Asparagine 394 in Putative Helix 11 of the Galactose-H⁺ Symport Protein (GalP) from Escherichia coli Is Associated with the Internal Binding Site for Cytochalasin B and Sugar*

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The galactose-H⁺ symport protein (GalP) of Escherichia coli is very similar to the human glucose transport protein, GLUT1, and both contain a highly conserved Asn residue in predicted helix 11 that is different in a cytochalasin B-resistant member of this sugar transport family (XylE). The role of the Asn394 residue (which is predicted to be in putative trans-membrane α-helix 11) in the structure/activity relationship of the D-galactose-H⁺ symporter (GalP) was therefore assessed by measuring the interaction of sugar substrates and the inhibitory antibiotics, cytochalasin B, and forskolin with the wild-type and Asn394 → Gln mutant proteins. Steady-state fluorescence quenching experiments show that the mutant protein binds cytochalasin B with a Kd 37-53-fold higher than the wild type. This low affinity binding was not detected with equilibrium binding or photolabeling experiments. In contrast, the mutant protein binds forskolin with a Kd similar to that of the wild type and is photolabeled by 3-¹₁⁴C-azido-phenethyl-amido-7-O-succinyl-desacetyl-forskolin. The mutant protein displays an increased amount of steady-state fluorescence quenching with the binding of forskolin, suggesting that the substitution of the Asn residue has altered the environment of a tryptophan, probably Trp395, in a conformationally active region of the protein. Time-resolved fluorescence measurements on the mutant protein provided association and dissociation rate constants (k₂ and k₋₂), describing the initial interaction of cytochalasin B to the inward-facing binding site (T₁), that are decreased (9-fold) and increased (4.9-fold) compared with the wild type. This yielded a dissociation constant (Kd) for cytochalasin B to the inward-facing binding site 44-fold higher than that of the wild type. The binding of forskolin gave values for k₂ and k₋₂ 3.9- and 3.6-fold lower, respectively, yielding a Kd value for T₁ similar to that of the wild type. The low overall affinity (high Kd) of the mutant protein for cytochalasin B is due mainly to a disruption in binding to the T₁ conformation. It is proposed that Asn394 forms either a direct binding interaction with cytochalasin B or is part of the immediate environment of the binding site and that Asn394 is in the immediate environment, but not part, of the forskolin binding site. The ability of the mutant protein to catalyze energized transport is only mildly impaired with 4.8- and 2.1-fold reduction in V_max/Km⁴ values for D-galactose and D-glucose, respectively. In stark contrast, the overall Kd describing binding of D-galactose and D-glucose to the inward-facing conformation of the mutant and their subsequent translocation across the membrane is substantially increased (64-fold for D-galactose and 163.3-fold for D-glucose). These data indicate that Asn394 is associated with both the cytochalasin B and internal sugar binding sites. This conclusion is also supported by data showing that the sugar specificity of the mutant protein has been altered for D-xylose. This work powerfully illustrates how comparisons of the aligned amino acid sequences of homologous membrane proteins of unknown structure and characterization of their phenotypes can be used to map substrate and ligand binding sites.

The D-galactose-H⁺ symporter (GalP) from Escherichia coli is homologous to the L-arabinose-H⁺ symporter (AraE) and the D-xylose-H⁺ symporters (XylE) of E. coli, with 64 and 33% identity, respectively (1, 2). These transporters are also homologous to a family of mammalian passive facilitated glucose transporters (GLUT) (2–9). The inclusion of both passive and active transporters in the homologous transporter family implies they share common features of both structure and molecular mechanism. Furthermore, the E. coli and mammalian proteins are predicted to have a similar membrane topology, comprising 12 membrane spanning α-helices (12 TM), with helices 6 and 7 connected by a cytoplasmic domain containing 60–70 amino acids (Fig. 1) (2, 8, 10). The sugar specificities of the E. coli transporters differ, with GalP primarily transporting hexoses and AraE and XylE pentoses. However, the sugar specificity of GalP is very similar to that of the glucose transporters from human erythrocytes (GLUT1) and rat adipocytes (GLUT4) (11–14) giving rise to the suggestion that GalP is the bacterial equivalent of the mammalian glucose transporter (9).

This suggestion is reinforced by the observation that GalP-

* The abbreviations used are: GalP, the D-galactose-H⁺ symporter of E. coli; AraE, the L-arabinose-H⁺ symporter of E. coli; XylE, the D-xylose-H⁺ symporter of E. coli; GLUT1, the human erythrocyte glucose transportor; ANS, 8-anilino-1-naphthalene-sulfonate; TM, putative trans-membrane α-helix; Kd and V_max are apparent values as it was not known whether the co-substrate, H⁺, was at saturating amounts; MES, 24-morpholinethanesulfonic acid; IAPS-forskolin, 3-¹₁⁴C-azido-phenethylamido-7-O-succinyl-desacetyl-forskolin; APS, azido-phenethylamido-7-O-succinyl.
mediated sugar transport is inhibited by the antibiotics cytochalasin B and forskolin (13–17), which are also potent inhibitors of glucose transport mediated by GLUT1, GLUT2, GLUT3, and GLUT4 (18–29). The structures of these antibiotics are shown in Fig. 1. Both the GLUT1 and GalP proteins can be photolabeled by cytochalasin B and a derivative of forskolin (IAPS-forskolin) in a substrate-protectable manner (2, 13, 15, 16, 30–34). The binding of both cytochalasin B and forskolin induces a quench in the intrinsic protein fluorescence of both GLUT1 and GalP (14, 16, 22, 35–37). This phenomenon has allowed the kinetics of the binding of cytochalasin B to GLUT1 and cytochalasin B and forskolin to GalP to be time-resolved using stopped-flow fluorescence spectroscopy; the binding of cytochalasin B and forskolin to GalP and of cytochalasin B to GLUT1 occurs by similar mechanisms, with at least three conformational states of the transport proteins identified (14, 16, 38). The identification of transporter amino acid residues involved in the interaction with antibiotics will help elucidate the mechanism of recognition of both antibiotics and substrates. To this end, we have exploited the homology of the bacterial and mammalian transporters and compared their aligned amino acid sequences with their reported sensitivities to inhibition by cytochalasin B; it is probable that conserved residues are important in maintaining a common structure and mechanism and that differences are associated with variations in sugar specificity and antibiotic binding. In previous studies the covalent photolabeling of GLUT1 with cytochalasin B has been localized, by peptide mapping experiments, to a region containing amino acids Phe389 to Trp412 (30–33, 39), which is proposed to form part of TM11 and the cytoplasmic loop joining it to TM10. Site-directed mutagenesis experiments on GLUT1 (40) led to a model where cytochalasin B can photolabel either Trp389 or Trp412. These regions of the aligned sequences of GalP, AraE, XylE, GLUT1, GLUT2, GLUT3, GLUT4, GLUT7, and a high affinity D-fructose transporter, GLUT5 (41), were compared. The transporters GalP, AraE, GLUT1, GLUT2, GLUT3, and GLUT4 have a conserved Asn residue and are all susceptible to inhibition by cytochalasin B. In contrast, the transporters XylE, GLUT5, and GLUT7 do not have a conserved Asn residue and show no (XylE2 and GLUT5 (41)) or little (GLUT7 (42)) sensitivity to inhibition by cytochalasin B. We therefore predicted that the conserved Asn residue would be involved in cytochalasin B binding. To test this hypothesis Asn394 of GalP, which is predicted in a three-dimensional model to be in the middle of TM11 (Fig. 1), was changed by site-directed mutagenesis to Gln. The ability to over-express wild-type and mutant GalP proteins in high yield in appropriate E. coli strains has enabled us to achieve a rigorous characterization, which shows that changing Asn394 to Gln markedly diminishes sensitivity to the antibiotic cytochalasin B, but not to forskolin, and also diminishes binding of sugar to the inward-facing side of the protein.

