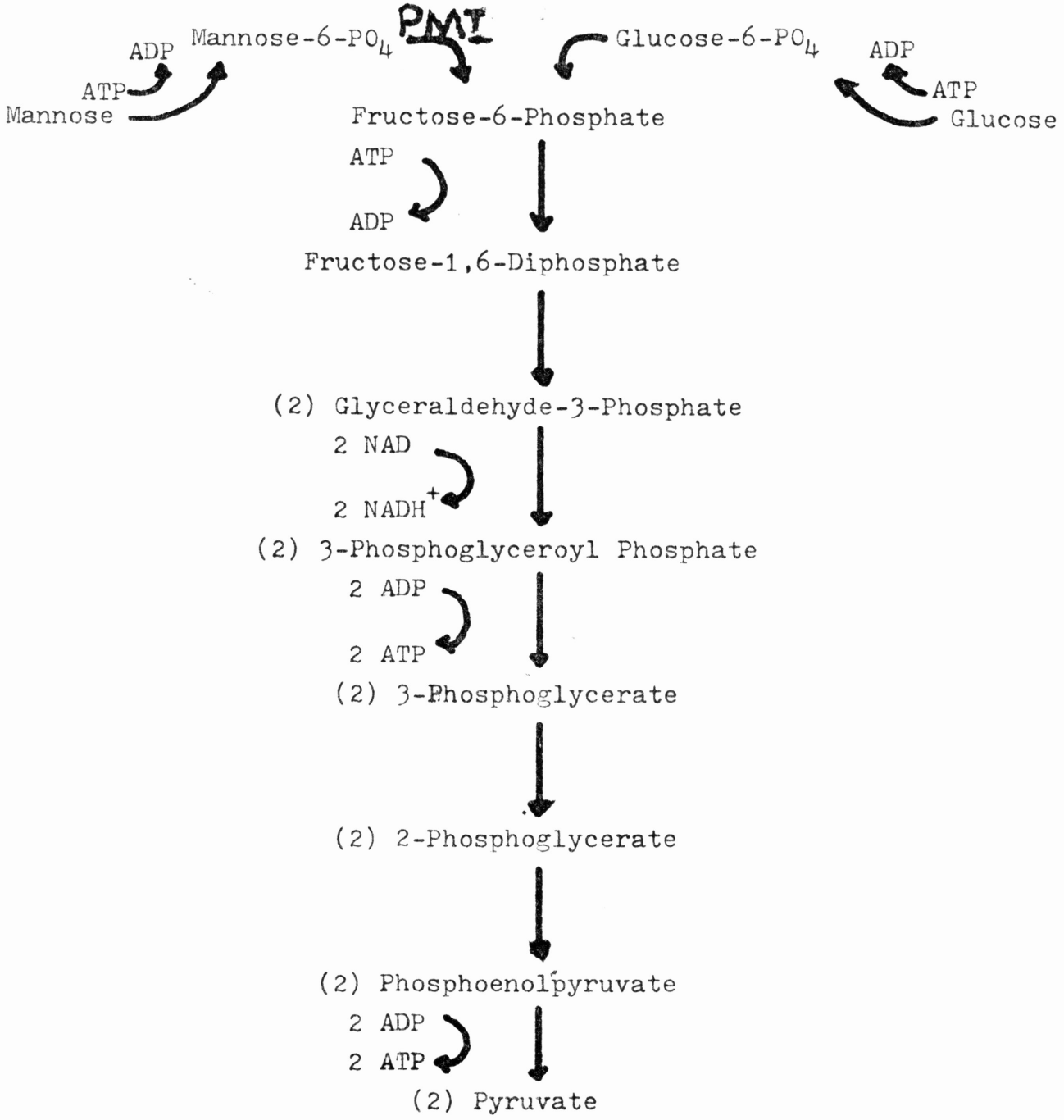
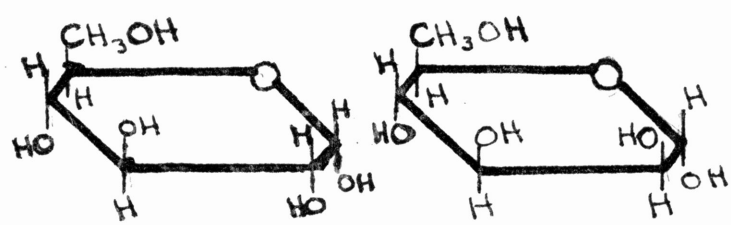
# APPENDIX I

