The galactokinase from *Saccharomyces cerevisiae* (ScGal1p) is a bifunctional protein. It is an enzyme responsible for the conversion of α-D-galactose into galactose 1-phosphate at the expense of ATP but can also function as a transcriptional inducer of the yeast GAL genes. For both of these activities, the protein requires two ligands; a sugar (galactose) and a nucleotide (ATP). Here we investigate the effect of these ligands on the stability and conformation of ScGal1p to determine how the ligands alter protein function. We show that nucleotide binding increases the thermal stability of ScGal1p, whereas binding of galactose alone had no effect on the stability of the protein. This nucleotide stabilization effect is also observed for the related proteins *S. cerevisiae* Gal3p and *Kluyveromyces lactis* ScGal1p and suggests that nucleotide binding results in the formation of, or the unmasking of, the galactose-binding site. We also show that the increase in stability of ScGal1p does not result from a large conformational change but is instead the result of a smaller more energetically favorable stabilization event. Finally, we have used mutant versions of ScGal1p to show that the galactokinase and transcriptional induction functions of the protein are distinct and separable. Mutations resulting in constitutive induction do not function by mimicking the more stable active conformation but have highlighted a possible site of interaction between ScGal1p and ScGal80p. These data give significant insights into the mechanism of action of both a galactokinase and a transcriptional inducer.

The yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* are both capable of utilizing galactose as a source of carbon. To do so, they activate the genes encoding the enzymes of the Leloir pathway, collectively termed the GAL genes (1). The transcriptional switch controlling GAL gene expression is composed of an activator (Gal4p), an inhibitor (Gal80p), and a ligand sensor/transcriptional inducer (Gal3p in *S. cerevisiae* or Gal1p in *K. lactis*). The GAL regulatory proteins from the two yeasts are at least in part, interchangeable. For example, Gal4p from both *S. cerevisiae* (ScGal4p) and *K. lactis* (Klgal4p) will complement a gal4 mutation in either yeast (2, 3) despite the two proteins sharing comparatively little overall sequence similarity (28% amino acid identity and 57% similarity over their entire length). Gal80p from either yeast are highly related (58% amino acid identity and 82% similarity) and will inhibit the transcriptional activity of either version of Gal4p (4). However, although Klgal1p can complement both a Scgal1 (galactokinase-defective) and Scgal3 (ligand sensor-defective) mutation (5), Scgal3p cannot complement the non-inducible phenotype of a Klgal1 deletion mutant unless the Klgal80 gene is also replaced by ScGal80 (6).

ScGal1p and ScGal3p are extraordinarily similar proteins (~70% amino acid identity and ~90% similarity). Both proteins require galactose and ATP to function. ScGal1p utilizes the ligands as a galactokinase that converts galactose into galactose 1-phosphate at the expense of ATP. ScGal3p, on the other hand, requires the ligands to promote an interaction with ScGal80p. This interaction ultimately results in the activation of GAL gene expression (1). The enzymatic mechanism of ScGal1p has been shown to proceed via an ordered tertiary complex mechanism in which ATP binds first (7). Although ScGal1p is primarily a galactokinase, it is able to weakly interact with ScGal80p (8) and serves as an inducer of GAL gene expression in the absence of ScGal3p. ScGal3p, however, does not possess a detectable galactokinase activity although ScGal3p can be converted into a galactokinase through the insertion of two amino acids (a serine and an alanine immediately after amino acid 164) (9).

Recently, the three-dimensional structures of ScGal1p, ScGal80p, and Klgal80p have been solved (10–12). In addition, the structure of Klgal80p in the presence of a peptide representing Gal4p has been solved (13). ScGal1p, whose structure was solved in the presence of galactose and a non-hydrolysable ATP analogue, displays a marked bilobal appearance, with the active site located between distinct N- and C-terminal domains (11). The nucleotide and galactose binding sites are buried between the two domains, and this led to the proposal of a mechanism by which the binding of the ligands results in a conformational change. It has not been possible to crystallize ScGal1p in the absence of its ligands.2

Here we probe the mechanism by which ScGal1p is converted into an active galactokinase/transcriptional inducer by the presence of its ligands. Using a variety of biophysical techniques, we show that in the presence of its ligands ScGal1p becomes a substantially more stable protein. This effect is also observed for the related proteins ScGal3p and Klgal1p. Analysis of the conformation of ScGal1p in the absence and presence of ligands by velocity analytical ultracentrifugation (AUC)3 demonstrated that the binding of ligands does not introduce a wholesale conformational

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2 J. B. Thoden and H. M. Holden, unpublished data.