MATERIALS AND METHODS

Bacterial Strains—E. coli strain JM1100 HfrC his-gnd2 thyA galK ptsM galP mglP mglF ptsG (43), containing plasmids with wild-type or mutated galP genes, was the host strain for over-production of the GalP protein. After the mutagenic reaction (see below) the recombinant bacteriophage M13 DNA was transfected into E. coli strain TG1 lacI (lac-proAB) supF thi hsdR51 F’[traDS6 proAB’ lacI qZ M15]. The modification (−) and restriction (−) phenotype of strain TG1 necessitated that the mutant plasmids constructed from DNA isolated from strain

FIG. 1. Two-dimensional model of the galactose-H+ symport protein. The Asn394 residue of GalP mutated to Gln in this study is indicated. The Asn residue is predicted to be in the middle of TM11. The structures of the sugar substrate D-galactose and of the inhibitory antibiotics cytochalasin B and forskolin are also shown.
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TG1 were transformed into strain DH1 (modification (+), restriction (−)) before introduction into E. coli strain JM1100 (modification (+), restriction (−) phenotype), to prevent restriction of unmodified plasmid DNA. E. coli strain DH1 has the genotype supE44 thi-1 relA1 gyrA96 thi-1 hsdR17 (rK- mK-) F−

Growth of the Over-producing E. coli Strains—The E. coli strain JM1100(pPER3) (wild-type GalP (2)) and JM1100(pTPM6) (GalP with the Asn394→Gln mutation, this work) were used for the constitutive over-expression of the wild-type and mutated GalP proteins. To gain maximum expression of GalP, strains were grown overnight in minimal media with 15 μg/ml tetracycline, supplemented with l-histidine (80 μg/ml) and thymine (20 μg/ml). For sugar transport and sugar-H+ symport experiments, requiring lower levels of expression, cells were grown on rich media (2TY supplemented with 20 mM glycerol, 20 μg/ml thymine, and 15 μg/ml tetracycline).

Preparation of Inner Membranes Containing Over-expressed Protein—The E. coli cells were disrupted by explosive decomposition in a French Press at 137.5 MPa, and the inner membranes were prepared essentially as described by Osborn et al. (44). This procedure yields predominantly inside-out vesicles (45).

Quantification of Over-expressed Protein—A sample of the membrane preparation (30 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis. After staining with Coomassie Blue, gels were scanned with a Molecular Dynamics 100A Computing Densitometer to determine the proportion of GalP protein was measured. The levels of expression of the wild-type and mutant proteins were determined by comparison of the intensities of the bands corresponding to the GalP proteins on Coomassie-stained SDS-polyacrylamide gels. The expression of the Asn394→Gln mutant protein was 107% that of the wild-type protein.

Protein Assays—The concentration of protein in the membrane preparations was assayed by the method of Schaffner and Weissman (46). Oligonucleotide-directed, Site-specific Mutagenesis—The codon (AAC) for asparagine 394 of the GalP protein was replaced by that of glutamine (CAG) as follows. The single-stranded DNA template for mutagenesis was prepared by subcloning a 2.46-kilobase pair Alul-Alul DNA fragment coding for the C-terminal half of GalP from plasmid pPER3 (which confers elevated expression of GalP (2)) into bacteriophage M13mp18. Mutagenesis was carried out with the Amersham Corp. in vitro mutagenesis kit and transferred to quartz cuvettes containing 0.4 nM 3-125I-APS-forskolin by autoradiography. The wild-type and mutant proteins were grown in 2 TY supplemented with 20 mM glycerol until A600 = 0.6. The cells were harvested and washed twice in the growth volume of 150 mM KCl, 5 mM MES, pH 6.6, and finally resuspended to a density of 0.68 mg of dry cell mass/ml. Aerated samples (0.25 ml) were incubated at 25 °C for 3 min with 25 mM glycerol before addition of 6.25 μl of radiolabeled sugar to give the indicated concentration range (10–300 μM). The filtered and washed four times in 2 ml of the above medium. The initial rates of sugar uptake (nmol/min per dry mass) were calculated and displayed as a Lineweaver-Burk plot. The graph is a typical example of the uptake of 1-(4-[3H]glucosylcatalyzed by wild-type and mutant proteins. The data yielded K$_v$ values of 58.9 ± 8.6 and 107.6 ± 34.0 μM and V$_{max}$ values of 65.4 ± 2.3 and 28.5 ± 2.2 nmol/mg/min for wild-type and mutant proteins, respectively. Similar experiments were carried out on the uptake of D-glucose and D-xylose, and the results are shown in Table I.

8.0, 1 mM EDTA. After 30 min, 100 volumes of 150 mM KCl, 5 mM MES, pH 6.5, 10 mM MgSO$_4$ were added and the cells harvested by centrifugation and resuspended in the same buffer.

Photoaffinity Labeling with 4-[3H]Cytochalasin B and IAPS-Forskolin—Ligands were incorporated into membrane proteins by irradiation with ultraviolet light (13, 49). Inner membrane vesicles were preincubated with or without 500 mM n-galactose at 4 °C in photolabeling buffer (50 mM sodium-potassium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4) and transferred to quartz cuvettes containing 0.4 nM 3-[3H]APS-forskolin or 0.5 μM 4-[3H]cytochalasin B. The samples were flushed with argon to free reduce free radical production and irradiated with ultraviolet light for 10 min. After irradiation, noncovalently bound ligand was removed by diluting the sample in photolabeling buffer containing 1% mercaptoethanol (for 3-[3H]APS-forskolin) or unlabelled cytochalasin B (for 4-[3H]cytochalasin B) and centrifugation (130,000 g for 2 h at 4 °C). The resulting membrane pellet was resuspended in 15 mM Tris/ HCl, pH 7.5, and assayed for protein. The proteins were separated by SDS-polyacrylamide gel electrophoresis. The incorporation of 3-[3H]cytochalasin B was monitored by fluorography and the incorporation of 4-[3H]cytochalasin B was monitored by autoradiography.

