The crystal structure of the yeast galactokinase, Gal1p, in the presence of its substrates has been solved recently. We systematically mutated each of the amino acid side chains that, from the structure, are implicated to be involved in direct contact with the hydroxyl groups of the galactose ring. One of these mutations, D62A, abolished all detectable galactokinase activity but retained the ability to use D-glucose as a substrate. Mutation of Asp-62 to either leucine, phenylalanine, or histidine resulted in the formation of protein with similar characteristics to D62A. Yeast galactokinase is highly similar to Gal3p, the ligand sensor and transcriptional inducer of the GAL genes. Equivalent mutations in Gal3p also abolished its ability to respond to galactose and uncovered its ability to respond to D-glucose. It therefore appears that Gal1p and Gal3p respond to their substrates in a similar, perhaps identical, fashion. This work also validates the approach of screening for mutants in an easily assayable system prior to mutant analysis in a more experimentally difficult transcriptional regulator.

The yeast Saccharomyces cerevisiae contains within its genome two galactokinase-like genes. The first of these, GAL1, encodes Gal1p, a bona fide galactokinase whose major role in the cell is to convert α-D-galactose into galactose 1-phosphate at the expense of ATP as part of the Leloir pathway, which is responsible for the conversion of β-D-galactose to the more metabolically useful glucose 6-phosphate (1). The enzyme adopts a bilobal appearance with the active site being wedged between distinct amino- and carboxyl-terminal domains. The sugar is held in the active site by a network of potential hydrogen bond interactions (Fig. 1). This network, composed of residues Arg-53, Glu-59, His-60, Asp-62, Asp-217, and Tyr-274, is capable of contacting each of the five hydroxyl groups of the sugar ring. To explore the requirement of this hydrogen bond network for interaction with the sugar, we undertook a mutational analysis of the amino acid side chains that form part of this network. We identified that Asp-62 is important for the recognition of galactose by the enzyme. Mutation of this residue resulted in the formation of proteins that had lost the ability to phosphorylate galactose but retained the ability to phosphorlyate D-glucose. In addition, the equivalent residue in a highly related protein, the ligand sensor of the yeast GAL genetic switch, Gal3p, displayed a similar phenotype.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—MC2 cells (MATa, ura3-52, leu2-3, prc1-407, prb1-112, pep4-3) (3) were used for protein expression and purification. β-Galactosidase assays were performed in JPY5 cells (MATa ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385) (6). Disruption of GAL3 and GAL80 from JPY5 was achieved by using PCR-generated blaster cassettes (7).

**Protein Expression and Purification**—Wild type Gal1p was produced in and purified from MC2 yeast cells. MC2 cells were transformed with pYEX-BX (Clontech) containing the GAL1 gene pAP60 (3) and grown in 1.5 liters of Leu- selective medium at 30 °C to an A600 of 1.0. The cells were then induced with 0.5 mM CuSO4 for 24 h. The induced cells were pelleted by centrifugation at 1,800 × g for 5 min, resuspended in Buffer g for 5 min, and centrifuged at 18,000 × g for 5 min, resuspended in Buffer A (20 mM HEPES-KOH (pH 7.8), 300 mM NaCl, 10% (v/v) glycerol), frozen in liquid nitrogen, and lyzed by grinding in a mortar with a pestle (8). The cells were thawed at room temperature and centrifuged at 75,000 × g for 20 min. The supernatant was then loaded onto a 2-ml Ni2+−nitrilotriacetic acid-agarose column (ProBond resin; Invitrogen) pre-washed with Buffer A. The column was then washed with Buffer A and Buffer A containing 500 mM NaCl, 10 mM β-mercaptoethanol, 30 mM imidazole. Protein was eluted from the column with Buffer A containing 250 mM imidazole. The protein was then dialyzed into several changes of Buffer B (20 mM HEPES-KOH (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1.4 mM β-mercaptoethanol, 10% (v/v) glycerol) at 4 °C overnight.

**Mutagenesis**—Mutations were introduced into pAP60 (3) using the QuikChange method (9). Oligonucleotide sequences are available on request. The GAL1 open reading frame of each mutated plasmid was sequenced to confirm the presence of the mutation and that no other
mutations had been introduced during the PCR. All mutant proteins were expressed and purified using the same method as described for the wild type protein above.