3 AUC, analytical ultracentrifugation; DSC, differential scanning calorimetry.
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change within the protein. Instead, it simply stabilizes the structure of the protein. We also investigated the effect of introducing ScGal3p constitutive mutations into ScGal1p on the stability of the protein to determine whether constitutive activation results from the formation of an active conformation in the absence of ligands. We demonstrate that four of these mutations, when introduced into ScGal1p, result in constitutive transcriptional activation but that these mutations have negative effects on the galactokinase activity of the protein. There is, however, still a difference in their stability in the presence and absence of ligands, which would suggest that constitutive transcriptional induction does not occur through the stabilization of the protein in the absence of ligands.

EXPERIMENTAL PROCEDURES

Escherichia coli and Yeast Strains—The production of ScGal1p, ScGal80p, and KiGal80p was performed in HMS174(DE3), BL21(DE3), and Rosetta(DE3) E. coli cells (obtained from Novagen), respectively. MC2 yeast cells (MATa, trp1, ura3-52, leu2-3, prc1-407, prb1-112, pep4-3) (9) were used for Gal3p production and purification. β-Galactosidase assays were performed in JPY5:ΔGAL1::pGAL3::RY131 cells (MATα ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385) (9). Disruption of GAL1 and GAL3 from JPY5 was achieved by using PCR-generated blaster cassettes (14).

Protein Expression and Purification—Protein purification from E. coli was performed after the appropriate gene was cloned into pET28a (Novagen). E. coli cells harboring these plasmids were grown in 3 liters of LB medium supplemented with 30 mg/liter kanamycin at 37 °C to an _A_<sub>660</sub> of 0.8. The cells were then induced with 1 mM isopropyl _β_-D-thiogalactopyranoside for 16 h at 16 °C. The induced cells were pelleted by centrifugation at 1800 × _g_ for 5 min, resuspended in buffer A (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 30 mM imidazole, 10% (v/v) glycerol), and lysed by sonication (3 × 30 s). The lysate was clarified by centrifugation at 75,000 × _g_ for 20 min. The supernatant was then loaded onto a 2 ml Ni<sup>2+</sup>-NTA-agarose column (ProBond resin; Invitrogen) prewashed with buffer A. The column was then washed with buffer A, and protein was eluted from the column with buffer C containing 250 mM imidazole. The protein was then dialyzed into several changes of buffer B (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1.4 mM _β_-mercaptoethanol, 10% (v/v) glycerol) at 4 °C overnight.

For the purification of Gal3p, MC2 yeast cells were transformed with pAP45 (pYEX-BX (Clontech) containing the GAL3 gene (15)) and grown in 6 liters of Leu<sup>−</sup>-selective medium at 30 °C to an _A_<sub>660</sub> of 1.0. The cells were then induced with 0.5 mM CuSO<sub>4</sub> for 24 h. The induced cells were pelleted by centrifugation at 1800 × _g_ for 5 min, resuspended in buffer A, frozen in liquid nitrogen, and lysed in a SPEX CertiPrep 6850 Freezer/Mill using 5 cycles with 2 min of grinding and 2 min of cooling per cycle (16). The cells were thawed at room temperature, and the lysate was clarified by centrifugation at 75,000 × _g_ for 20 min. The protein was purified on a 2-ml Ni<sup>2+</sup>-NTA-agarose column as described above.

Mutagenesis—Mutations were introduced into pET28-GAL1 using the QuikChange method (17). 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 using an enzyme-linked assay system (7, 18). Briefly, reaction mixtures (150 μl) were set up in 96-well microtiter dishes containing 20 mM HEPES-KOH (pH 8.0), 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 400 μM phosphoenol pyruvate, 1 mM dithiothreitol, 1 mM NADH, 1.1 units pyruvate kinase, 1.5 units 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 using a Multiskan Ascent plate reader.

Differential Scanning Calorimetry (DSC)—DSC was performed using a VP-DSC instrument (MicroCal Inc., Amherst, MA) with a scan rate of 60 °C/h from 15 to 100 °C. Protein samples (0.3 mg/ml) were dialyzed into buffer C (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% (v/v) glycerol) and run with buffer C as a reference. Ligands were added at a concentration of 2.5 mM. All thermal transitions were irreversible under the conditions used in this study. Data were analyzed using ORIGIN software (Microcal).