Fluorescence Studies—Fluorescence spectra were measured in a Perkin-Elmer LS50B spectrophotofluorimeter. The protein was excited at either 280 or 297 nm and the fluorescence emission monitored between 300 and 400 nm. Rapid reactions were followed using an Applied Photophysics (London, UK) spectrophotofluorimeter, operated at 20 °C, as described by Walsley et al. (14, 17, 50). 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. Routinely the membranes were used at a protein concentration of 200 μg/ml in the above buffer.

Equilibrium Dialysis—Equilibrium dialysis was carried out as described by Walsley et al. (50). The binding of 12-[3H]forskolin and 4-[3H]cytochalasin B to inner membranes (1.0 mg/ml) containing GalP was measured over the range of 0.05–80 μM ligand at 4 °C. Ratios of bound to free ligand were calculated from the equilibrium distribution of the radiolabeled ligand and used to determine both the $K_d$ and the number of ligand binding sites by an unweighted nonlinear least-squares fit of the data to an hyperbola using the Biosoft program Ultrafit 2.1.

Statistical Analyses—The apparent $K_v$ values, with their standard deviations, for transport of radiolabeled sugar were obtained by a least-squares fit of the unweighted initial rate data directly to a hyperbola.
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**RESULTS**

**Substitution of Asn\textsuperscript{394} by Gln Does Not Greatly Impair the Sugar Transport Activity of GalP**—The transport of D-galactose by the wild-type GalP protein, under energized conditions, was characterized by a hyperbolic increase in the initial rate with increasing concentrations of sugar. A least-squares fit yielded apparent \( K_m \) and \( V_{\text{max}} \) values of 42.4–58.9 \( \mu \)M and 59.1–65.4 nmol/mg/min (Fig. 2 and Table I). The \( K_m \) and \( V_{\text{max}} \) values quoted here and subsequently are apparent as it was not known whether the co-substrate, H\textsuperscript{+}, was at saturating amounts. The substitution of Asn\textsuperscript{394} by Gln led to a 2-fold increase in the \( K_m \) for D-galactose and a 2-fold decrease in the \( V_{\text{max}} \) compared with the wild type (Fig. 2 and Table I). The data indicated a relatively small (4.8-fold) reduction in the specificity (as defined by \( V_{\text{max}}/K_m \)) of the mutant protein for D-galactose relative to the wild type. The uptake of 50 \( \mu \)M D-galactose was accumulated by the mutant to 42-fold that of the external media, compared with 88-fold by the wild type. These data demonstrated that the mutant protein can catalyze energized transport and that the gross conformation of the protein is not significantly impaired.

D-Glucose was transported into the mutant with a \( V_{\text{max}} \) 1.5-fold higher than that of the wild-type protein, although the \( K_m \) was 3.3-fold higher (Table I). The transport of D-xyllose was characterized by a 4.3-fold increase in the \( K_m \) and a 0.8-fold decrease in the \( V_{\text{max}} \) compared with that of the wild type. The \( V_{\text{max}}/K_m \) values were 2.1-fold (D-glucose) and 5.3-fold lower (D-xyllose) than that of the wild type.

**The Asn\textsuperscript{394} → Gln Mutant Protein Still Catalyzes D-Galactose-Proton Symport**—The sugar-H\textsuperscript{+} proton symport activities of the wild-type and mutant proteins were assayed by monitoring D-galactose-promoted alkaline pH changes (data not shown), which are diagnostic of sugar-H\textsuperscript{+} symport (51, 52). The extent of D-galactose-H\textsuperscript{+} symport catalyzed by the mutant protein (5.19 ± 2.7 nmol of H\textsuperscript{+} per mg (n = 5)) was not significantly different from that of the wild-type protein (4.87 ± 3.88 nmol of H\textsuperscript{+} per mg (n = 8)). However, the rate of D-galactose-H\textsuperscript{+}-symport catalyzed by the mutant (1.16 ± 0.41 nmol of H\textsuperscript{+} per mg (n = 5)) was 2.8-fold lower that of the wild type (3.31 ± 1.54 nmol of H\textsuperscript{+} per mg (n = 8)). Again this implies that the gross conformation and transport competence of the mutant protein are not seriously affected by the Asn\textsuperscript{394} → Gln mutation.

**D-Galactose Transport Catalyzed by GalP with the Asn\textsuperscript{394} → Gln Mutation**

**TABLE I**

| Parameter               | Wild-type     | Asn\textsuperscript{394} → Gln |
|-------------------------|---------------|--------------------------------|
| \( K_m \) (D-galactose) | 42.4–58.9 \( \mu \)M | 107.6 ± 3 \( \mu \)M          |
| \( V_{\text{max}} \) (D-galactose) | 59.1–65.4 nmol/mg/min | 28.5 ± 2.2 nmol/mg/min |
| \( V_{\text{max}}/K_m \) (D-galactose) | 1.4–1.1 nmol/mg/min/\( \mu \)M | 0.26 nmol/mg/min/\( \mu \)M |
| \( K_m \) (D-glucose)    | 10.2 ± 3.5 \( \mu \)M | 34.2 ± 3.2 \( \mu \)M          |
| \( V_{\text{max}} \) (D-glucose) | 15.6 ± 1.4 nmol/mg/min | 23.6 ± 0.9 nmol/mg/min |
| \( V_{\text{max}}/K_m \) (D-glucose) | 1.5 nmol/mg/min/\( \mu \)M | 0.7 nmol/mg/min/\( \mu \)M |
| \( K_m \) (D-xyllose)    | 3800 ± 600 \( \mu \)M | 16500 ± 7900 \( \mu \)M        |
| \( V_{\text{max}} \) (D-xyllose) | 297.5 ± 25.5 nmol/mg/min | 241 ± 89.5 nmol/mg/min |
| \( V_{\text{max}}/K_m \) (D-xyllose) | 0.0768 nmol/mg/min/\( \mu \)M | 0.0146 nmol/mg/min/\( \mu \)M |

**Fig. 3.** Energized uptake catalyzed by GalP with the Asn\textsuperscript{394} → Gln mutation is inhibited by forskolin but not cytochalasin B.

