Characterization of the *Saccharomyces cerevisiae* galactose mutarotase/UDP-galactose 4-epimerase protein, Gal10p

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UDP-glucose; Leloir pathway; SDR family enzyme; galactose metabolism; bifunctional enzyme; aldose epimerase.

**Abstract**

*Saccharomyces cerevisiae* and some related yeasts are unusual in that two of the enzyme activities (galactose mutarotase and UDP-galactose 4-epimerase) required for the Leloir pathway of d-galactose catabolism are contained within a single protein—Gal10p. The recently solved structure of the protein shows that the two domains are separate and have similar folds to the separate enzymes from other species. The biochemical properties of Gal10p have been investigated using recombinant protein expressed in, and purified from, Escherichia coli. Protein–protein crosslinking confirmed that Gal10p is a dimer in solution and this state is unaffected by the presence of substrates. The steady-state kinetic parameters of the epimerase reaction are similar to those of the human enzyme, and are not affected by simultaneous activity at the mutarotase active site. The mutarotase active site has a strong preference for galactose over glucose, and is not affected by simultaneous epimerase activity. This absence of reciprocal kinetic effects between the active sites suggests that they act independently and do not influence or regulate each other.

**Introduction**

D-Galactose is metabolized by the Leloir pathway to produce the metabolically more useful D-glucose 1-phosphate (Caputto *et al*., 1949; Leloir, 1951; Cardini & Leloir, 1953; Frey, 1996; Holden *et al*., 2003). Four enzymatic activities are required to catalyze the reactions of this pathway. The equilibrium between the α- and β-anomers of galactose is maintained by galactose mutarotase (EC 5.1.3.3). Galactokinase (EC 2.7.1.6) phosphorylates galactose using ATP to produce β-D-galactose 1-phosphate (Wilkinson, 1949; Howard & Heinrich, 1965). The reaction of this product with UDP-β-D-glucose (catalyzed by galactose-1-phosphate uridylytransferase; EC 2.7.7.12) produces glucose 1-phosphate and UDP-β-D-galactose. Glucose 1-phosphate is then converted to β-D-glucose 6-phosphate, which can enter glycolysis by the action of phosphoglucomutase (EC 5.4.2.2). UDP-glucose is regenerated from UDP-galactose in a reaction catalyzed by UDP-galactose-4-epimerase (EC 5.1.3.2). Mutations in the genes coding for all the Leloir pathway enzymes except galactose mutarotase have been shown to cause the disease galactosemia (Elsas & Lai, 1998; Petry & Reichardt, 1998; Novelli & Reichardt, 2000; Holden *et al*., 2004; Sidjianin *et al*., 2005; Timson, 2006).

In the budding yeast *Saccharomyces cerevisiae*, Gal10p contains both galactose mutarotase (referred to here as mutarotase) and UDP-galactose-4-epimerase (referred to as epimerase) activities (Majumdar *et al*., 2004). This dual activity appears to be unique to *Sac. cerevisiae* and other yeasts such as *Kluyveromyces fragilis*, *K. lactis*, *Pachysolen tannophilus* and *Schizosaccharomyces pombe* (Brahma & Bhattacharrya, 2004). It is not present in more distantly related fungi such as *Neurospora crassa* (Seiboth *et al*., 2002). Indeed, it is unusual to see two nonsequential enzymatic activities encoded in the same protein, and it is not clear why the two activities are linked in this way in some yeasts. One possibility is that the Leloir pathway enzymes assemble into a multienzyme complex (or metabolon) similar to that seen in other pathways (Ovadi, 1988; Watford, 1989; Mitchell, 1996; Velot *et al*., 1997; Abadjiieva *et al*., 2001; Kindzelskii *et al*., 2004). This would have the advantage of sequestering galactose 1-phosphate, which is toxic to both yeasts and mammals (Tsakiris *et al*., 2002). Evidence for the *in vivo* association of the Leloir pathway enzymes in yeasts was provided by experiments showing that green fluorescent protein-tagged Gal7p is localized to discrete spots within the cytoplasm, and that this localization was abolished when either the *GAL1* or *GAL10* genes were deleted (Christacos *et al*., 2000).
Limited proteolysis experiments suggested that the two activities of \textit{K. fragilis} Gal10p have separate active sites located in different parts of the protein (Brahma & Bhattacharya, 2004). This was confirmed by the crystal structure of Gal10p, which shows an N-terminal epimerase domain and a C-terminal mutarotase domain (Thoden & Holden, 2005). These domains are structurally similar to human and bacterial epimerases and mutarotases (Thoden et al., 1996, 2000, 2001, 2002, 2004; Thoden & Holden, 2002). They are joined by a short linker, and there are limited noncovalent interactions between them. The possibility that there is communication between the two active sites has not been addressed experimentally before. In order to do this, the steady-state kinetic parameters of both domains of Gal10p were determined, the sugar specificity of the mutarotase domain was investigated, and the effects of the activities on each other were probed.