Velocity AUC—ScGal1p was further purified by gel filtration on a Sephadex 200 10/300 GL column (GE Healthcare) which had been pre-equilibrated in buffer D (20 mM Tris-HCl (pH 8.0), 150 mM NaCl). The column was run at 0.5 ml/min with 0.5-ml fractions collected, and the peak fractions, as determined by SDS-PAGE, were pooled. Samples were loaded into two-sector cells in either a Beckman XLA or XL1 rotor at concentrations ranging between 0.3 and 1.7 mg/ml. Sedimentation of the proteins was achieved at 115,000 × _g_ collecting data for 200 scans at 280 nm at 20 °C. Both interference optics and absorbance were used to determine concentration dependence of the sedimentation coefficient (s), and the sedimenting boundaries were analyzed using both _ls_<sup>g</sup>(s) and Lamm-equation solution modeling with the program Sedfit (19).

β-Galactosidase Assays—Yeast cells (JPY5:ΔGAL1::pGAL3::RY131) were transformed with pJR374, which contains the GAL1 coding sequence under the control of the GAL3 promoter or versions of the same plasmid containing mutated versions of the GAL1 gene. The cells were grown in 3 ml of the appropriate yeast selective dropout medium at 30 °C to an _A_<sub>600</sub> of 0.8–1.0. The cells were pelleted by centrifugation at 4000 × _g_ for 5 min and resuspended in 250 μl of buffer E (100 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 20% (v/v) glycerol). The β-galactosidase assays were performed as described previously (20).

RESULTS

The Galactokinase Activity of ScGal1p Was Inhibited by Gal80p, and This Inhibition Was Relieved by Gal4p—ScGal1p can function both as a galactokinase and a transcriptional inducer. We investigated the effect of the interaction of ScGal1p with ScGal80p on its galactokinase activity (Fig. 1). The presence of ScGal80p inhibits the galactokinase activity of ScGal1p. The galactokinase activity of ScGal1p was reduced by 50% at a 1:1.5 molar excess of ScGal80p (Fig. 1A). It is possible that the interaction with ScGal80p locks ScGal1p into a confor-
mation that prevents it from catalyzing the formation of galactose 1-phosphate or that it blocks product release. The inhibition of ScGal1p galactokinase activity in the presence of ScGal80p is similar to that previously reported for KlGal1p in the presence of KlGal80p (21). The same effect is, however, not observed if the experiment is repeated using KlGal80p (Fig. 1B). The lack of effect seen with KlGal80p is perhaps expected as KlGal80p is able to interact with and inhibit ScGal4p, but this inhibition cannot be relieved by ScGal3p (6). This indicates that ScGal1p is also unable to interact with KlGal80p.

The inhibition of ScGal1p galactokinase activity by ScGal80p is relieved efficiently by ScGal4p (Fig. 1C) but only modestly by ScGal3p (Fig. 1D). An equimolar concentration of ScGal4p was found to completely alleviate the inhibitory effect of ScGal80p upon the galactokinase activity of ScGal1p (Fig. 1C), whereas a 2-fold molar excess of ScGal3p only increased the relative galactokinase activity from 0.27 to 0.5 (Fig. 1D). Taken together, these data indicate that the form of ScGal1p that interacts with ScGal80p is incapable of performing its normal enzymatic role. Disrupting the interaction between ScGal1p and ScGal80p (by the addition of ScGal4p, which will favor the formation of the more stable Gal4p-Gal80p complex) leads to the restoration of galactokinase activity. The weak restoration of activity observed in the presence of ScGal3p presumably results from the competitive formation of Gal3p-Gal80p complexes, thereby allowing ScGal1p to again function as an enzyme.

