The Role of Tryptophans 371 and 395 in the Binding of Antibiotics and the Transport of Sugars by the D-Galactose-H\(^+\) Symporter (GalP) from Escherichia coli*

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The interactions between the D-galactose-H\(^+\) symporter (GalP) from Escherichia coli and the inhibitory antibiotics, cytochalasin B and forskolin, and the substrates, D-galactose and H\(^+\), have been investigated for the wild-type protein and the mutants Trp-371 → Phe and Trp-395 → Phe, so that the roles of these residues in the structure-activity relationship could be assessed. Neither mutation prevented photolabeling by either [\(^{3}H\)]cytochalasin B or by 3-[\(^{125}\)I]iodo-4-azidophenethylamido-7-O-succinylforskolin ([\(^{125}\)I]JAPS-forskolin). However, measurements of protein fluorescence show that both residues are in structural domains, the conformations of which are perturbed by the binding of cytochalasin B or forskolin. Moreover, both mutations cause a substantial decrease in the affinity of the inward-facing site of the GalP protein for cytochalasin B, 10- and 43-fold, respectively, but have little effect upon the affinity of this site for forskolin, 0.8- and 2.6-fold reductions, respectively. Both these mutations change the equilibrium between the putative outward- (T\(_{o}\)) and inward-facing (T\(_{i}\)) conformations, so that the inward-facing form is more favored. They also stabilize a different conformational state, “T\(_{i}\)-antibiotic,” in which the initial interactions between the protein and antibiotics are tightened. Overall, this has the effect of compensating for the reduction in affinity for cytochalasin B, so that the respective overall K\(_{d}\) values are 0.74- and 3.5-fold that of the wild type, while causing a slight increase, 1.5- and 3.2-fold, respectively, in affinity of the inward-facing site of the GalP protein for cytochalasin B or forskolin. Moreover, both mutations cause a 15-fold reduction in the affinity of the inward-facing site for D-galactose, suggesting that this residue forms part of the sugar binding site. In contrast, the Trp-395 → Phe mutation has no effect upon the affinity of the inward-facing site for D-galactose. These effects may be related to the reduction in galactose-H\(^+\) symport activity only in the Trp-371 → Phe mutant, although it still effects active transport to the same extent as the Trp-395 → Phe mutant. However, there is a 10–20-fold increase in the K\(_{m}\) values for energized transport of d-galactose for both mutants.

The D-galactose-H\(^+\) symporter (GalP)\(^{3}\) from Escherichia coli is homologous to a family of mammalian glucose transporters (GLUT) (Maiden et al., 1987; Baldwin and Henderson, 1989; Henderson and Maiden, 1990; Henderson, 1990; Henderson et al., 1992; Griffith et al., 1992). The GalP and GLUT proteins are, therefore, predicted to have a similar membrane topology, comprising 12 membrane-spanning \(\alpha\)-helices, with helices 6 and 7 connected by a cytoplasmic domain containing 60–70 amino acids (Mueckler et al., 1985; Griffith et al., 1992; Roberts, 1992). Moreover, GalP has many properties in common with mammalian glucose transporters. The sugar specificity of GalP is very similar to that of the human erythrocyte (GLUT1) and the rat adipocyte (GLUT4) glucose transporters (Barnett et al., 1973; Rees and Holman, 1981; Cairns et al., 1991; Walmsley et al., 1994b).

Another similarity is that GalP-mediated sugar transport is inhibited by the antibiotics cytochalasin B and forskolin (Henderson and Maiden, 1990; Cairns et al., 1991; Walmsley et al., 1993; Martin, 1993; Martin et al., 1994, 1995; Walmsley et al., 1994a, 1994b), which are potent inhibitors of glucose transporters (Baldwin et al., 1980; Baldwin and Lienhard, 1981; Shanahan, 1982; Gorga and Lienhard, 1981, 1982; Walmsley, 1988; Carruthers and Helgerson, 1991; Helgerson and Carruthers, 1987; Carruthers, 1990; King et al., 1991; Burant and Bell, 1992). The GLUT1 and GalP proteins can be photoabeled by these antibiotics, or derivatives of them, in a D-glucose-inhibitable manner (Cairns et al., 1984, 1987, and 1991; Holman et al., 1986; Holman and Rees, 1978; Karim et al., 1987; Wadzinski et al., 1990; Roberts, 1992; Martin 1993; Martin et al., 1994). The binding of either cytochalasin B or forskolin induces a quench in the protein fluorescence of both GLUT1 and GalP (Gorga and Lienhard, 1982; Carruthers, 1986; Pawagi and Deber, 1990; Chin et al., 1992; Walmsley et al., 1994, 1995; Martin, 1993; Martin et al., 1995). This phenomenon allowed the kinetics of the binding of cytochalasin B to GLUT1 and GalP to be monitored by stopped-flow fluorescence spectroscopy, showing that the mechanisms of binding of cytocha-

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*The abbreviations used are: GalP, the D-galactose-H\(^+\) symporter of E. coli; AraE, the L-arabinose-H\(^+\) symporter of E. coli; GLUT1, the human erythrocyte glucose transportor; CB, cytochalasin B; T\(_{o}\), conformational form of transporter; DNP, 2,4-dinitrophenol; MES, 4-morpholineethanesulfonic acid; [\(^{3}\)H]JAPS-forskolin, 3-[\(^{125}\)I]iodo-4-azidophenethylamido-7-O-succinylforskolin.
larin B to GLUT1 or GalP were similar (Walmsley et al., 1994a). Also, the mechanism for the binding of forskolin to GalP has been analyzed and is similar to that for cytochalasin B (Martin et al., 1995). All these data identified at least three conformational states of the transport proteins, changes in which were detected by measuring protein fluorescence.