Galactokinase Kinetics—Galactokinase activity was measured by using an enzyme-linked assay system (4, 10). Briefly, reaction mixtures (150 μl) were set up in microtiter wells containing 20 mM HEPES-KOH (pH 8.0), 150 mM NaCl, 5 mM MgCl₂, 400 μM phosphoenolpyruvate, 1 mM dithiothreitol, 1 mM NADH, 1.1 units of pyruvate kinase, 1.5 units of lactic dehydrogenase (Sigma). Reactions were supplemented with Gal1p or mutant Gal1p and various concentrations of galactose and ATP. The plates were incubated at 30 °C, and the decrease in absorbance at 340 nm was measured by using a Multiskan Ascent plate reader.

β-Galactosidase Assays—JYP5:ΔGAL3 or JYP5:ΔGAL3::ΔGAL80 yeast cells containing either the integrated reporter construct pJP184 or pJP185 were transformed with YEplac112His (11) or a version of the same plasmid containing the GAL3 gene expressed from its native promoter pAP25 (3). The cells were grown in 7 ml of the appropriate yeast medium containing 2% galactose and 1% maltose until an A₆₀₀ of 0.8–1.0. The cells were pelleted by centrifugation at 4,000 × g for 5 min and resuspended in 200 μl of Buffer C (100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 20% (v/v) glycerol). An equal volume of glass beads (0.25–0.5 mm) was added, and the cells were lysed by vortexing (6 times for 30 s). A further 250 μl of Buffer C was added and the supernatant removed. The supernatant was clarified by centrifugation at 20,000 × g for 15 min. β-Galactosidase levels in the extract were measured with the Galacto-Light Plus chemiluminescent reporter gene assay system (PE Biosystems). In a luminometer tube, 20 μl of extract was mixed with 100 μl of Reaction Buffer (PE Biosystems) and incubated at 30 °C for 30 min. 70 μl of accelerator (PE Biosystems) was then added and vortexed briefly, and after a 1–2-s delay, the sample was read in a luminometer (Turner Designs). The β-galactosidase units were normalized by dividing the number of units by the protein concentration, which was calculated using 10 μl of extract in a Bradford assay (12).

RESULTS

Mutation of Amino Acids in the Galactose-binding Site—In order to characterize the binding of galactose by Gal1p, the amino acids that were identified in the crystal structure as being part of the galactose-binding site were mutated to alanine (see Fig. 1). This enzyme has been reported as having a high degree of specificity for galactose (13); hence the mutations were also tested for their ability to phosphorylate other sugars to determine whether the mutations would relax the specificity of the sugar binding. The residues mutated were Arg-53, Glu-59, Asp-62, L-arabinose, 2-deoxy-d-galactose, d-fucose, d-glucose, l-glucose, and d-talose. As expected, Gal1p was able to phosphorylate d-galactose, and the kinetic constants for each sugar are indicated. All reactions were carried out at 30 °C as described under “Experimental Procedures.” ND indicates not detectable.

TABLE 1

| Sugar         | kcat/s⁻¹ | Km mM | ATP | kcat/Km s⁻¹ | ATP |
|---------------|----------|-------|-----|-------------|-----|
| d-Galactose   | 22.3 ± 1.4 | 0.539 ± 0.119 | 0.057 ± 0.002 | 41.34 ± 11.72 | 391 ± 38 |
| 2-Deoxy-d-galactose | 4.01 ± 0.34 | 29.21 ± 9.27 | 0.046 ± 0.002 | 0.137 ± 0.055 | 87.17 ± 11.18 |
| d-Glucose     | 0.034 ± 0.002 | 0.225 ± 0.101 | 0.389 ± 0.046 | 0.149 ± 0.076 | 0.086 ± 0.015 |
| l-Arabinose   | ND       | ND    | ND  | ND           | ND  |
| d-Fucose      | ND       | ND    | ND  | ND           | ND  |
| l-Glucose     | ND       | ND    | ND  | ND           | ND  |
| d-Talose      | ND       | ND    | ND  | ND           | ND  |