## Mannose Incorporation Pathways in Yeast

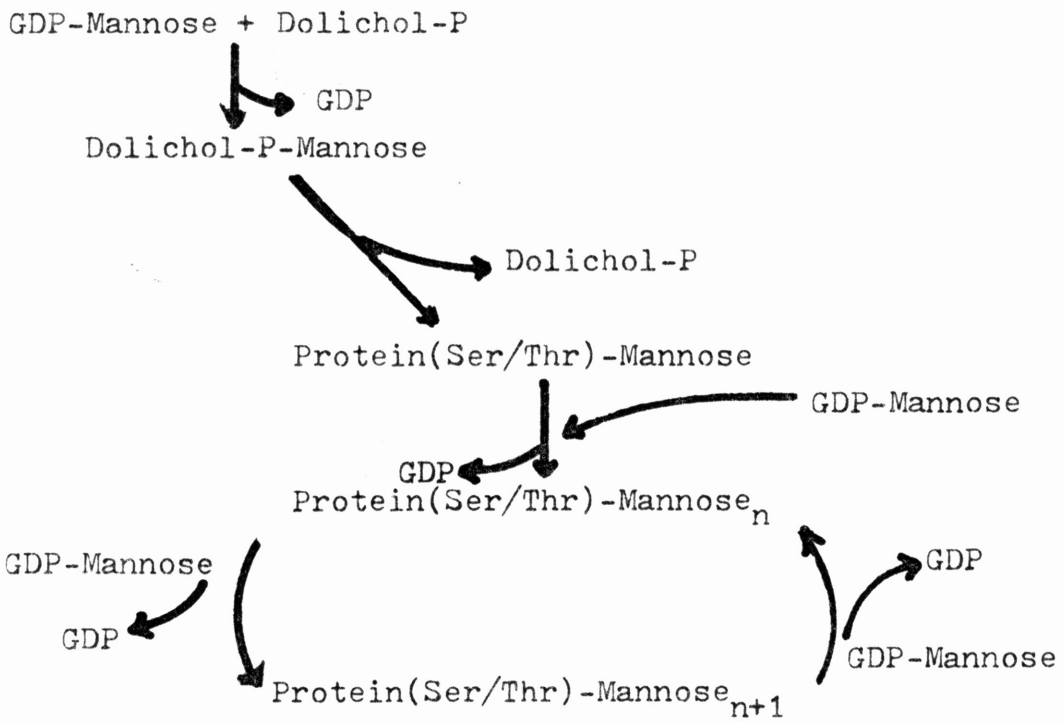
# METABOLIC PATHWAY OF MANNOSE IN YEAST



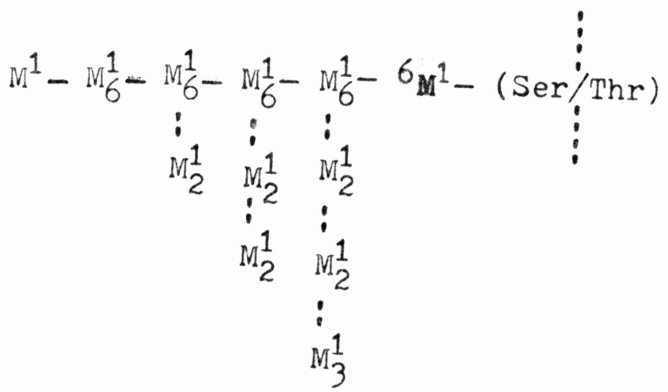




GLUCOSE MANNOSE

INCORPORATION OF MANNOSE INTO YEAST GLYCOPROTEIN




Mannosylation of the Glycoprotein Backbone in Saccharomyces




Mannosylated Glycoprotein in Yeast