The efficiency of photolabeling GLUT1 with antibiotics is maximal at 280 nm, suggesting that a tryptophan residue may be activated prior to the covalent attachment of the antibiotic. Moreover, the binding of antibiotics induces a conformational change in GLUT1, where the environment around one or more of the tryptophans must be perturbed. The sites of covalent attachment for cytochalasin B and forskolin in GLUT1 have been localized to putative α-helices 10 and 11 by identifying the photolabeled proteolytic fragments from GLUT1 (Cairns et al., 1984, 1987; Holman et al., 1986; Holman and Rees, 1987; Karim et al., 1987; Wadzinski et al., 1990). The tryptic digestion of the GalP protein, which has been photolabeled with [4-3H]cytochalasin B, also produces a labeled fragment (M, 17,000–19,000) of almost identical M, to that produced by digestion of labeled GLUT1 (Cairns et al., 1991). There are two tryptophan residues in GLUT1 that lie within this region, Trp-388 and Trp-412, toward the cytoplasmic ends of putative helices 10 and 11, respectively (Mueckler et al., 1985). Furthermore, cytochalasin B is thought to bind to the inward or cytoplasmic facing site (Deves and Krupka, 1978; Gorga and Lienhard, 1981), so that these tryptophan residues seem likely candidates for forming a dynamic segment of the transporter.

The role of these residues has been investigated in several studies in which mutant proteins, produced by site-directed mutagenesis, were tested for their ability to bind antibiotics and transport sugars (Katagiri et al., 1991, 1993; Garcia, et al., 1992; Schurmann et al., 1993). The eukaryotic expression systems used did not provide sufficient amounts of the GLUT1 mutant proteins to allow them to be characterized in detail. The only firm conclusion reached by these studies is that neither Trp-388 nor Trp-412 is essential for photolabeling with [4-3H]cytochalasin B (Martin et al., 1994a). The alignment of the amino acid sequence of GalP with that of GLUT1 indicates that these tryptophan residues are conserved in GalP and indeed within most members of this extensive family of sugar transport proteins (Griffith et al., 1992). It seems highly likely that these residues, Trp-371 and Trp-395, respectively, in the GalP sequence (Roberts, 1992), play similar roles in the function of the GalP and GLUT proteins. The present investigation has achieved a rigorous characterization of the role(s) of these residues in the binding of antibiotics, β-galactose, and protons, and in the subsequent translocation events. This investigation was only possible because the wild-type and mutant GalP proteins could be produced in high yield, from a strain of E. coli that overexpresses GalP (Roberts, 1992), which is amenable to detailed transient kinetic studies (Walmsley et al., 1994a, 1994b; Martin et al., 1995).

In this paper we have determined the precise changes in the kinetic constants caused by mutagenesis of the two highly conserved tryptophan residues in GalP on β-galactose transport. Changes in the inhibition by, and binding of, cytochalasin B and forskolin were rigorously quantified. Also, the effect of these mutations on photolabeling with [4-3H]cytochalasin B and [125I]APS-forskolin, and on galactose- H + symport activities, were determined. Moreover, we have identified these changes with different conformational states of the protein and with the distribution between inward- and outward-facing forms.
Tryptophan Mutants of GalP

TABLE I

A comparison of the steady-state kinetic parameters for the uptake of D-galactose mediated by wild-type and mutant GalP proteins.

| Protein | $V_{max}$ (nmol/mg min) | $K_m$ (μM) | $V_{max}/K_m$ (nmol/mg min/μM) | Ratio of D-galactose accumulation (inside/outside) |
|---------|-------------------------|-------------|-------------------------------|-----------------------------------------------|
| Wild-type | 65.4 ± 2.3 | 58.9 ± 8.6 | 1.14 | 116.67 |
| Trp-371 → Phe | 11.1 ± 0.4 | 564.7 ± 58.3 | 0.02 | 7.71 |
| Trp-395 → Phe | 4.75 ± 3.3 | 1127.4 ± 188.0 | 0.04 | 7.69 |

150 mM KCl, 2 mM glycyglycine, pH range 6.3–6.5, as detailed by Henderson and Macpherson (1986).

Mutations in Trp-371 and Trp-395 Impair D-Galactose Transport Activity of GalP

The transport of D-galactose by wild-type GalP, under energized conditions, was characterized by an hyperbolic increase in the initial rate with increasing concentrations of sugar. A least squares fit yielded $K_m$ and $V_{max}$ values of 42–59 μM and 59–65 nmol/mg/min, respectively (Table I).

The substitution of Trp-371 by Phe in the D-galactose-H$^+$ symporter (GalP) seriously impaired its ability to transport D-galactose under energized conditions, with more than a 10-fold increase in the $K_m$ and a 6-fold decrease in the $V_{max}$ compared with the wild-type (Table I). We can assume that Δ$p$ is unaffected at the low levels of GalP expression applying in these experiments (see "Materials and Methods"). While the $V_{max}$ term is largely governed by the rate constants for reorientation, the $K_m$ term is governed by both the rate constants for translocation and the true affinity of the sugar binding site (Walmsley, 1988; Lowe and Walmsley, 1986). The rate of sugar translocation will be governed by the rate constants for reorientation of the unloaded and sugar-loaded transporter. The increase in the $K_m$ for this mutant may simply reflect a reduction in the rate constant for reorientation of the loaded transporter, rather than any change in affinity. An increase in the rate constant for reorientation of the unloaded transporter would lead to a decrease in $K_m$.