We attempted to express and purify the mutant proteins from yeast. Only Gal1(D62A) of the single mutations was able to be purified, and the protein levels obtained were comparable with the wild type protein (data not shown). Both the double mutants also produced soluble protein at similar levels to the wild type protein. Interestingly, both the double mutants were purified, however, in all but one of the cases (Asp-62), each of the mutations on their own resulted in no protein purification. It is possible that the mutations by themselves disrupt the structure of the enzyme, whereas the addition of two alanine residues allows sufficient flexibility in the protein chain for the protein to fold appropriately.

The substrate specificity of the three purified mutants (D62A, R53A/ D217A, and D62A/Y274A) and wild type Gal1p was analyzed by testing their ability to phosphorylate d-galactose, l-arabinose, 2-deoxy-d-galactose, d-fucose, d-glucose, l-glucose, and d-talose. As expected, Gal1p was able to phosphorylate d-galactose, and the kinetic constants

![FIGURE 1. The galactose-binding site of Gal1p.](image)

The amino acids that are presumed to be involved in the interaction with galactose are shown. The dashed black lines indicate distances of 2.3–3.3 Å between the protein and the ligands and might indicate potential hydrogen bond interactions. The positions of the AMP-PNP (adenosine 5′-(β,γ-imino) triphosphate) molecule and the magnesium ion are also indicated.
obtained (see Table 1) were similar to those reported previously (4). It was also found to be able to phosphorylate 2-deoxy-D-galactose (14) and D-glucose, which has not been reported previously (see Table 1). None of the other sugars were phosphorylated by Gal1p at a detectable level. It is notable that Gal1p was able to phosphorylate D-glucose but not L-glucose, which suggests that the enzyme is specific for the particular stereochemistry at position 5 of the sugar ring. The turnover number (kcat) for 2-deoxy-D-galactose was 4 s−1, which is less than 6-fold lower than the kcat for galactose, and showed that the enzyme was moderately active with this sugar. The Km,ATP was essentially unchanged from that for galactose, whereas the Km,2-DG was over 60-fold higher than the Km value observed with galactose binding. D-Glucose was phosphorylated at a very low rate with a turnover number (0.034 s−1), which is 650-fold lower than that observed for the natural substrate (galactose). The Km value for the D-glucose was within the same order of magnitude as galactose; however, the Km value for ATP was 7-fold higher. Because the binding of the sugar and ATP is ordered with ATP binding first (4), it suggests that the protein may undergo a conformational change in order to accept D-glucose into its sugar-binding pocket and that the D62A mutation may stabilize this conformation.

Mutagenesis of Aspartate 62—Because mutation of aspartate 62 to alanine results in loss of D-galactose and 2-deoxy-D-galactose phosphorylation without affecting the phosphorylation of D-glucose, this residue was mutated sequentially to all of the other amino acids. Seven of the mutations resulted in the purification of protein from yeast (Aia, Glu, Phe, His, Leu, Lys, and Asn). The Km,ATP for each of the mutants was found to be essentially unchanged in comparison to the wild type protein (Table 2). By assuming the mutant proteins retain the kinetic mechanism of the wild type protein, this indicates that ATP binding is not affected. In addition, the specificity constant (kcat/Km) for each of the soluble mutant proteins was found to be similar to the wild type galactokinase when functioning as a D-glucose kinase (Table 2).