Permeabilized cells from E. coli strain JM1100 expressing wild-type or mutant GalP were equilibrated with either 50 \( \mu \)M cytochalasin B, 80 \( \mu \)M forskolin, or ethanol (solvent control) and 25 mM glycerol for 3 min. The uptake of 50 \( \mu \)M 1\textsuperscript{[3H]}D-galactose was determined by taking a sample 120 s after the addition of the sugar as described by Henderson et al. (43). The graph shows % transport compared with ethanol control. Wild-type samples with the addition of cytochalasin B are the mean of 12 uptakes in three separate experiments; samples with the addition of cytochalasin B are the mean of 6 uptakes in two separate experiments, and samples with the addition of forskolin are the mean of 3 samples in a single experiment. The mean values ± the S.D. are shown. Energized uptake catalyzed without the addition of antibiotic was 7.08 ± 0.54 nmol/mg/120 s\textsuperscript{-1} for the wild type and 1.84 ± 0.19 nmol/mg/120 s\textsuperscript{-1} for the Asn\textsuperscript{394} → Gln mutant.

Gln Mutation Was Inhibited by Forskolin but Not by Cytochalasin B—The abilities of cytochalasin B or forskolin to inhibit the energized uptake of 50 \( \mu \)M D-galactose catalyzed by wild-type and mutant GalP into E. coli cells were investigated. The cell walls were rendered permeable to the antibiotics by prior treatment with Triton-EDTA, pH 8.0 (see "Materials and Methods"). In the case of the wild type, both cytochalasin B (80 \( \mu \)M) and forskolin (80 \( \mu \)M) inhibited the transporter, cytochalasin B being a slightly more potent inhibitor (Fig. 3). However, the transport catalyzed by the mutant protein was not significantly inhibited by cytochalasin B, although it demonstrated a marked increase in sensitivity to inhibition by forskolin.

**The Asn\textsuperscript{394} → Gln Mutant Protein Was Photolabeled by IAPS-Forskolin but Not by Cytochalasin B**—The wild-type GalP protein was susceptible to photolabeling with 4\textsuperscript{[3H]}cytochalasin B and 3-\textsuperscript{[35S]}APS-forskolin, and the protein was protected against this photolabeling by the physiological substrate, D-galactose, but not by L-galactose, which is not a substrate (Fig. 4, lanes 2 and 3). This protection by substrate showed that both cytochalasin B and IAPS-forskolin specifically label the GalP protein (13–15).
The Asn394 Glu mutant GalP protein was photolabeled by 3-125I-APS-forskolin, and D-galactose, but not L-galactose, protected the protein against the ligand; the extent of labeling and of protection were at levels similar to those of the wild-type protein (Fig. 4, B and D). However, no photolabeling of the mutant protein by cytochalasin B was detected (Fig. 4, A and C).

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FIG. 4. GalP with the Asn394 → Glu mutation is photolabeled by forskolin but not by cytochalasin B. Inside-out membrane vesicles were prepared from E. coli strain JM1100 expressing the wild-type and mutant protein using a French press as described under “Materials and Methods.” These were photolabeled with 4-[3H]cytochalasin B (0.5 μM) or 3-125I-APS-forskolin (0.4 nM) in the presence of either 500 mM D- or L-galactose. The photolabeled samples (30 μg) were separated on a 12.5% SDS-polyacrylamide gel and stained overnight with Coomassie Blue (cytochalasin B (A) and forskolin (B)) and subjected to fluorography (cytochalasin B (C)) and autoradiography (forskolin (D)). The characteristic appearance of the GalP protein is at 34–36 kDa. The lane order for both photolabeling experiments is as follows: lane 1, standards; lane 2, wild type + D-galactose; lane 3, wild type + L-galactose; lane 4, Asn394 → Glu + D-galactose; lane 5, Asn394 → Glu + L-galactose.
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The binding of either forskolin or cytochalasin B to the GalP protein was previously analyzed by stopped-flow fluorescence spectroscopy in terms of the following three-step Model A (14, 16, 38).

\[
\begin{align*}
T_o & \longrightarrow T_i \longrightarrow T_{i-o-antibiotic} \\
& \longrightarrow T_{i-o-antibiotic} \text{antibiotic} \\
& \longrightarrow T_{o-antibiotic}
\end{align*}
\]

Equation 1

FIG. 5. The binding of forskolin, but not of cytochalasin B, to the Asn 394 → Gln mutant protein was detected by equilibrium dialysis. Inner membrane vesicles containing over-expressed Asn 394 → Gln mutant GalP was subjected to microdialysis against a range of cytochalasin B or forskolin concentrations (0.05–80 μM), and the proportions of antibiotic bound to GalP and free in solution were measured using 12-[3H]forskolin or 4-[3H]cytochalasin B. The data were analyzed by a least-squares fit to a hyperbola from which the overall dissociation constant \( K_d \) for forskolin binding was calculated in two separate experiments to be 1.28 ± 0.34 and 2.16 ± 0.69 μM and the number of binding sites to be 8.1 ± 0.66 and 6.04 ± 0.4 nmol/mg membrane protein. The illustrated graph shows the mean data of two experiments in the form of a "Scatchard plot." The horizontal line for cytochalasin B shows that the small amount of bound 4-[3H]cytochalasin B was not displaced by increasing amounts of unlabeled antibiotic and is indicative of low level nonspecific binding.

To assess the importance of Asn 394 in the binding of antibi-
otic, the intrinsic fluorescence of the mutant protein was ti-
trated with antibiotic over a range of concentrations up to 80 μM (Figs. 6 and 7). A fit of the data to a hyperbola yielded an overall dissociation constant \( K_d \) for forskolin binding of 1.48 ± 0.5 μM \((n = 3)\), similar to that of 1.83 ± 0.56 μM determined for the wild type (16). This is consistent with the equilibrium dialysis data (above), which showed similar \( K_d \) values for the binding of forskolin to both wild-type and mutant proteins. In contrast to the equilibrium dialysis and photolabeling experiments described above, some low affinity cytochalasin B bind-
ing to the mutant protein was detected using the fluorescence method; however, this was shown to be with an affinity 37- and 53-fold lower than the wild-type protein \( (K_f 41.4 \) and 58.5 μM) in two separate experiments.

The extent of the quenching of fluorescence by saturating amounts of forskolin (10 μM) was compared between the wild-
type and Asn 394 → Gln mutant proteins. Measurements were made with \( \lambda_{em} \) at 280 and 297 (\( \lambda_{em max} \) 330–340 nm) to detect any differences due to light absorption by tryrosine and/or tri-
pyrrole residues and at two protein concentrations (50 and 100 μg/ml) to detect any differences due to the light scattering properties of the membrane preparations; no differences in the level of quench was found at either wavelength or between the two protein concentrations in either preparation. A 1.4-fold increase in the fluorescence quench of the mutant protein, relative to the wild type (wild type 8.09 ± 0.32% \((n = 7)\); Asn 394 → Gln 11.36 ± 0.88% \((n = 6)\)), was observed with \( \lambda_{em} \) 280 and 1.8-fold increase with \( \lambda_{em} \) 297 (wild type 6.16 ± 0.49% \((n = 5)\); Asn 394 → Gln 11.06 ± 0.14% \((n = 4)\)). A similar quantification of the amount of fluorescence quench concomitant with the binding of cytochalasin B to the mutant protein could not be made as saturating amounts of ligand could not be obtained.