In contrast, for the Trp-395 → Phe mutant, there was about a 20-fold increase in the $K_m$, but the $V_{max}$ was only reduced to about half that of the wild type (Table I). This suggests that this mutation has a more pronounced effect on the affinity of the transporter for sugar, rather than on the rate constants for translocation.

In both cases the data indicate a significant reduction in the specificity ($V_{max}/K_m$) of the mutant transport proteins for D-galactose relative to the wild-type transport protein. The specificity of the Trp-371 mutant was reduced 56-fold and that of the Trp-395 mutant 26-fold, relative to the wild type (Table I). However, it was demonstrated that the mutant proteins catalyze energized transport by measuring the maximum accumulation of 0.05 mM D-galactose in the presence and absence of the uncoupler DNP (2 mM). The intracellular concentration of D-galactose was measured at 4.2, 0.216, and 0.277 mM for the cells expressing wild type Trp-371 → Phe and Trp-395 → Phe protein, respectively (using an intracellular volume of 2.059 μl dry weight of cells). The concentration of intracellular D-galactose for cells treated with DNP was measured at 0.036 mM, 0.028 mM and 0.036 mM for cells expressing the wild type, Trp-371 → Phe and Trp-395 → Phe proteins, respectively, indicating that the cells were completely uncoupled. A comparison of the intracellular D-galactose concentrations of cells treated with and without DNP showed that D-galactose was accumulated 116.67–7.71-, and 7.69-fold by cells expressing the wild-type Trp-371 → Phe and Trp-395 → Phe proteins, respectively (Fig. 1). The use of the host strain JM1100, which has a GalK phenotype and is therefore unable to metabolize D-galactose, ensured that any effects of DNP on the metabolic enzymes would have no effect on the accumulation of D-galactose.

We conclude that both mutants are still capable of energized sugar uptake against a concentration gradient, albeit at an efficiency 1% of wild type.

Photoaffinity Labeling with Cytochalasin B and [125I]APS-forskolin

Fluorescence Studies—Fluorescence spectra were measured in a Jasco FP777 spectrofluorimeter. The protein was excited at 297 nm and the fluorescence emission monitored between 300 and 550 nm. Rapid fluorescence measurements were carried out in 50 mM potassium phosphate, 100 mM NaCl, and 1 mM EDTA, buffered to pH 7.4 at 20°C.

The wild-type GalP protein was susceptible to photolabeling with [4-3H]cytochalasin B and [125I]APS-forskolin, which could be significantly inhibited by D-galactose, but not L-galactose, as

\[ \Delta p \] The values quoted throughout the text for $K_m$ or $V_{max}$ are only apparent values. The true values cannot be calculated, because the affinity for the second substrate, H$^+$, is not known.

\[ \text{Ratio of D-galactose accumulation (inside/outside)} \]
shown in Fig. 2 (Cairns et al., 1991; Martin et al., 1994). The hexose \( \alpha \)-galactose is the physiological substrate for the GalP protein, which has little, if any, affinity for \( \beta \)-galactose (Cairns et al., 1991; Martin et al., 1994; Walmsley et al., 1994b). This protection by substrate indicated that both cytochalasin B and \(^{125}\)IAPS-forskolin specifically label the GalP protein. Previous studies showed that the wild-type GalP protein is not susceptible to photolabeling with forskolin (Martin et al., 1994), but this antibiotic is a transport inhibitor (Martin, 1993; Martin et al., 1994, 1995).

The labeled mutant GalP proteins were found to migrate to identical positions to the wild-type GalP protein on the gels (Fig. 2), confirming their identity.

The Trp-371 mutant GalP protein was less readily photolabeled with \(^{3}H\)cytochalasin B, but more readily photolabeled with \(^{125}\)IAPS-forskolin than the wild-type protein (Fig. 2); \( \alpha \)-galactose was less effective at protecting the mutant against photolabeling, by either inhibitor, than the wild-type protein (Fig. 2).

In the case of the Trp-395 \( \rightarrow \) Phe mutant the extent of photolabeling with cytochalasin B was moderately less than wild-type, whereas photolabeling with \(^{125}\)IAPS-forskolin was greater for the mutant than the wild type. However, substrate protected the Trp-395 \( \rightarrow \) Phe mutant against photolabeling by both compounds better than its protection of the Trp-371 \( \rightarrow \) Phe mutant.

We conclude that neither Trp-371 nor Trp-395 is essential for photoaction with \(^{3}H\)cytochalasin B and \(^{125}\)IAPS-forskolin, although we cannot rule out the possibility that the Phe residues are susceptible to photolabeling.

Inhibition of \( \alpha \)-Galactose Transport into Wild-type and Mutant GalP Strains by Antibiotics

The abilities of (80 \( \mu \)M) cytochalasin B or (80 \( \mu \)M) forskolin to inhibit the uptake of (50 \( \mu \)M) \( \alpha \)-galactose by energized cells, from wild-type and mutant GalP strains of E. coli, were determined. The cell walls of the intact cells were rendered permeable to the antibiotics by prior treatment with Tris-EDTA, pH 8 (see “Materials and Methods”).