In Vivo Analysis of the Effect of the Mutations on Gal3p—Gal3p, the inducer of the GAL pathway, is highly similar to Gal1p (≈70% identity and ≈90% similarity), and in the absence of Gal3p the galactokinase can act as a transcriptional inducer. Given this and the homology in the galactose and ATP binding domains, two of the Asp-62 Gal1p mutations were introduced into Gal3p to assess whether similar effects would be seen with the activation of GAL gene transcription. The equivalent residue to Asp-62 in Gal3p is Asp-56, and this was mutated to alanine and leucine. The transcription from a reporter plasmid was assayed under galactose (raffinose/galactose) and glucose conditions. The GAL1-GAL10 promoter (16). These sites are −44 to −52 bases and −117 to −125 bases from the GALI TATA box, respectively. In an attempt to relieve this repression, two integrated reporter constructs were tested. These constructs contain the GALI-GAL10 promoter upstream of the lacZ gene; however, two consensus Gal4p-binding sites...
were introduced into the GAL1-GAL10 promoter at 50 or 100 bp upstream of the GAL1 TATA box (pJP184 and pJP185, respectively). A schematic diagram of pJP184 relative to the wild type promoter is shown in Fig. 2A. The effectiveness of each construct at relieving the glucose repression was tested using cells deleted for GAL3 and GAL80, thus allowing constitutive activation of Gal4p. It was found that pJP184 had 3-fold greater levels of expression than pJP185, indicating that pJP184 was more effective at relieving glucose repression (data not shown). This is likely to result from the disruption of the Mig1p-binding site at −44, which occurred when the Gal4p-binding sites were introduced 50 bp upstream of the TATA box. The activation in glucose grown cells is still 12-fold lower than in raffinose/galactose-grown cells (Fig. 2, B and C; compare levels in column 6); therefore, glucose repression is not completely relieved, either due to the presence of the undisturbed Mig1p-binding site at −117 or other factors, and possibly both. This fold reduction in glucose compared with raffinose/galactose was observed in each experiment.

The experiment was performed with cells deleted for GAL3, and plasmid-borne copies of either wild type or mutant GAL3 were then transformed into the cells. The level of expression in each variant was then tested in triplicate (with three independent colonies) using a β-galactosidase assay. As expected wild type Gal3p gave high levels of expression in raffinose/galactose (Fig. 2B, column 3), and small reproducible levels of expression were detected with glucose as the sole carbon source (Fig. 2C, column 3). Therefore, Gal3p can bind D-glucose, and this binding, together with that of ATP, can induce Gal4p-mediated transcription albeit at a low level.

Analysis of the mutants showed that expression levels in cells producing Gal3(D56L) did not increase above background in the presence of D-galactose (Fig. 2B, column 5), which was the same as observed in the Gal1(D62L) mutant. This demonstrates that the D56L mutation abolishes the ability of Gal3p to bind D-galactose. However, Gal3(D56A) had a 2-fold increase in levels of expression compared with the empty vector control (Fig. 2B, compare columns 2 and 4), which indicates that it has a very low level of D-galactose induction compared with wild type Gal3p (Fig. 2B, compare columns 3 and 4). This interaction was not observed in the Gal1p equivalent that had no detectable D-galactose phosphorylation. This may be due to slight differences between the Gal3p and Gal1p galactose-binding sites and the surrounding amino acids. In media containing glucose both the mutant proteins displayed wild type levels of D-glucose expression, which correlated with the results seen with Gal1p in which the mutants were able to bind D-glucose (Fig. 2C, columns 3–5). This showed that the mutations abolished or severely reduced the ability of Gal3p to respond to the presence of D-galactose without effecting the interaction between Gal3p and D-glucose. The overall levels of expression in these cells were reduced compared with cells deleted for GAL3 and GAL80 (Fig. 2C, column 6) because in cells containing Gal80p, Gal4p will be repressed, and this repression can only be relieved by Gal3p. When D-glucose is the sole carbon source, it would be necessary for Gal3p to bind D-glucose, together with ATP, in order for it to induce expression. Because it is likely that this would occur at a low level, the relative levels of expression compared with cells without Gal80p would be markedly reduced. This experiment demonstrates that mutation of Asp-56 in Gal3p to either alanine or leucine has similar, although not identical, effects to the same mutations of Asp-62 in Gal1p.