Measurement of the Transient Kinetic Parameters for the Binding of Antibiotics to the Asn 394 → Gln Mutant Protein—

The binding of either forskolin or cytochalasin B to the GalP protein was previously analyzed by stopped-flow fluorescence spectroscopy in terms of the following three-step Model A (14, 16, 38).

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& \longrightarrow T_{i-o-antibiotic} \text{antibiotic} \\
& \longrightarrow T_{o-antibiotic}
\end{align*}
\]

Equation 1

Where \( K_1 = k_{-1}/k_1, K_2 = k_{-2}k_2, \) and \( K_3 = k_{-3}/k_3. \)

Asn 394 → Gln GalP mutant protein was characterized by a concentration-dependent linear increase in the rate of the fast phase of forskolin binding to the \( T_1 \) conformation of the protein (Fig. 6), indicating association \( (k_3) \) and dissociation rate constants \( (k_{-3}) \) of 1.51 ± 0.1 μM \(^{-1}\) s \(^{-1}\) and 2.37 ± 0.96 s \(^{-1}\), respectively; the dissociation constant \( (K_2 (k_{-2}k_2)) \) was calculated to be 1.57 ± 0.64 (Table II). The rates of association and dissociation of forskolin with the mutant were 4- and 3.6-fold slower, respectively, than for the wild type. Thus, the dissociation constant \( (K_2) \) of the mutant is almost identical to the wild type, demonstrating that the affinity of the \( T_1 \) or putative inward-facing transporter of the mutant protein for forskolin is unchanged. As \( K_f \) and overall \( K_v \) values are similar for both wild-type and mutant proteins there is no evidence to suggest changes to either \( K_f \) or \( K_v \).

Cytochalasin B also bound to the mutant protein in a linear, concentration-dependent manner (Fig. 7), indicating an associ-
ation rate constant of 0.67 ± 0.28 μM \(^{-1}\) s \(^{-1}\). In previous work with the wild-type GalP protein the dissociation rate constant for cytochalasin B was measured by directly displacing ligand, bound to the transporter, by adding sugar substrate (49). The transport protein was previously equilibrated with 80 μM ligand and mixed with 500 mM sugar in the stopped-flow appara-
atus to displace the bound ligand. The increase in protein fluorescence resulting from displacement of the bound cytochal-
asin B by d-galactose could be fitted to a single exponential function revealing a dissociation rate constant of 12.18 ± 2.78 s \(^{-1}\). The dissociation constant \( K_2 (k_{-2}k_2) \) for the \( T_1 \) confor-
mation of the mutant protein was calculated as 18.1 ± 8.78 μM. This value is 44-fold higher than that of the wild type and is similar to the 37–53-fold decrease in the overall affinity (in-
crease in overall \( K_v \)) for cytochalasin B.

The Binding of d-Galactose and d-Glucose to the Internal Binding Site Is Greatly Impaired in the Asn 394 → Gln Mutant of GalP—8-Anilino-1-naphthalene sulfonate (ANS) can be used as a fluorescent probe to monitor conformational changes in the GalP protein induced by the binding of sugar (17). Transported sugars such as d-galactose and d-glucose cause an enhance-
ment in the ANS fluorescence; non-transported sugar ana-
alogues and antibiotics produce only a slight quench in the ANS fluores-
cence, but they can reverse the enhancement in fluorescence induced by transported sugars. The increase in ANS fluorescence is attributable to the sugar-induced reorientation
of the transporter from an inward to an outward-facing conformation. Non-transported ligands are thought to reverse the fluorescence enhancement by recruiting the transporter to an inward-facing conformation. Before interpreting the effects of the Asn394 → Gln mutation, we first considered the kinetic model for the wild-type protein, which was derived from measurements of ANS Fluorescence (17).

The rate of enhancement in ANS fluorescence produced by D-galactose was measured by stopped-flow spectrofluorimetry (17) and was found to increase in a biphasic manner as [sugar] increased. The fast phase was attributed to the initial binding of the sugar to the inward-facing conformation of the protein and the slow phase to a subsequent conformational change. The rate of the fast phase increased hyperbolically with increasing D-galactose concentration indicative of the rapid binding of the transporter that was rate-limited by a slower conformational change at near saturating concentrations (see Equation 2).

$$K_{p} \text{ sugar} + T_1 \text{sugar} \rightarrow K_{n} \text{sugar} - T_0 \text{sugar} - T_n \text{ (Eq. 2)}$$

Where $T_1$ and $T_0$ are inward- and outward-facing conformational forms of the transporter, respectively; $K_{p}$ is the dissociation constant for the inward-facing GalP-D-galactose complex and $K_2 = k_d/k_{-2}$, and $k_2$ and $k_{-2}$ are rate constants for the conformational change subsequent to sugar binding and were attributed to transporter reorientation.

The change in fast phase fitted to the following hyperbolic function as shown in Equation 3.

$$k_{obs} = \frac{k_d [\text{gal}]}{K_{gal} + [\text{gal}]} + k_{-2} \quad \text{(Eq. 3)}$$

Where $k_{obs}$ is the change in the fast rate; $[\text{gal}]$ is D-galactose concentration; $K_{gal}$ is apparent dissociation constant for D-galactose. This yielded an apparent $K_{gal}$ of 7.22 ± 1.49 M with minimum ($k_{-2}$) and maximum ($k_2$) rates of 1.36 ± 0.18 s⁻¹ and 4.06 ± 0.16 respectively.

The overall $K_d$ for D-galactose binding was calculated at 1.8 M from the measured values for $K_{gal}$ and $K_2$ by the relationship shown in Equation 4.

$$K_{d,overall} = \frac{K_{gal} K_2}{K_{gal} + K_2} \quad \text{(Eq. 4)}$$

However, it was also measured from the total fluorescence change since the sum of the fast and slow fluorescence phases also exhibited hyperbolic dependence upon the D-galactose concentration with an overall $K_2$ of 0.66 ± 0.03 M. As the calculated value for the overall $K_2$ is greater than the measured value, a further conformational change following the formation of $T_1$ D-gal causing additional tightening of the binding of D-galactose was proposed. The calculated $K_d$ for the binding of glucose (0.33 mM) was also greater than the measured value (0.18 mM). These results led to the suggestion that the kinetic scheme shown above be expanded to give Equation 5.