In the case of the wild type, both cytochalasin B and forskolin inhibited the transport of \( \alpha \)-galactose, but cytochalasin B was a more potent inhibitor (Fig. 3). The reason for this difference is not clear, since titration of the protein fluorescence of GalP, with these antibiotics, indicates that the GalP protein has a similar overall affinity (K, ) for both of them (Table I).

Interestingly, cytochalasin B and forskolin were more potent inhibitors of the Trp-371 \( \rightarrow \) Phe mutant mediated \( \alpha \)-galactose transport, relative to the wild type. This is most likely due to the greatly impaired \( \alpha \)-galactose binding capability of the Trp-371 \( \rightarrow \) Phe mutant protein (higher K, ) together with the higher affinity (lower K, ) that the mutant has for both cytochalasin B and forskolin compared with the wild-type protein (Table I).

Furthermore, forskolin was more potent than cytochalasin B with the Trp-371 \( \rightarrow \) Phe mutant (Fig. 3), consistent with the higher affinity (lower K, ) of the mutant for forskolin compared with cytochalasin B (Table I).

In contrast, both cytochalasin B and forskolin were less effective inhibitors of the transport of \( \alpha \)-galactose mediated by the Trp-395 \( \rightarrow \) Phe mutant, than by the wild-type, and were much less potent against the Trp-395 \( \rightarrow \) Phe than against the Trp-371 \( \rightarrow \) Phe mutant (Fig. 3). The antibiotic forskolin had a significantly greater inhibitor potency than cytochalasin B, with the Trp-395 \( \rightarrow \) Phe mutant. This probably reflects the substantial difference in the overall affinity (K, ) of the Trp-395 mutant GalP for forskolin and cytochalasin B (Table I).

Fluorescence Studies of the Binding of Antibiotics

Comparisons of the Steady-state Protein Fluorescence Changes Induced by the Binding of Cytochalasin B and Forskolin—The ability to overexpress the wild-type and mutant GalP proteins enabled a much more rigorous analysis of the kinetics of the binding of cytochalasin B and forskolin by monitoring the associated changes in tryptophan fluorescence of GalP (Walmsley et al., 1994a; Martin et al., 1995).

In order to assess the importance of Trp-371 and Trp-395 in the binding of cytochalasin B and forskolin, the intrinsic fluorescence levels of the wild-type and mutant proteins were titrated with these antibiotics in the stopped-flow apparatus. Although the changes in tryptophan fluorescence of the mutant proteins were less than for the wild-type protein, they were sufficient to allow detection of the binding of both cytochalasin B (Figs. 4B and 5B) and forskolin (Fig. 4D and 5D) to both mutants. In each case, the stopped-flow records were best fitted to a double-exponential function. The total amplitudes of the fluorescence changes were determined from the sum of the amplitudes of the two phases and are plotted as a function of each antibiotic concentration in Figs. 4, B and D, and 5, B and D. A fit of each set of data to a quadratic equation for a second-order binding process (Walmsley et al., 1993) yielded the K, values, and maximal fluorescence changes, for cytochalasin B and forskolin given in Table I.

The wild-type protein has a similar overall affinity for cytochalasin B and forskolin, with K, values of about 1.8–1.9 \( \mu \)M (Table I). The binding of cytochalasin B causes a larger quenching of the tryptophan fluorescence of the wild-type protein than does forskolin, although we cannot rule out the possibility that the Phe residues are susceptible to photolabeling.
in the protein fluorescence, of 7.6%, than forskolin, which only causes a 4.2% quench (Table II).

There was a slight increase in the overall affinity of the Trp-371 → Phe mutant GalP protein for both cytochalasin B and forskolin, which was concomitant with a small decrease in the fluorescence quench, relative to the wild type. However, the differences from the wild type are small.

The Trp-395 → Phe mutation had a much more pronounced
effect, causing a 3.5-fold reduction in the overall affinity for cytochalasin B and 3.2-fold increase in the affinity for forskolin (Table I). Indeed, while the wild-type protein has similar affinities for cytochalasin B and forskolin, the Trp-395 → Phe mutant has an 11–12-fold difference in affinities for these antibiotics. In addition, the fluorescence quenches with these antibiotics were reduced to about half those for the wild type (Table II).

Comparisons of the maximal fluorescence changes, corrected for the relative levels of expression, indicated that both Trp-371 and Trp-395 contribute to the fluorescence changes associated with the binding of cytochalasin B and forskolin to the GalP protein. Trp-395 contributes much more to the protein fluorescence signal, with cytochalasin B and forskolin, than Trp-371. Thus, although it is likely that neither residue is photolabeled with the binding of cytochalasin B and forskolin, indicating that they form part of a dynamic segment, or segments, of the transporter, which might be part of the antibiotic binding site. Although neither residue is solely responsible for the fluorescence changes associated with the binding of cytochalasin B and forskolin, together, they are probably responsible for most, if not all, of the signals.