DISCUSSION

The crystal structure of the yeast galactokinase enzyme, Gal1p, in the presence of galactose and a nonhydrolyzable ATP analogue has yielded

![Figure 2](https://example.com/figure2.png)
Sugar Interactions in Gal1p and Gal3p

intriguing insights into both its mechanism of action and into the mechanism of small molecule recognition by its close homologue, the transcriptional inducer Gal3p (5). To explore these topics further, we undertook a mutational analysis of all the amino acid side chains in Gal1p that are directly implicated in participating in sugar binding. S. cerevisiae galactokinase displays a strong preference for D-galactose as its preferred substrate (Table 1). The protein is able to utilize 2-deoxy-D-galactose, but the $K_m$ is $\sim$50-fold higher with this alternative substrate. In addition, Gal1p can also utilize D-glucose, but not L-glucose, as a substrate. Interestingly, the $K_m$ value for D-glucose is similar to that for D-galactose. However, the $K_m$ value for ATP using the same assay is some 7-fold higher when D-glucose is the sugar, and the $K_m$ value for the enzyme when using D-glucose as a substrate is some 650-fold lower than when D-galactose is utilized. We have not been able to detect any kinase activity for the protein using other sugars (see Table 1).

In an attempt to dissect the relative contribution of each amino acid implicated in the structure of Gal1p to the overall ability to utilize sugar substrates, we mutated the following residues of Gal1p to alanine: Arg-53, Glu-59, Asp-62, Asp-217, and Tyr-274. In addition, we also made double mutations (R53A/D217A and D62A/Y274A) where different side chains had the potential to form a hydrogen bond with one of the sugar hydroxyl groups. Rather disappointingly, only D62A of the single mutants was able to be purified in a soluble form for subsequent analysis. Both of the double mutants (R53A/D217A and D62A/Y274A) were also purified, but both of these failed to show any sugar kinase activity in our assays with any of the sugars that we tested. It is therefore likely that the galactose-binding site of Gal1p plays a role in the overall stability of the enzyme. The D62A mutant protein did not display any detectable galactokinase activity, but it did retain the ability to use D-glucose as a substrate (Table 2). The kinetics of this utilization were similar to that observed for the wild type protein. D62A has thus specifically lost its ability to phosphorylate galactose.

To explore the role of Asp-62 further, we then mutated this residue to all other possible amino acids. Again we found that the majority of changes made resulted in proteins that could not be purified, either as a consequence of being insoluble or simply not accumulating to appreciable levels within yeast cells. For the proteins that we were able to purify, D62E, D62K, and D62N failed to show any kinase activity with any of the sugars tested. D62L, D62F, and D62H all failed to phosphorylate galactose but, like D62A, retained the ability to use D-glucose as a substrate. These data show that Asp-62 is specifically required for the utilization of galactose as a substrate. Even comparatively conservative changes (e.g. D62E) show no galactokinase activity. The changes that allow Gal1p to retain its ability as a D-glucose kinase are more difficult to interpret. The replacement of Asp-62 with either Ala, Leu, Phe, or His results, in the main, with the acidic side chain being replaced by a hydrophobic one. The size of this hydrophobic chain seems unimportant because alanine, leucine, or phenylalanine are all approximately equally adept at allowing the phosphorylation of D-glucose to occur. Attempts to model D-glucose into the Gal1p structure (data not shown) have not revealed a satisfactory explanation as to why these particular mutants will allow the protein to continue its low level activity with D-glucose as a substrate.

The mutation of the equivalent residue to Asp-62 to alanine in the human form of the galactokinase (Asp-46) also renders the enzyme inactive in terms of galactokinase activity (17). However, unlike the yeast enzyme, human galactokinase does not possess the ability to phosphorylate D-glucose even at a low level. In addition, the conversion of Glu-43 to alanine (equivalent to the yeast E59A mutation; Fig. 1) results in the formation of a protein with essentially wild type properties, whereas the mutation of His-44 (equivalent to His-60 in yeast) results in a nonsoluble protein (17). Combined with the work presented here, these data may indicate slight differences in sugar binding during the catalytic cycle that may not be immediately apparent from the crystal structures (5, 18).