ScGal1p Is Stabilized in the Presence of ADP and Galactose—When the crystal structure of ScGal1p was solved, we suggested that ligand binding resulted in a conformational change in the protein leading to a more stable structure (11). To investigate the effect of ligand binding on ScGal1p we used DSC on highly purified ScGal1p to estimate the temperature at which the protein unfolds in the presence and absence of ligands (Fig. 2A). In the absence of nucleotide and galactose, the protein was found to have a melting temperature (\(T_m\)) of 56.4 °C. The peak consisted of two distinct and separate unfolding events that most likely result from the N- and C-terminal domains of the protein unfolding at different temperatures. One domain unfolds at 54.3 °C and the other at 57.2 °C (Table 1, ScGal1p data); however, it was not possible to determine which domain unfolds first. In the presence of ADP (used instead of ATP to prevent enzyme catalysis) and galactose the \(T_m\) of ScGal1p was increased to 59.1 °C, an increase of 2.9 °C, indicating significant stabilization of the protein structure in the presence of ligands (Fig. 2A and Table 1, ScGal1p + ADP + Gal data). Similar experiments performed with highly purified KlGal1p also showed an increase in the \(T_m\) of the protein in the presence of its ligands from 55.9 °C to 60.4 °C (Table 1, KlGal1p and KlGal1p + ADP + Gal data). The presence of ligands also stabilizes ScGal3p. In the absence of ATP and galactose, the protein consistently precipitated during DSC analysis, making a determination of the \(T_m\) impossible. However, in the presence of the

![FIGURE 1. Inhibition of ScGal1p galactokinase activity by ScGal80p. A, the addition of ScGal80p to ScGal1p inhibits ScGal1p galactokinase activity. B, the presence of KlGal80p has no inhibitory effect on ScGal1p galactokinase activity. C, ScGal4p is able to relieve the inhibition by ScGal80p although to a lesser extent than ScGal4p. D, ScGal3p is also able to relieve the inhibition by ScGal80p although to a lesser extent than ScGal4p. A 1:5 ratio of ScGal1p:ScGal80p was used in each case.](image-url)
Ligand-induced Stabilization of Gal1p

ligands, ScGal3p does not precipitate and has a \( T_m \) of 57.1 °C (Table 1, ScGal3p + ADP + Gal data). This demonstrates that this family of proteins adopts a more stable conformation upon ligand binding, whether it is for galactokinase activity or for transcriptional induction (which is the only role of ScGal3p).

**ScGal1p** is converted to a more stable form in the presence of ADP alone—It has previously been shown that the catalytic mechanism of yeast galactokinase proceeds through the ordered formation of a tertiary complex in which ATP binds to the enzyme first and galactose second (7). This suggests that the binding of the nucleotide is essential either for the formation of, or the unmasking of, the galactose-binding site. DSC with ScGal1p was repeated in the presence of either galactose or ADP alone. In the presence of galactose the \( T_m \) remained essentially unchanged (Fig. 2B and Table 1, ScGal1p + Gal data). However, in the presence of ADP the \( T_m \) was increased to 59.1 °C (Table 1, ScGal1p + ADP data), which was the same as the increase observed in the presence of both ligands (Fig. 2B). This suggests that the binding of ADP does indeed result in the formation or the unmasking of the galactose-binding site and also demonstrates that the binding of galactose does not have a major effect on the stability or conformation of the protein.

**The Formation of the Kinetically Active Form of ScGal1p Is Not Mediated by a Significant Conformational Change**—The increased stability of ScGal1p in the presence of ligands suggested that ScGal1p may undergo a conformational change upon ligand binding which results in the kinetically active form of the protein seen in the crystal structure (11). To investigate this, ScGal1p in the absence or presence of one or both ligands was analyzed by velocity AUC. Velocity sedimentation of ScGal1p at a concentration of 1 mg/ml gave a sedimentation value \( (s_{app}) \) of 2.34 in the absence or presence of ligands (Fig. 3). Sedimentation of ScGal1p using various protein concentrations and extrapolation to zero concentration gave a \( s_{0w}^0 \) value of 3.98 S, and a hydrodynamic radius of 3.3 nm. By modeling the crystal structure of ScGal1p (11) with beads of known hydrodynamic parameters (SOLution MOdeler) (22), we were able to determine whether the crystal structure matched the hydrodynamic properties of ScGal1p in solution. The bead model suggests that the crystal structure would have a \( s_{0w}^0 \) value of 4.16 S and a hydrodynamic radius value of 3.1 nm, which is similar to, if slightly more compact than, the protein in solution. The addition of galactose and/or ADP had no effect on the sedimentation value of the protein, which clearly demonstrates that the protein does not undergo a large conformational change upon ligand binding (Fig. 3). It is likely, therefore, that ligand binding results in a small, more energetically favorable conformational change that stabilizes the protein and forms or unmask the galactose-binding site. There is an unpublished structure of an unliganded galaktokinase in the Protein Data Bank (accession number 2CZ9) arising from the crystallization of the *Pyrococcus horikoshii* enzyme (23). This structure appears to be very similar to the liganded versions (11, 24, 25), which supports the notion that a large conformational change does not occur within the galaktokinases upon ligand binding.