Materials and methods

Expression and purification of \textit{Sac. cerevisiae} Gal10p

A plasmid for the high-level expression of Gal10p was kindly supplied by Jim Thoden and Hazel Holden (University of Wisconsin). Protein was expressed according to their published protocol (Thoden & Holden, 2005), except that Luria–Bertani (Miller) medium was used throughout for bacterial culture. Following expression, cells were pelleted by centrifugation (10 min at 4200 g at 4 °C), resuspended in c. 20 mL of 50 mM Hepes–NaOH (pH 7.5), 150 mM NaCl, and 10% (v/v) glycerol, and stored at −80 °C. The protein was purified using the same method as previously described for human UDP-galactose-4-epimerase (Timson, 2005). Protein concentrations were estimated by the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.

UDP-galactose-4-epimerase assay

Epimerase activity was assayed as previously described (Timson, 2005), except that all assays were carried out at 30 °C. The concentration of Gal10p was 10 nM in all assays.

Galactose mutarotase assay

The galactose mutarotase activity of Gal10p was assayed by coupling this reaction to that catalyzed by the glucose/galactose dehydrogenase from \textit{Thermoplasma acidophilum} (EC 1.1.1.47, Sigma). The enzyme is specific for the β-anomers of d-glucose and d-galactose, and requires NADP\(^+\) as a cofactor (Smith et al., 1989). Reactions were carried out at 30 °C in 50 mM Tris–HCl (pH 8.8) and 3 mM NADP\(^+\). The total reaction volume was 1 mL, 9 U (manufacturer’s definition) of glucose/galactose dehydrogenase were used per reaction, and Gal10p was present at a final concentration of 1 nM (galactose reactions) or 10 nM (glucose reactions). Reactions were initiated by the addition of freshly dissolved galactose or glucose (Sigma) to a final concentration of 300 μM. The absorbance at 340 nm was monitored for 60 min, and the data were corrected such that the curve passed through the origin and fitted to the equation \(A_{340\text{ nm}} = k_1(1 - \exp(-k_2t))\), where \(k_1\) and \(k_2\) are constants. The initial rate was estimated from the product \(-k_1k_2\). Initial rates in the absence of Gal10p were subtracted from rates in the presence of Gal10p to give the rate of the reaction catalyzed by Gal10p (\(v_i\)). At low substrate concentrations, the Michaelis–Menten equation reduces to \(v_i = \frac{k_{cat}}{[\text{Gal10p}][\text{sugar}]/K_m}\). From this relationship, the specificity constant \((k_{cat}/K_m)\) was determined.

Crosslinking of Gal10p

Recombinant Gal10p (8 μM) was incubated for c. 5 min at 30 °C. Suberic acid \(\text{bis}(3\text{-sulfo-N-hydroxysuccinimide ester})\) (BS\(^3\)) (Partis et al., 1983) was added (final concentration, 100 μM), and the reaction was allowed to proceed for 30 min at the same temperature. Reactions were stopped by the addition of an equal volume of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer [125 mM Tris–HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate, 20% (w/v) glycerol, 1% (w/v) dithiothreitol, 0.002% (w/v) bromophenol blue] and heating at 95 °C for 5 min. Products were analyzed by 8% SDS-PAGE.

Results and discussion

Gal10p is a dimer in solution

Gal10p can be expressed in and, purified from, \textit{Escherichia coli} (Fig. 1a). The crystal structure of the protein (Thoden & Holden, 2005) suggests that it is a homodimer, with the dimerization occurring through the epimerization domains. All UDP-galactose-4-epimerases so far characterized are dimers (Thoden et al., 1996; Roper & Ferguson, 2003; Timson, 2005). The situation with galactose mutarotases is more varied. The first mutarotase to have its structure solved, the \textit{Lactococcus lactis} enzyme, is a dimer (Thoden & Holden, 2002; Thoden et al., 2002). However, the human enzyme is a monomer (Mulhern et al., 1979; Timson & Reece, 2003; Thoden et al., 2004). Protein–protein crosslinking, using the amine-specific crosslinker BS\(^3\) (Partis et al., 1983), was used to assess the oligomeric state of Gal10p in solution. The protein clearly forms dimers under these conditions. As in the human enzyme (Timson, 2005), small amounts of higher-order oligomers were also observed with this method. The amount of dimer produced does not
appear to be affected by the presence of UDP-galactose, galactose, or a mixture of both substrates (Fig. 1b).