The transcriptional inducer of the yeast GAL genes, Gal3p, is $\sim$70% identical and $\sim$90% similar to the galactokinase Gal1p (2). Rather than acting as a galactokinase, Gal3p senses the presence of galactose and ATP within the cell and, under appropriate conditions, binds to the transcriptional repressor Gal80p. In turn, this allows Gal4p to activate the transcription of the GAL genes. The high degree of similarity between Gal1p and Gal3p prompted us to ask whether similar mutations that we have described for Gal1p would be capable of functioning in the context of Gal3p. We therefore mutated the equivalent residue to Gal1p Asp-62 (Asp-56 in Gal3p) and tested the ability of the mutant protein to activate GAL gene expression. It is not straightforward to monitor the ability of Gal3p to utilize D-glucose in vivo. Catabolite repression results in the down-regulation of the GAL genes (19). This repression occurs at several levels, e.g. the repression of Gal4p activity by Gal80p, the down-regulation of GAL4 expression in the presence of glucose (20), and the binding of Mig1p to the GAL promoters and subsequent recruitment of the Snf6p-Tup1p co-repressor complex (21). The result of this repression is that glucose repression of the GAL genes dominates, even in the presence of galactose (22). Therefore, to test whether glucose was able to affect the activity of mutant forms of Gal3p, glucose repression effects needed to be reduced or eliminated. We did this by utilizing a modified GAL1-lacZ fusion in which two consensus Gal4p-binding sites were inserted into the GAL1 promoter (at $\sim$50 bp from the TATA box) to disrupt the Mig1p-binding site. This reporter construct was still inducible by galactose (Fig. 2B). In the absence of GAL80, this reporter was highly active with either a mixture of raffinose and galactose or D-glucose as the available carbon source. However, unlike the wild type protein, neither Gal3pD56A nor Gal3pD56L were able to support efficient transcription of the lacZ gene in the presence of galactose (Fig. 2B, columns 2–3). These data show that Asp-62 is specifically required for the utilization of galactose as a substrate. Even comparatively conservative changes (e.g. D62E) show no galactokinase activity. The changes that allow Gal1p to retain its ability as a D-glucose kinase are more difficult to interpret. The replacement of Asp-62 with either Ala, Leu, Phe, or His results, in the main, with the acidic side chain being replaced by a hydrophobic one. The size of this hydrophobic chain seems unimportant because alanine, leucine, or phenylalanine are all approximately equally adept at allowing the phosphorylation of D-glucose to occur. Attempts to model D-glucose into the Gal1p structure (data not shown) have not revealed a satisfactory explanation as to why these particular mutants will allow the protein to continue its low level activity with D-glucose as a substrate.

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Through the production of mutant proteins with impaired galactose function, we have uncovered a weak affinity of both Gal1p and Gal3p for D-glucose. Combined, the data in this study support a kinetic model for galactokinase function in which ATP binds to the protein first and sugar binding occurs subsequently (4). From a structural perspective, this is hard to explain. Yeast galactokinase is a bilobal protein with the sugar- and nucleotide-binding sites sandwiched between the two domains of the protein (5). From the structure with both substrates bound, it would appear that galactose should enter the complex first. However, because the structure of any galactokinase has not been solved in the absence of ligands (5, 18, 23, 24), it is difficult to speculate as to the state of the apoprotein. Recently, however, crystals of the galactokinase from Pyrococcus horikoshii in both the apo- and ligand-bound forms have been reported (25).
Attempts to change or broaden the substrate specificity range of galactokinases have been undertaken using the *Escherichia coli* enzyme through the application of *in vitro* glyco-randomization (26, 27). During these studies, it was observed that *E. coli* GalK M173L displayed moderate D-glucose activity (27). The equivalent residue in the yeast enzyme (Met-216) is located in the active site (next to Asp-217 in Fig. 1), and its mutation may provide the space for the altered orientation of the hydroxyl group attached to C-4 of the sugar. The wild type *E. coli* galactokinase enzyme does not, however, appear to have a natural affinity for D-glucose itself. The affinity for D-glucose is enhanced if the M173L mutation is combined with Y371H. The equivalent residue to Tyr-371 (Ile-515 in Gal1p) is located some 16 Å from the sugar ring, and the mode of action of this change is unclear.

It is clear from the work presented here that Gal1p and Gal3p interact with their substrates in a very similar, and perhaps identical, fashion. The ability to make equivalent mutations in a readily assayable enzyme and a transcriptional inducer has greatly aided the understanding of the molecular mechanism of the latter.

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