![Table II](image)

| Parameter | Wild-type | Asn394 → Gln |
|-----------|-----------|-------------|
| $K_p$ (forskolin) | 1.83 ± 0.56 μM | 1.48 ± 0.5 |
| $K_{f}$ (forskolin) | 1.36–1.43 μM | 1.28–2.61 |
| $k_{-2}$ (forskolin) | 8.62 ± 2.49 s⁻¹ | 2.37 ± 0.96 |
| $k_{2}$ (forskolin) | 5.95 ± 0.36 μM⁻¹ s⁻¹ | 1.51 ± 0.1 |
| $K_{gal}$ (forskolin) | 1.45 ± 0.43 μM | 1.57 ± 0.64 |
| $D_{max}$ (forskolin) | 6.16 ± 0.99 μM | 11.90 ± 0.14 |
| $K_{gal}$ (cytochalasin B) | 1.1 ± 0.19 | 41.4–55.8 |
| $k_{-2}$ (cytochalasin B) | 2.5 ± 0.2 s⁻¹ | 12.18 ± 2.75 |
| $k_{2}$ (cytochalasin B) | 6.1 ± 0.14 μM⁻¹ s⁻¹ | 0.67 ± 0.28 |
| $K_{gal}$ (cytochalasin B) | 0.41 ± 0.03 μM | 18.1 ± 8.78 |

* The overall dissociation constants (Kf) were determined by titration of the protein fluorescence (ΔFmax).

b The overall dissociation constants (Kf) were determined by equilibrium dialysis.

c Association and dissociation rate constants (k2 and k−2) were determined from the linear plot of the observed rate of antibiotic binding versus antibiotic concentration.

d The dissociation constant for antibiotic binding to the inward-facing antibiotic site (Kf) was calculated from the measured association (k2) and dissociation (k−2) rate constants (k2/Kf).

ΔFmax values were measured at steady state with saturating amounts of forskolin using right-side-out inner membrane vesicles with Asn394 Gln and wild-type proteins induced by the binding of forskolin, fitted by least-squares to a hyperbolic equation. Three separate experiments yielded an overall dissociation constant (Kf) of 1.48 ± 0.5 (n = 3) compared with 1.83 ± 0.56 (n = 3) of the wild type. B shows the concentration dependence of the total quench in the Asn394 → Gln mutant and wild-type protein. Linear regression analysis of the data shown yielded values of 1.5 ± 0.099 μM⁻¹ s⁻¹ (Asn394 → Gln mutant) and 6.16 ± 0.511 μM⁻¹ s⁻¹ (wild type) for the association rate constant (k2) and 2.37 ± 0.36 μM⁻¹ s⁻¹ (Asn394 → Gln mutant) and 10.43 ± 3.23 s⁻¹ (wild type) for the dissociation rate constant (k−2). These values yield 1.57 ± 0.64 μM (Asn394 → Gln mutant) and 1.71 ± 0.55 μM (wild type) for the apparent dissociation constant (Kf) of forskolin binding.

![FIG. 6. Fluorescence titration demonstrates that the Asn394 → Gln mutant binds forskolin with an affinity similar to wild type.](image)
Where \( T_0^* \) is an outward-facing conformation, slightly different to that of \( T_o \).

Hence the \( K_d \) would be as shown in Equation 6.

\[
K_d = \frac{K_{D-gal}}{1 + K_{D-gal} + K_2} \quad \text{(Eq. 6)}
\]

In contrast to the wild-type protein, binding of \( \beta \)-galactose and \( \beta \)-glucose to the mutant protein was characterized by a monophasic increase in the ANS fluorescence with time. The rate was apparently independent of sugar concentration, and it was not possible to determine rate constants, \( k_2 \) and \( k_2' \), and the dissociation constant, \( K_{D-gal} \). It is probable that \( k_2 \) and \( k_2' \) are very similar. The total fluorescence enhancement, which was 8.5 times lower in the case of \( \beta \)-galactose and 3.6 times lower in the case of \( \beta \)-glucose, did, however, exhibit a hyperbolic dependence upon the concentration of sugar (Fig. 8). The concentration dependence of the total fluorescence enhancement for \( \beta \)-galactose and \( \beta \)-glucose fitted to a hyperbolic function and from three separate experiments yielded overall \( K_d \) values of 42.3 \( \pm \) 11.8 mM (Asn394 → Gln) and 29.4 \( \pm \) 3.5 mM (wild type) for \( \beta \)-galactose and 42.3 \( \pm \) 11.8 mM (Asn394 → Gln) and 0.66 \( \pm \) 0.16 mM (wild type) for \( \beta \)-glucose.

**Fig. 7.** Fluorescence titration demonstrates that the Asn394 → Gln mutant binds cytochalasin B with much reduced affinity. A shows the concentration dependence of the total quench in the Asn394 → Gln and wild-type proteins induced by the binding of cytochalasin B, fitted by least-squares to a hyperbolic equation. Repeated experiments yielded an overall dissociation constant (\( K_{D} \)) of 41.4 \( \pm \) 38.5 \( \mu \)M for the Asn394 → Gln protein compared with 1.1 \( \pm \) 0.19 (n = 4) for the wild type. B shows the concentration dependence of the observed rate of binding of cytochalasin B to the Asn394 → Gln mutant and wild-type protein. Linear regression analysis of the data shown yielded values of 0.673 \( \pm \) 0.282 \( \mu \)M \( \cdot \) s \( \text{)}^{-1} \) (Asn394 → Gln mutant) and 6.14 \( \pm \) 0.102 \( \mu \)M \( \cdot \) s \( \text{)}^{-1} \) (wild type) for the association rate constant (\( k_2 \)); 12.18 \( \pm \) 2.78 s \( \text{)}^{-1} \) (Asn394 → Gln mutant) and 2.51 \( \pm \) 0.65 s \( \text{)}^{-1} \) (wild type) for the dissociation rate constant (\( k_{2'} \)); with 18.1 \( \pm \) 8.78 \( \mu \)M (Asn394 → Gln mutant) and 0.41 \( \pm \) 0.106 \( \mu \)M (wild type) for the apparent dissociation constant (\( K_d \)).

**Fig. 8.** The dissociation constant for sugar binding and translocation is drastically altered by the Asn394 → Gln mutation. The data show the ANS fluorescence enhancement as a function of the concentration of \( \beta \)-glucose (A) and \( \beta \)-galactose (B) fitted by least squares to a hyperbolic equation. Repeated experiments yielded an overall dissociation constant (\( K_{D} \)) of 29.4 \( \pm \) 3.5 (n = 4) (Asn394 → Gln) and 0.18 \( \pm \) 0.01 mM (wild type) for \( \beta \)-glucose and 42.3 \( \pm \) 11.8 mM (n = 3) (Asn394 → Gln) and 0.66 \( \pm \) 0.16 mM (wild type) for \( \beta \)-galactose. Substrate Specificity of the Asn394 → Gln Mutant of GalP—To determine the substrate specificity of the Asn394 →
Gln mutant protein, an extensive kinetic analysis was carried out in which increasing concentrations of the unlabeled sugar under investigation were used to inhibit labeled $1^\text{[3H]}$D-galactose transport over a range of concentrations of both sugars.