The epimerase activity of Gal10p has similar characteristics to that of the human enzyme and is not affected by the mutarotase activity

The recombinant enzyme is an active UDP-galactose-4-epimerase (Fig. 2a). The kinetic parameters \( K_m = 89 \pm 13 \, \mu M \); \( k_{cat} = 13 \pm 1 \, s^{-1} \); \( k_{cat}/K_m = (1.4 \pm 0.2) \times 10^5 \, \text{L mol}^{-1} \text{s}^{-1} \) are of a similar order of magnitude to those obtained with recombinant human enzyme (Wöhlers & Fridovich-Keil, 2000; Timson, 2005). To test whether the activity of the epimerase active site is affected by events at the mutarotase active site, the epimerase activity was measured at a constant UDP-galactose concentration (80 \( \mu M \); chosen so as to be approximately equal to \( K_m \)) in the presence of increasing concentrations of freshly dissolved galactose. Although this concentration range reached 100 mM [typical \( K_m \) values for mutarotases are in the range 10–100 mM (Li et al., 1964; Bailey et al., 1966; Mulhern et al., 1973; Thoden et al., 2002; Beebe et al., 2003; Timson & Reece, 2003)], there was essentially no effect on the epimerase rate (Fig. 2b).

The mutarotase site of Gal10p shows a preference for galactose over glucose, and the activity is not affected by the epimerase activity

The protein is an active mutarotase and, in common with other mutarotases that have been characterized, shows a preference for galactose over glucose (Table 1). Indeed, the activity with glucose was so low under these conditions as to

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**Fig. 1.** Expression, purification and dimerization of Gal10p. (a) 10\% SDS-PAGE showing the expression and purification of Gal10p. The protein was expressed as a hexahistidine-tagged fusion protein in *Escherichia coli* and purified on nickel agarose resin. Typical yields were 5 mg of protein per liter of bacterial culture. (b) Dimerization of recombinant Gal10p was assessed by protein–protein crosslinking using the amine bifunctional reagent BS3. In this experiment, equal amounts of protein (8 pmol) were loaded in each lane. The results were analyzed by 8\% SDS-PAGE.
be difficult to measure above the rate of uncatalyzed mutarotation. The ratio of the specificity constants for these two substrates is $c.100$. This is a higher value than that seen for the human enzyme ($c.4$) (Timson & Reece, 2003), and is consistent with the GAL10 gene being induced in low glucose and high galactose concentrations. In Sac. cerevisiae, the GAL genes, which encode the proteins of the Leloir pathway, are under tight transcriptional control. When glucose is present in the growth medium, the action of the repressor Mig1p ensures that the GAL genes are switched off (Frolova et al., 1999). However, when galactose is the sole carbon source, the action of the gene regulatory proteins Gal4p, Gal80p and Gal3p ensure that the GAL genes are transcribed rapidly and to a high level (reviewed by Sellick & Reece, 2005). Thus, in vivo, the enzyme would be unlikely to encounter glucose as a substrate. Aldose epimerase activity can be detected in extracts from Sac. cerevisiae cells that have been grown in media containing 2% (w/v) glucose (Brahma & Bhattacharyya, 2004). Presumably, this is due to one of the two other mutarotase-like sequences in the yeast genome (YHR210c and YNR071c) (Goffeau et al., 1996) – most likely YHR210c, as YNR071c lacks the active site base present in all other known mutarotases. The mutarotase reaction appears to be unaffected by activity at the epimerase active site: addition of UDP-galactose to the reaction mix results in essentially no change in the mutarotase activity.

### Conclusions

The absence of kinetic interactions between the active sites suggests that the two activities operate independently and do not influence or regulate each other. The enzymes of the Leloir pathway may associate (Christacos et al., 2000) to form a metabolon. One advantage of this would be to enable communication between the various active sites and thus regulation at a pathway level. That no communication is observed between the mutarotase and epimerase active sites is thus an interesting result. The possibility of supramolecular organization of the Leloir pathway enzymes in Sac. cerevisiae is currently being investigated.

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