**TABLE 1**

Summary of the thermodynamic data for ScGal1p

| Sample          | \( T_m \) (°C) | \( T_m \) \(_{110} \) (°C) | \( 
\Delta H \) (kcal/mol) | \( T_m \) \(_{220} \) (°C) | \( 
\Delta H \) (kcal/mol) |
|-----------------|----------------|--------------------------|------------------------|--------------------------|------------------------|
| ScGal1p         | 56.40 ± 0.12   | 54.34 ± 0.52             | 60.4 ± 13.1            | 57.15 ± 0.12             | 49.6 ± 12.7            |
| ScGal1p + ADP   | 59.17 ± 0.06   | 57.02 ± 0.38             | 65.0 ± 11.9            | 59.57 ± 0.09             | 56.2 ± 11.5            |
| ScGal1p + Gal   | 59.25 ± 0.05   | 57.18 ± 0.29             | 73.5 ± 9.2             | 59.70 ± 0.06             | 57.4 ± 8.9             |
| ScGal1p + Gal   | 56.78 ± 0.05   | 55.07 ± 0.40             | 51.6 ± 11.3            | 57.51 ± 0.16             | 35.3 ± 11.1            |
| ScGal3p         | ND             | ND                       | ND                     | ND                       | ND                     |
| ScGal3p + ADP   | 57.11 ± 0.20   | ND                       | 49.6 ± 12.2            | 56.16 ± 0.15             | 25.4 ± 12.4            |
| ScGal3p + Gal   | 55.90 ± 0.11   | 53.02 ± 0.32             | 93.4 ± 4.81            | 69.91 ± 0.21             | 80.7 ± 14.7            |

Figures and Table:

**FIGURE 2.** DSC analysis of ScGal1p. Protein samples in the absence or presence of galactose and/or ADP were heated from 15 to 100 °C. A, DSC of ScGal1p in the absence and presence of ligands showing the maximum of thermal transition (\( T_m \)) and Hv in each case. B, DSC of ScGal1p in the absence or presence of one or both ligands. For clarity, only the \( T_m \) is indicated. Full details of the Hv values are shown in Table 1.

**TABLE 1**

Summary of the thermodynamic data for ScGal1p

\( T_m \) indicates the melting temperature from the non-deconvoluted data. \( T_m \) \(_{110} \) and \( T_m \) \(_{220} \) indicate the melting temperature from the two deconvoluted curves. The enthalpy of unfolding (\( \Delta H \)) for the deconvoluted curves is also shown. The data were analyzed using ORIGIN software (Microcal). ND indicates not detectable.
Constitutive Gal3p Mutations Affect the Galactokinase Activity of ScGal1p

Previously, a number of constitutive mutants of ScGal3p have been isolated (26–28). These mutant proteins (V69E, V203I, F237Y, D368V, V396A, S509P, and S509L) were found to be able to interact with ScGal80p in the absence of galactose, whereas the wild-type protein only interacts with ScGal80p in the presence of galactose. We surmised that these mutations could either directly alter the ScGal3p-ScGal80p interaction site, or they could mimic the ligand-induced stabilization that occurs upon binding of galactose and ATP. The latter theory was based on the observation that when the constitutive ScGal3p mutations were mapped onto the homology model they were located around the interface between the N- and C-terminal domains (11), and as expected, this was also the case when the equivalent mutations were mapped onto the structure of ScGal1p (Fig. 4). In an effort to distinguish between these possibilities, we made the equivalent changes to ScGal1p bearing constitutive ScGal3p mutations. Each of the mutants was assayed for galactokinase activity at 30 °C as described in “Experimental Procedures.” WT, wild type. B, transcriptional induction by ScGal1p bearing ScGal3p constitutive mutations. The expression levels of lacZ were inferred by measured β-galactosidase activity in cells grown in either 2% raffinose (R) or 2% raffinose/galactose (RG). JPY5::GAL1::GAL3::RY131 cells were transformed with either the empty vector or a version of the vector containing the GAL1 gene or ScGal3p constitutive mutants introduced into the GAL1 gene under the control of the GAL3 promoter. A schematic diagram of the integrated reporter construct (RY131), which contains the GAL1 promoter upstream of the lacZ gene, for measuring transcriptional induction by Gal1p is shown above.
Ligand-induced Stabilization of Gal1p