Initial velocities of D-galactose uptake catalyzed by the wild-type and mutant proteins into *E. coli* were measured. The $V_\text{max}$ against [I] plots (Dixon plots) for D-galactose were linear, as expected for simple hyperbolic binding, in the presence of unlabeled D-galactose, 2-deoxy-D-glucose, D-talose, 6-deoxy-D-glucose, 6-deoxy-D-galactose, and D-mannose. At higher concentrations of D-xylene, 6-deoxy-D-galactose (wild type only), D-glucose, and L-arabinose the Dixon plots were non-linear and deviated systematically from hyperbolic binding (data not shown). However, the data points at the lower concentrations of these inhibiting substrates did display linearity, and extrapolated estimates of $K_i$ were determined. It was reported previously (13) that the kinetics of transport of D-glucose, 2-deoxy-D-glucose, and 6-deoxy-D-glucose, and 6-deoxy-D-galactose transport by GalP were non-hyperbolic. In order that changes in substrate specificity of the mutant protein could be readily detected, a ratio of mutant $K_i$ to wild-type $K_i$ was calculated, which was then normalized by dividing with the ratio for D-galactose (Table III). The recognition by the mutant protein of most of the sugar substrates was only altered from that of the wild type between 0.5–2.0-fold, indicating little change in sugar selectivity. Surprisingly, the specificity for D-xylene was seven times lower with the mutant protein compared with the wild type. There is no obvious, simplistic interpretation of this effect such as an altered substrate recognition in terms of a single H-bonded sugar-protein interaction. Clearly the mutation of the Asn$^{394}$ residue to Gln has, in terms of D-xylene, selectively affected the substrate specificity of the GalP protein.

### DISCUSSION AND CONCLUSIONS

An alignment of the amino acid sequences of those proteins that are homologous to GalP, together with a knowledge of the ability of these transporters to bind cytochalasin B, suggested that the Asn$^{394}$ residue might be involved in the recognition of cytochalasin B as a part of the cytochalasin B binding site or pocket. For the purposes of this work we will define the binding site as comprising those amino acids that bind directly with the antibiotic, and the binding pocket is defined as amino acids in the immediate environment of the antibiotic that do not necessarily bind directly to it. To investigate the involvement of Asn$^{394}$ the residue was altered by mutagenesis to Gln.

Inward D-galactose transport catalyzed by the mutant protein, as defined by the parameters $K_m$ and $V_{\text{max}}$, was relatively unimpaired, implying that the gross conformation of the protein was intact and the transport mechanism conserved. Similarly, the mutant protein was able to catalyze sugar proton symport, to an extent similar to wild type albeit at a 2.8-fold lower rate, indicating that coupling between proton and sugar transport was maintained.

Previous equilibrium binding experiments on the wild-type protein revealed mutual competition between cytochalasin B and forskolin for the antibiotic binding site (16). It was therefore of interest to investigate the effects of the Asn$^{394}$ → Gln substitution on the interaction of GalP with both antibiotics. The mutant protein was photolabeled by IAPS-forskolin at levels similar to that of the wild-type protein, and the reaction was protected by D-galactose. These data showed that Asn$^{394}$ is not essential for photolabeling by forskolin. However, it cannot be ruled out that Asn$^{394}$ of wild-type GalP is the photolabeled amino acid residue and that Gln$^{394}$ is being photolabeled in the mutant protein. Positive identification must wait for the direct sequencing of protein fragments generated from photolabeled transporter. A Scatchard analysis demonstrated that the overall affinity for forskolin binding to the mutant protein and the number of forskolin binding sites were almost identical to those of the wild-type protein; this unchanged overall affinity of the mutant protein for forskolin was confirmed by the titration of the protein’s fluorescence. D-Galactose transport catalyzed by the mutant protein demonstrated a marked increase in sensitivity to inhibition by forskolin compared with the wild type (wild type 58.0 ± 2.9% and Asn$^{394}$ → Gln 7.3 ± 2.9% compared with the ethanol control). This increase probably reflects a decrease in the level of competition by D-galactose. The maximal fluorescence quench induced by forskolin binding was 1.8-fold greater with the mutant protein than the wild type ($\lambda_{\text{em}}$ max 330–340 nm), although the amount of GalP was the same. This indicates that the substitution of Asn$^{394}$ by Gln altered the environment of one or more tryptophan residues.

Characterization of a Trp$^{395}$ → Phe mutant GalP protein indicated that Trp$^{395}$ is in a conformationally active region of the protein and that this residue is the main reporter of forskolin binding in fluorescence quenching experiments (38). The increase in the maximal fluorescence quench due to the binding of forskolin to the Asn$^{394}$ → Gln mutant can be rationalized by suggesting that the presence of Gln$^{394}$ causes a localized conformational change altering the environment of the neighboring Trp$^{395}$ residue. Although the overall affinity for forskolin binding to the mutant protein is unaffected, transient fluorescence measurements showed that the rates of association ($k_+$) and dissociation ($k_-$) to the inward-facing conformation of the protein ($T_1$) are reduced (3.9- and 3.6-fold for $k_+$ and $k_-$, re-

### Table III

| Sugar                | Wild-type $K_i$ ($\mu$M) | Asn$^{394}$ → Gln $K_i$ ($\mu$M) | Asn$^{394}$ Gln → ($K_i$) Wild-type ($K_i$) | Comparison normalized to D-Galactose |
|----------------------|-------------------------|----------------------------------|------------------------------------------|------------------------------------|
| D-Glucose            | 34.1 ± 11               | 84.0 ± 24                        | 2.46                                     | 1.17                               |
| D-Galactose          | 103.6 ± 12.6            | 306 ± 73                         | 2.9                                      | 1                                  |
| 2-Deoxy-D-glucose    | 236 ± 79                | 322 ± 98                         | 1.3                                      | 0.45                               |
| D-Talose             | 352 ± 42.2              | 1392 ± 136                       | 3.95                                     | 1.36                               |
| 6-Deoxy-D-glucose    | 413 ± 144               | 662 ± 150                        | 1.6                                      | 0.55                               |
| 6-Deoxy-D-galactose  | 513 ± 80                | 3853 ± 622                       | 7.5                                      | 2.1                                |
| 2-Deoxy-D-galactose  | 630 ± 201               | 1844 ± 529                       | 2.9                                      | 1                                  |
| D-Mannose            | 789 ± 160               | 4944 ± 1697                      | 6.26                                     | 2                                  |
| D-Xylose             | 1271 ± 330              | 28128 ± 545                      | 22                                       | 7.6                                |
| L-Arabinose          | 6649 ± 453              | 47193 ± 19837                    | 7                                        | 2.4                                |
The affinity for the initial interaction with forskolin to the inward-facing conformation of the protein ($T_\text{i}$), as defined by the dissociation constant $K_D$ ($k_{\text{diss}}/k_{\text{ass}}$), was unchanged. As $K_D$ and overall $K_D$ values are similar for both wild-type and mutant proteins, there is no evidence to suggest changes to either $K_1$ or $K_2$.