Comparisons of the Transient Kinetics of the Binding of Cytochalasin B and Forskolin—Variations in the rate constants for the fast and slow phases in the binding of the antibiotics are informative of the kinetic mechanism (Walmsley et al., 1993, 1994a; Martin et al., 1995). We have previously shown for GalP, GLUT1, and AraE sugar transport proteins that the rate of the fast phase increases linearly with the antibiotic (A) concentration, while that of the slow phase is constant. The fast phase was attributed to the formation of the transporter-antibiotic complex (T2(A)), while the slow phase was attributed to reorientation of the transporter between inward and outward-facing conformations (T1–T3). The following kinetic scheme was proposed for the binding of antibiotics,

\[
T_1 \rightarrow T_2 \rightarrow T_2(A) \rightarrow T_3(A) \\
T_1 \rightarrow T_2 \rightarrow T_3(A)
\]

in which T1 and T2 are, respectively, the outward- and inward-facing forms of the unloaded transporters. In addition, for GLUT1 there was an additional state T2(CB) (Walmsley et al., 1994a). T2(A) and T3(A) are two different conformations of the transporter-antibiotic complex. For such a model the apparent dissociation constant of the transporter-antibiotic complex is given by the following equation,

\[
K_d = K_d(1 + K_i) \left(\frac{1}{1 + K_i}\right)
\]

with \(K_1, K_2\), and \(K_3\) defined as \(K_1 = k_{32}k_{21}/k_{12}\), \(K_2 = k_{32}k_{12}/k_{21}\), and \(K_3 = k_{32}/k_{12}\). The second-order association rate constant (\(k_{32}\)) and dissociation rate constant (\(k_{21}\)) for the transporter-antibiotic complexes were determined from the slope and intercept, respectively, of a plot of the antibiotic concentration dependence of the rate of the fast phase, and the dissociation constants for these complexes were calculated from the rate constants \(K_2 = k_{32}/k_{21}\) (Table II).

As shown in Table II, the wild-type GalP protein was characterized by similar association rates for cytochalasin B and forskolin, but the dissociation of cytochalasin B was significantly slower than forskolin. Thus, the T2, or putative inward-facing transporter, has a higher affinity for cytochalasin B than forskolin.

In the case of wild-type GalP, the measured overall \(K_d\) for cytochalasin B is 4.6-fold greater than \(K_d\) (Table II), indicating that at least 78.4% of the transporters are in the T1 (or putative outward-facing) conformation prior to the binding of cytochalasin B (\(K_1 \approx (K_d/K_2) - 1\)) (Walmsley et al., 1994a). As such, the kinetics of the binding of cytochalasin B do not lend any sup-

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**TABLE II**

The kinetic parameters for the binding of cytochalasin B and forskolin to wild type GalP and mutant GalP proteins

The kinetics of the binding of antibiotics to the wild-type and mutant GalP proteins were determined by stopped-flow fluorescence spectroscopy, monitoring the antibiotic induced quench in protein fluorescence. A fit of the amplitude data to a quadratic equation for a second-order binding process, as described under “Results,” yielded the maximum percent quench in fluorescence and the overall affinity \(K_d\). The association \(k_{32}\) and dissociation \(k_{21}\) rate constants were determined from the antibiotic concentration dependence of the rate of signal change (protein fluorescence quench) as described under “Results.” The rate constants are numbered so as to be consistent with kinetic Scheme I. In each case, the dissociation constant \(K_d\) for the putative inward-facing transporter \(T_2\) was calculated from the measured values for \(k_{32}\) and \(k_{21}\). The kinetic data for the binding of cytochalasin B and forskolin are given in Table II.

| Protein | Cytochalasin B | Forskolin |
|---------|----------------|-----------|
|         | \(K_d\) | Quench | \(k_{32}\) | \(s^{-1}\) | \(k_{21}\) | \(s^{-1}\) | \(K_d\) | Quench | \(k_{32}\) | \(s^{-1}\) | \(k_{21}\) | \(s^{-1}\) |
| Wild-type | 1.9 | 0.6 | 6.0 | 0.14 | 2.5 | 0.20 | 0.41 | 0.13 | 18.3 | 0.56 | 4.2 | 0.59 | 6.6 | 0.24 | 3.0 | 0.43 |
| Trp-371 → Phe | 1.4 | 0.5 | 6.1 | 0.17 | 10.5 | 0.18 | 4.2 | 0.13 | 12.5 | 0.37 | 3.9 | 0.76 | 7.0 | 0.14 | 11.0 | 0.60 |
| Trp-395 → Phe | 6.7 | 1.0 | 6.7 | 0.13 | 10.5 | 0.24 | 17.5 | 0.12 | 0.58 | 0.43 | 2.1 | >8.6 | >28.4 | 3.70 | 2.70 |
port to the existence of the postulated T_{3}(CB) conformation for GalP. However, there is kinetic evidence to support the existence of all four postulated states of the human erythrocyte glucose transporter (GLUT1) (Appleman and Lienhard, 1985, 1989; Lowe and Walmsley, 1986, 1987; Walmsley and Lowe, 1987; Walmsley, 1988; Walmsley et al., 1994a).

On the other hand, forskolin is bound 3.5-fold less tightly, to the wild-type GalP protein, in the initial complex (e.g. T_{2}(forskolin)), due to its faster rate of dissociation (Table II). Accordingly, for forskolin, the overall $K_2$ is only marginally greater than $K_3$. However, the binding of forskolin is biphasic, indicating that this process involves at least two steps. Since there is an equilibrium between the T_{2} and T_{3} conformations in the absence of bound ligand, $K_1$ and 1/$K_3$ must have similar values when forskolin is bound (Martin et al., 1995).