**TABLE 2**

Kinetic parameters for each mutant are indicated. All reactions were carried out at 30 °C as described under “Experimental Procedures.” ND indicates not detectable.

| Gal1p     | $k_{cat}$ for Galactose $K_m$ for ATP $k_{cat}/K_m$ for Galactose $K_m$ for ATP $k_{cat}/K_m$ for ATP |
|-----------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Wild type | 12.68 ± 0.43                                     | 0.537 ± 0.14                                    | 0.299 ± 0.036                                   | 0.099 ± 0.017                                   | 42.41 ± 6.54                                    |
| F75E      | 4.27 ± 0.10                                     | 0.807 ± 0.232                                   | 5.29 ± 1.65                                     | 43.13 ± 8.42                                   | 116.05 ± 19.50                                  |
| V211I     | 9.4 ± 0.32                                      | 0.883 ± 0.305                                   | 0.081 ± 0.013                                   | 0.097 ± 0.031                                   | 4.64 ± 1.79                                     |
| F245Y     | 0.45 ± 0.03                                     | 0.652 ± 0.216                                   | 0.200 ± 0.18                                    | 93.95 ± 7.95                                   | 93.95 ± 7.95                                   |
| D376V     | 4.04 ± 0.06                                     | 2.024 ± 0.151                                   | 0.111 ± 0.012                                   | 93.95 ± 7.95                                   | 93.95 ± 7.95                                   |
| V404A     | 1.42 ± 0.03                                     | 0.362 ± 0.128                                   | 3.92 ± 1.46                                     | 12.79 ± 1.65                                   | 12.79 ± 1.65                                   |
| S517P     | ND                                              | ND                                              | ND                                              | ND                                              | ND                                              |
| S517L     | ND                                              | ND                                              | ND                                              | ND                                              | ND                                              |

**TABLE 3**

Summary of the thermodynamic data for the ScGal1p mutants bearing ScGal3p constitutive mutations in the absence or presence of its ligands, galactose, and ADP.

| ScGal1p  | $T_m$ (°C) |
|----------|------------|
| Wild type| 56.4 ± 0.1 |
| F75E     | 52.3 ± 1.1 |
| V211I    | 56.1 ± 0.8 |
| F245Y    | 50.9 ± 1.7 |
| D376V    | ND         |
| V404A    | ND         |
| S517P    | ND         |
| S517L    | ND         |

**DISCUSSION**

The crystal structure of ScGal1p in the presence of galactose and a nonhydrolyzable ATP analogue has allowed speculation on the mechanism by which it is converted into an active galactokinase and/or transcriptional inducer (11). The work presented here shows that ScGal1p and KlGal1p as well as ScGal3p
form a more stable structure in the presence of ligands. Moreover, this change in stability is observed in the presence of the nucleotide alone. Velocity sedimentation analysis of ScGal1p in the presence and absence of ligands demonstrated that there are no large structural changes in the protein upon ligand binding, which suggests that the protein is activated via a far more subtle stabilization event. Finally, the effects of constitutive ScGal3p mutations were investigated to explore the theory that this phenotype was caused by the protein maintaining the active conformation under both inducing and non-inducing conditions. These experiments were carried out by introducing the mutations into ScGal1p, assaying for constitutive transcriptional induction, and finally analyzing the conformational state by DSC.

Because the crystal structure of ScGal1p was solved in the presence of ligands, it has been suggested that ligand binding results in a conformational change that stabilizes the structure of the protein (11). This was based on the observations that, despite numerous attempts under a large range of different crystallization conditions, it was not possible to crystallize ScGal1p in the absence of ligands. To investigate this theory, the protein was analyzed by DSC to determine the temperature at which the protein structure was disrupted in the presence or absence of ligands. The results showed ligand-induced stabilization of ScGal1p with the $T_m$ increasing by 2.9 °C in the presence of ligands. The galactokinase from *K. lactis* (KiGal1p) showed a 4.5 °C increase in the temperature at which thermal unfolding occurs in the presence of ligands. The increase in stabilization was observed with the addition of the nucleotide alone, whereas the addition of galactose alone had no effect on the stability of the protein. Given that the enzymatic mechanism of ScGal1p has been shown to proceed via an ordered tertiary complex mechanism in which ATP binds first (7), these results provide further evidence that ATP binding occurs before galactose is bound and suggests that binding of the nucleotide is required to either form or unmask the galactose-binding site. Analysis of the peak obtained when the protein unfolded showed that the protein unfolds as two distinct events that likely represent the separate unfolding of the N- and C-terminal domains of the protein. The temperatures at which the two domains unfold differs by ~3 °C in the absence of ligands, and this reduces to ~2.5 °C in the presence of ligands (Fig. 2). Although it is likely that the entire protein is stabilized by the presence of the ligands, the peaks for the more stable domain in the absence of ligands and the less stable domain in the presence of ligands unfold at the same temperature so it is conceivable that only one domain is stabilized in the presence of ligands; however, it has not been possible to define which domain unfolds first under each condition.