In stark contrast to the binding of forskolin, cytochalasin B was shown, in fluorescence quenching experiments, to bind to the mutant protein with an overall affinity 37–53-fold lower than that of the wild-type protein. The displacement of cytochalasin B from the mutant protein by sugar substrate provided corroborating evidence of ligand binding. This low affinity cytochalasin B binding was not detectable at all in photolabeling or equilibrium dialysis experiments, most likely due to an insufficient concentration or specific activity of the radiolabeled cytochalasin B.

Transient fluorescence measurements showed that the affinity for the initial interaction with cytochalasin B to the inward-facing conformation of the protein ($K_D$, as defined by $k_{\text{diss}}/k_{\text{ass}}$) was also much lower than wild type (40-fold lower). The reduction in overall affinity for cytochalasin B cannot be attributed to stabilization of the $T_\text{o}$ (outward-facing) conformation of the mutant transporter, otherwise a similar effect should be observed with forskolin binding; thus, it is most likely to be entirely due to a reduction in affinity of the $T_\text{i}$ conformation.

Cytochalasin B was shown to have a rate of association to the mutant protein ($k_{\text{ass}}$) lower than that of the wild type (9-fold) and as such is similar to forskolin. However, in direct contrast with the rate of forskolin dissociation, that of cytochalasin B from the mutant protein is higher (4.9-fold) than that of the wild type.

Possible explanations for these data are that the amino acid substitution has 1) altered the shape of the forskolin binding pocket making it more difficult for the antibiotic to bind, but once bound it is held within the pocket for longer, and (2) disrupted a single H-bonded cytochalasin B-protein interaction resulting in a reduction of overall affinity for the ligand. The reduction in affinity for cytochalasin B and alteration of forskolin binding provides evidence that the cytochalasin B and forskolin binding sites are closely associated. We speculate that Asn394 forms part of the cytochalasin B binding site and part of the forskolin binding pocket. However, the possibility that the disruption in cytochalasin B binding is due to steric hindrance by the larger Glu residue cannot be discounted.

In contrast to the energized transport of sugar, which is relatively unimpaired, ANS fluorescence measurements show that the overall $K_D$ describing the binding of d-glucose to d-glucose to the inward-facing conformation of the mutant protein and their subsequent translocation across the membrane were drastically higher than that for the wild-type protein (64.1-fold for d-galactose and 163.3-fold for d-glucose). The fact that energized transport catalyzed by the mutant transporter is relatively unimpaired suggests that the translocation mechanism is intact. It is therefore most likely that the decrease in overall affinity is largely due to loss of affinity for these sugars at the inward-facing binding site.

The substitution of the Asn394 residue by Glu has, at least, at the primary structural level made the GalP protein more like XylE. Such single residue differences may be the means by which proteins are able to discriminate between different substrates, e.g., between hexoses and pentoses. The sugar specificity of the wild-type GalP protein has been extensively investigated (2, 13, 15, 17), which showed the importance of the C-3 hydroxyl and C-6 on the pyranose ring for hexose recognition by GalP and the lack of importance of the C-2 and C-4 hydroxyl groups. These observations parallel the requirements for sugar recognition by the passive glucose transporters of human erythrocytes (GLUT1) and rat adipocytes (GLUT4) (11, 12), confirming the functional similarity of the bacterial and mammalian transporters. The substrate specificity study carried out for energized uptake of sugars in this work did not reveal a general role for the Asn394 residue in the recognition of external sugar substrates, but, surprisingly, the specificity for d-xylene was significantly reduced (7.6-fold compared with the wild type); there is no obvious, simplistic interpretation of this effect, such as an altered substrate recognition in terms of a single H-bonded sugar-protein interaction. Overall, these observations support the other data that indicate Asn394 is part of the internal binding site for sugars.

Cytochalasin B has been shown by kinetic analysis to compete with d-glucose for binding to the inward-facing conformation of the GLUT1 protein (55). Our data, which demonstrate that the disruption of sugar binding to the inward-facing conformation of GalP (as opposed to the outward-facing conformation) is coupled to the disruption of cytochalasin B binding by the Asn394 $\rightarrow$ Glu mutation, provide further evidence of the functional and structural similarity of the GLUT1 and GalP proteins. These data contribute to the ongoing debate as to whether cytochalasin B and sugar bind to the same site of the inhibitor susceptible proteins and suggest, at least with GalP, that this is the case.

An Asn415 $\rightarrow$ Asp mutant of the GLUT1 transporter has been isolated (56). Asn415 is conserved in all the GalP, AraE, XylE, and GLUT transporters and is the 4th residue after Asn394 in GalP. Characterization of the Asn415 $\rightarrow$ Asp mutant GLUT1 protein showed 40% less cytochalasin B photolabeling although the inhibition of labeling by ethylene glucose, which preferentially binds to the outer glucose binding site (57), inhibited cytochalasin B similar to the wild type. This led to the suggestion that the Asn415 residue is likely to reside close to the inner glucose and cytochalasin B binding sites of GLUT1. It is interesting to note that to make a complete turn of an $\alpha$-helix, the polypeptide backbone must traverse 3.6 amino acids. Thus amino acids three or four amino acids apart (as in the case of Asn412 (aligned with Asn394 of GalP) and Asn415 of GLUT1) would be expected to project from the same side of an $\alpha$-helix. It has also been shown (38) that the affinity for the initial interaction of cytochalasin B to a Trp395 $\rightarrow$ Phe mutant as described by the apparent dissociation constant ($K_D$) is 43-fold lower than that for the wild type while the $K_D$ for forskolin binding to the mutant is similar to wild type. These data suggest that the residue adjacent to Asn394 (Trp395) is also associated with the cytochalasin B but not the forskolin binding site. Interestingly, the affinity for d-galactose of the inward-facing binding site of the Trp395 $\rightarrow$ Phe mutant is similar to wild type and is therefore unlikely to be involved in d-galactose binding (38). It is by the collation of such data from many mutants that structural and mechanistic models for these related sugar transporters will begin to emerge, and the binding sites of sugar and antibiotic will be determined.

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