The Trp-371 → Phe mutation was also characterized by a concentration-dependent linear increase in the rate of the fast phase of the binding of cytochalasin B (Fig. 4A) and forskolin (Fig. 4C), yielding the kinetic parameters given in Table II. For cytochalasin B, this mutation decreases the association rate constant, while the dissociation rate constant is increased, so that the affinity of the T_{2} conformation was reduced by 10.2-fold, relative to the wild type. The overall affinity of the mutant for cytochalasin B is similar, or slightly greater, to that of the wild-type ($K_2 > K_3$ for mutant), which indicates that, following the formation of the initial complex, there is a further tightening in the binding of cytochalasin B, as the mutant GalP protein undergoes the T_{2}(CB)-T_{3}(CB) transition. Thus, the

**Fig. 4. The binding of antibiotics to the Trp-371 → Phe mutant GalP protein.** The concentration dependence of the observed rate of binding of cytochalasin B (A) and forskolin (C) to the Trp-371 → Phe mutant GalP protein. The concentration dependence of the total quench in the protein fluorescence of the Trp-371 → Phe mutant GalP protein induced by the binding of cytochalasin B (B) and forskolin (D).
The T₃(antibiotic) state is now detectable in this mutant.

In contrast, the binding of forskolin to the Trp-371→Phe mutant GalP protein was characterized by only small changes in the association and dissociation rate constants; these were marginally higher and lower, respectively, than the wild-type values, so that the mutant protein had a higher overall affinity (lower Kᵅ) for forskolin (Fig. 5C, Table II).

More significantly, the Trp-395→Phe mutant was characterized by a substantial reduction in the cytochalasin B association rate constant and a moderate increase in the dissociation rate constant, so that the affinity of the T₂ conformation was dramatically reduced by 42.7-fold, relative to the wild-type (Fig. 5A, Table II). Again, the reduction in the affinity of the T₂ conformation was not matched by a similar reduction in the overall affinity for cytochalasin B, indicative of the formation of the T₃(CB) conformation.

Surprisingly, the Trp-395→Phe mutant was characterized by an apparently hyperbolic increase in the rate of the fast phase of binding of forskolin (Fig. 5C). These data provide clear evidence that the transporter-forskolin complex undergoes a further conformational change, the T₂(forskolin) to T₃(forskolin) transition. Presumably, for the wild-type protein, this transition occurs at a rate that is too fast to be measured by stopped-flow fluorescence spectroscopy. The data in Fig. 5C were fitted to a hyperbolic function yielding respective minimal and maximal rates of 3.6 (± 7.0) s⁻¹ and 28.4 (± 5.8) s⁻¹ and an apparent Kᵅ of 3.7 (± 2.7) μM. These values will correspond to those for k₊, k₋, and Kₛ, respectively, in Scheme I. Using
these values and the measured $K_d$, $K_3$ can be calculated as 0.41 from Equation 1.

The Trp-395 → Phe mutation has caused a large shift in the equilibrium between $T_2$ and $T_3$, so that about 29% of the transporters are in the $T_3$ (or outward-facing) conformation in the absence of ligands, while stabilizing the $T_2$ (forskolin) complex ($K_2 = (T_2(forskolin)|(T_2(forskolin)) = 7.7$). After equilibration with forskolin, the GalP-forskolin complex is present as 11.3% $T_2$(forskolin) and 88.7% $T_3$(forskolin). Furthermore, the affinity of the $T_3$ conformation for forskolin was reduced 2.5-fold. The above data also provide minimal values for $K_2$ ($K_3 + K_{k_3})/(K_D)$ and $K_{k_2} - (K_2 + K_{k_2})$ of 8.6 s$^{-1}$ and 28.4 s$^{-1}$, respectively, indicating that the reduction in the affinity of the $T_2$ conformation is largely attributable to an increase in the dissociation rate constant.

Accordingly, the substitution of Trp-395 for Phe causes a destabilization of the $T_1$ (or outward-facing) conformation ($K_1 \geq 3.63$) for the wild-type GalP protein (Walmsley et al., 1994a), $K_1 = 0.41$ for Trp-395 → Phe mutant GalP protein, while stabilizing the $T_3$(antibiotic) conformation. A value of 1.2 can be calculated for $K_3$, in the binding of cytochalasin B to Trp-395 → Phe mutant protein, since $K_3$ is known. Thus, at equilibrium, the GalP-cytochalasin B complex is present as 54.5% $T_2$(CB) and 45.5% $T_3$(CB). This value for $K_3$ for the $T_3$(cytochalasin B) complex compares with a value of 7.7 for the $T_3$(forskolin) complex, so that this state is more stable with bound forskolin.

### The Binding of d-Galactose to the Trp-371 → Phe Mutant Is Substantially Reduced

We have shown previously that the affinity of the GalP protein for d-galactose can be determined by measuring the d-galactose inhibition of the apparent rate of binding of cytochalasin B; this technique provides a measure of the affinity of the inward-facing site for the sugar (Walmsley et al., 1993, 1994a, 1994b). When the rate data, determined as a function of both the d-galactose and cytochalasin B, were fitted to an equation for competitive inhibition, this procedure yielded a value of 5.8 m\text{M} for wild-type GalP (Walmsley et al., 1993, 1994a). An alternative procedure for determining the $K_m$ for d-galactose was also developed during the course of the present studies, which had the advantage of being simpler. The vesicles were equilibrated with cytochalasin B, at a concentration above the $K_m$, and then mixed with varying concentrations of d-galactose in the stopped-flow. The displacement of bound cytochalasin B, presumably from the inward-facing site, by the sugar led to an increase in the protein fluorescence. The signal amplitude increased with the sugar concentration in a hyperbolic manner and a fit of the data to a hyperbolic function indicated a $K_m$ for d-galactose of 6.42 m\text{M}, after correction for competitive displacement by the cytochalasin B (Table III).