In the case of ScGal3p, the ligands were found to inhibit protein precipitation at the temperatures used in the DSC experiments (Table 1, ScGal3p and ScGal3p + ADP + Gal data). The transcriptional inducer was found to be very unstable in the absence of ligands, and as such it was not possible to obtain a reliable $T_m$ value. However, in the presence of ligands, ScGal3p stabilized to give a $T_m$ of 57.1 °C. This demonstrates that the stabilization event is associated with the active conformation of the protein for both the galactokinase activity of ScGal1p and the transcriptional induction function of ScGal3p. In the case of ScGal1p it is possible that different conformations are adopted depending on whether it is acting as a galactokinase or as a transcriptional inducer, where it is required to interact with ScGal80p. The observations that both ScGal1p and ScGal3p are stabilized in the presence of ligands reinforces how similar these proteins are (9) and suggests that they act via similar mechanisms. The velocity AUC data demonstrated that the binding of ligands does not induce a gross conformational change within the protein.

To determine whether constitutive activation results from the mutations stabilizing the protein structure and mimicking the active conformation in the absence of ligands, we also investigated the effect of introducing ScGal3p constitutive mutations on the stability of ScGal1p. Four of the mutant proteins (F75E, F245Y, D376V, and S517L) were found to be constitutive inducers, and of these, two had less than 10% galactokinase activity. The fact that F245Y and S517L have almost no galactokinase activity while still retaining close to wild-type levels of transcriptional induction clearly demonstrates that the galactokinase activity and transcriptional induction functions of ScGal1p are distinct and separable. This is also indicated by the repression of galactokinase activity resulting from interaction of ScGal1p with ScGal80p, although it may also result from ScGal80p blocking release of the products. Analysis of the thermostability of the mutant proteins showed that there is still a difference in their stability in the presence and absence of ligands. This would indicate that constitutive transcriptional activation is not mediated through formation of the active conformation in the absence of ligands. However, all of the proteins that had mutations that resulted in constitutive transcriptional activity had a greater difference between the $T_m$ in the presence and absence of ADP and galactose; indeed some mutants did not have a $T_m$ that could be defined in the absence of ligands. This is similar to the results seen with wild-type ScGal3p and, given that most of these mutants have lost their galactokinase activity, may represent the difference between the galactokinase and transcriptional induction functions of ScGal1p. We have shown that ScGal1p in the absence of ligands has a similar shape as the protein in the presence of ligands; therefore, it may be that the mutations oblate the requirement for the formation of the stable conformation. The fact that three of the four mutations are clustered together (Phe-75, Phe-245, and Ser-517, see Fig. 4) suggests that this may be the site of interaction and that the mutations mimic the stabilized state, thus allowing the interaction to occur in the absence of ligands. Alternatively, introduction of the constitutive mutations into ScGal1p has been demonstrated to make the protein more likely to aggregate, and it may be this phenotype that allows it to induce transcriptional activation in the absence of ligands.

From the data presented here and from previous studies (7, 11), a refinement of the model for galactokinase function and/or transcriptional induction can be proposed. ATP is bound first, and this binding event stabilizes the structure of the protein resulting in a small conformational change which forms (or unmask) the galactose-binding site. Subsequently, galactose is bound, and the protein is then able to either phosphorlyate the sugar (galactokinase activity) or interact with Gal80p
to alleviate its repression of Gal4p (transcriptional induction function). Understanding the mechanism by which transcriptional induction occurs is vital for full comprehension of the GAL genetic switch. The interaction of the ligand sensor with Gal80p is the least understood part of the switch, with very little being known about the site of interaction of the proteins. This paper demonstrates that binding of the ligands stabilizes the conformation of the protein but does not result in a large conformational change as previously predicted.

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