The rate of binding of cytochalasin B to the Trp-371 → Phe mutant GalP protein was determined as a function of both the d-galactose and cytochalasin B concentrations (Fig. 6a). The data were fitted to an equation for competitive inhibition (Walmsley et al., 1993, 1994a), yielding values for the cytochalasin B association and dissociation rate constants of 2.1 s$^{-1}$ and 12.0 s$^{-1}$, respectively, and an apparent $K_m$ for d-galactose of 88.7 m\text{M}. The association and dissociation rate constants compare well with those determined independently by monitoring only the binding of cytochalasin B (Table II). The $K_m$ for d-galactose can also be determined from a fit of the amplitude data (protein fluorescence quench) to an equation for competitive inhibition (Walmsley et al., 1993), yielding a value of 78.6 m\text{M} (Fig. 6b). In addition, the $K_m$ for d-galactose was also determined by cytochalasin B displacement, yielding a value of 71.7 m\text{M} (Table I). When these $K_m$ values for the mutant are compared with those for the wild-type, they indicate that the Trp-371 → Phe mutation causes a 15-fold decrease in affinity of the inward-facing conformation of GalP for d-galactose. This is consistent with the fact that d-galactose affords less protection against photobleaching of the mutant than the wild-type.

### Table III

A comparison of the dissociation constants for the binding of d-galactose to wild-type and mutant GalP proteins

| Protein          | $K_d$ (m\text{M}) |
|------------------|-------------------|
| Wild-type        | 6.42 ± 2.63       |
| Trp-371 → Phe    | 71.7 ± 39.8       |
| Trp-395 → Phe    | 6.26 ± 2.31       |

###  \text{d-Galactose-H}^+ Symport Activity Is Abolished in the Trp-371 → Phe GalP Mutant but Not in the Trp-395 → Phe Mutant

Sugar-H$^+$ symport activity can be assayed by measuring alkaline pH changes associated with the addition of high concentrations of sugar to anaerobic suspensions of de-energized intact bacteria (Karlin et al., 1987; Henderson and Macpherson, 1986). Representative traces shown in Fig. 7 illustrate that the addition of 3.3 m\text{M} galactose to cells carrying the Trp-395 →
The mutation of Trp-371 and Trp-395 to phenylalanines in GalP yielded rates of H\textsuperscript{+} transport (2.62 ± 0.97 (5) nmol H\textsuperscript{+}/min/mg) comparable with those for the wild-type protein transport (3.32 ± 1.54 (8) nmol H\textsuperscript{+}/min/mg).

In complete contrast, cells carrying the Trp-371 → Phe mutation did not show any sugar-H\textsuperscript{+} symport activity (Fig. 7 and four additional measurements in two separate cultures). This important difference from both the wild-type and the Trp-395 → Phe mutation is discussed further below. It is possible that much higher concentrations of D-galactose need to be added to reveal sugar-H\textsuperscript{+} symport, given the much reduced affinity of this mutant for sugar (see above), but this could not be achieved in the experimental system used for measuring the pH changes.

**DISCUSSION**

From the results in this paper we conclude that residues Trp-371 and Trp-395 are located in conformationally dynamic positions within the GalP symporter, with both contributing to the changes in tryptophan fluorescence induced by the binding of antibiotics. Although neither residue is photolabeled by cytochalasin B or forskolin, this does not preclude the possibility that these residues interact directly with the antibiotics, which would be consistent with the observations discussed below.

The mutation of Trp-371 and Trp-395 to phenylalanines reduces the affinities of the initial GalP-cytochalasin B complexes by at least an order of magnitude. However, this is compensated for by a further conformational change in which the binding of the cytochalasin B is tightened, so that the overall affinities of the two mutants for cytochalasin B are similar to that of the wild-type protein.

In contrast, the affinities of the initial GalP-forskolin complexes formed by the two mutant proteins are similar to that of the wild-type protein. But again, there is a further tightening in the interaction between the antibiotic and the transporter, so that forskolin is bound more tightly by the mutants than by the wild-type, indicating that these residues interact differentially with cytochalasin B and forskolin.

The differential interaction of cytochalasin B and forskolin with GalP is best illustrated by the Trp-395 → Phe mutant. The replacement of Trp-395 by Phe causes about a 43-fold reduction in the affinity of the initial complex for cytochalasin B, but only a 3-fold reduction for forskolin. There is about a 3.5-fold reduction in overall affinity for cytochalasin B, while the overall affinity for forskolin is enhanced by about 3.2-fold. The increase in the overall affinity relative to those of the initial complexes is largely due to destabilization of the outward-facing conformation (>78% of wild-type GalP molecules are outward facing in the absence of ligands), so that the inward-facing conformation of the Trp-395 → Phe mutant, to which the antibiotics bind, is favored in the absence of ligands (K\textsubscript{1} = 0.41 and 71% are inward-facing). This is similar to the situation that prevails for human erythrocyte glucose transporters (GLUT1), where the inward-facing conformation progressively predominates at subphysiological temperatures (K\textsubscript{1} = 0.25 and 80% of the transporters face inwards at 20°C; Lowe and Walmsley, 1987; Walmsley, 1988). Furthermore, in both the case of the Trp-395 → Phe mutant GalP and GLUT1, there is evidence that these proteins undergo a further transition following the binding of cytochalasin B (e.g, in each case K\textsubscript{d} < K\textsubscript{j}): the T\textsubscript{2}(CB) to T\textsubscript{3}(CB) transition that further stabilizes the protein-cytochalasin B interaction.

The nature of the T\textsubscript{i}(antibiotic) to T\textsubscript{j}(antibiotic) transition is not known, but one possibility is that it represents a partial reorientation of the transporter (Martin et al., 1995). The binding of antibiotics may induce the closure of the binding site, by
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acting as transition-state analogues, which do not allow complete reorientation. In the case of wild-type GalP, there is no kinetic evidence to support the existence of the T3(CB) state (Martin et al., 1995). This suggests that the reorientation process may proceed to a smaller extent with cytochalasin B than with forskolin (Martin et al., 1995). This suggestion is consistent with the present studies, which show that the Trp-395→Phe mutant binds forskolin more tightly than cytochalasin B (K_d = 1.2 and 7.7 for cytochalasin B and forskolin, respectively). Moreover, the T_2 to T_3 transition for the Trp-395→Phe mutant is at least an order of magnitude faster when cytochalasin B, rather than forskolin, is bound. In the case of wild-type GalP and the Trp-371→Phe mutant, this transition is too fast to be measured by stopped-flow fluorescence spectroscopy for both cytochalasin B and forskolin. Clearly, the T_2(CB) and T_3(forskolin) states are not equivalent and may be reached by different pathways.

The much higher value of the overall K_d for D-galactose (0.67 mM; Walmsley et al., 1994b) compared with K_m (0.06 mM, Table I) implies that energization increases the affinity of the sugar binding site (Cairns et al., 1991; Walmsley et al., 1994). The affinity of the inward-facing site for D-galactose was determined to be 5.8 mM by the inhibition of the rate of binding of cytochalasin B (Walmsley et al., 1994b). The affinity of this site was also determined by D-galactose titration of the fluorescence of 8-anilino-1-naphthalenesulfonate bound to GalP, yielding a K_d of 7.2 mM, from which the affinity of the outward-facing site was calculated to be 13.8 mM (Walmsley et al., 1994b). A comparison of the K_d values obtained for D-galactose binding to the mutants with that for the wild-type will be informative of any changes in the sugar binding site. On the other hand, a comparison with the K_m values for transport should be informative as to whether these mutations have affected the communication between the proton and sugar binding sites. The K_m for the binding of D-galactose to the inward-facing site of the Trp-371→Phe mutant GalP was 11–15-fold higher than for the wild-type, suggesting that this residue may be involved directly in the interaction with the internal sugar. The reduction in the affinity of the sugar binding site probably accounts for the decrease in the K_m for transport, which is about 10-fold higher than that for the wild-type. In contrast, the K_m for the binding of D-galactose to the inward-facing site of the Trp-395→Phe mutant GalP was similar to that of the wild-type, suggesting that this residue does not interact directly with the sugar. However, the transport K_m was increased about 20-fold relative to the wild-type, so that it is only about 5.6-fold lower than the K_m for sugar binding to the inward-facing site. This might have indicated that the primary effect of the Trp-395→Phe mutation was to decrease the coupling between the putative proton and sugar binding sites, but its sugar-H^+ symport activity was hardly diminished, while that of the Trp-371→Phe mutant was apparently abolished. However, both mutants were still able to accumulate galactose to about the same extent, in an uncoupler-sensitive manner. This implies that the Trp-371→Phe mutant is not fundamentally impaired in H^+ translocation, although its apparent affinity for protons may be diminished by changes in its binding of sugars. These intriguing observations are the subject of further investigations, aimed at determining the site(s) of H^+ binding, the participation of H^+ in the discrete kinetic events we have identified, and its possible pathway during translocation through the GalP protein.

Garcia et al. (1992) have mutated the corresponding tryptophan residues in GLUT1 (Trp-388 and Trp-412) to leucine and glycine residues. Essentially, they found that the GLUT1 Trp-388 mutants had a decreased sensitivity of transport to cytochalasin B, while the sensitivity of the Trp-412 mutants was unaltered. This contrasts with GalP, where the Trp-371 mutant-mediated D-galactose transport is more sensitive to cytochalasin B inhibition than the wild-type, which is more sensitive than the Trp-395 mutant. For GLUT1, the Trp-412 mutant, but not the Trp-388 mutant, was shown to have reduced transport activity. Both of the Trp mutants of GalP have reduced transport activities, with the Trp-371 mutant having the lowest specificity constant. On the other hand, Katagiri et al. (1991 and 1993) have reported that both the Trp-388→Leu and Trp-412→Leu mutants of GLUT1 have specificities (V_max/K_m) that are reduced to 20 and 15% of the wild type. However, the Trp-388→Leu mutation was reported to cause a decrease in the specificity constant (V_max/K_m) for zero-trans uptake that was not compensated for by a corresponding change in the specificity constant for zero-trans efflux (Katagiri et al., 1993); this contradicts the Haldane relationship for a passive transport system and is impossible (V_max/K_m(influx) = V_max/K_m(efflux); Lowe and Walmsley, 1987). Both of the mutants had reduced levels of photolabeling with antibiotics (Katagiri et al., 1991, 1993). Schurmann et al. (1993) have reported that the Trp-388→Leu mutation decreases both the photoaffinity labeling of GLUT1, with [125]APS-forskolin, and the glucose...
transport activity; while the Trp-412 → Leu mutation does not affect the photolabeling but abolishes transport activity. These later studies are more consistent with those reported herein on GalP, in which the transport activity (V_{max}/K_m) for the Trp-371 → Phe and Trp-395 → Phe mutants is reduced to 1.7 and 3.8% of the wild-type level, respectively. However, in contrast to GLUT1, the low specificity constant for the Trp-395

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