mine whether these residues play a role in charge transfer. In rabbit SGLT1 we have mutated Asp-176 to asparagine and to alanine and measured charge transfer. Dramatic changes occurred with the Asp176Ala mutant, i.e. $V_{1/2}$ decreased from $+1$ to $-46$ mV and the $r$ decreased with depolarization instead of increasing. This result alone would suggest that Asp-176 is a charged residue directly involved in sensing the membrane field, but the Asp176Asn mutation only produced modest changes in charge-transfer kinetics. We conclude that polar residues, and not necessarily charged residues, constitute the charge sensor in SGLT1.

We are presently extending our studies to other cloned transporters. In general, the results so far indicate that the steady-state and pre-steady-state kinetics of Na+-Ins, H+-glucose and Na+ -Cl- -taurine cotransporters have much in common with SGLT1.

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The pre-steady-state kinetics of conformational changes in sugar transporters
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Introduction
The $\alpha$-galactose-$H^+$ symporter (GalP) from Escherichia coli shares sequence similarity with the GLUT family of mammalian glucose transporters [1-6]. As a consequence the GalP and GLUT proteins are predicted to have a similar membrane topology, comprising 12 membrane-spanning $\alpha$-helices, with helices 6 and 7 connected by a cytoplasmic domain composed of 60-70 amino acids [6-8]. 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 [9-12] and GalP-mediated sugar transport can be inhibited by the antibiotics cytochalasin B and forskolin [3,11,13-15], which are potent inhibitors of glucose transporters [16-27]. The GLUT1 and GalP proteins can be photolabelled by these antibiotics, or derivatives of them, in a $\beta$-glucose-inhibitable manner [8,11,13,14,28-33]. The tryptic digestion of the GalP protein, which has been photolabelled with cytochalasin B, produces a labelled fragment (Mr 17000-19000) of almost identical Mr to that produced by digestion of GLUT1 [11].

The efficiency of photolabelling GLUT1 with antibiotics is maximal at 280 nm, suggesting that a tryptophan residue in one of these helices may be activated before 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 binding site(s) for cytochalasin B and forskolin have been localized between $\alpha$-helices 10 and 11 by identifying the photolabelled proteolytic fragments from GLUT1 [28-33]. Two tryptophan residues within this region, Trp-388 and Trp-412, lie towards the cytoplasmic end of helices 10 and 11 respectively [7]. Furthermore, cytochalasin B is thought to bind to the inward- or cytoplasmic-facing site [19,34] so that these tryptophan residues seem likely candi-
mechanisms and regulation of sugar transport.

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.

The alignment of the amino acid sequence for GalP with that of GLUT1 indicates that these tryptophan residues are conserved in GalP, and indeed within the family of GLUT proteins. It seems highly likely that these residues, Trp-371 and Trp-395 respectively in the GalP sequence, will play similar roles in the function of the GalP and GLUT proteins.

Here we describe the analyses of the transient kinetics of the binding of antibiotic and sugar to inside-out vesicles, produced from a strain of E. coli that over-expresses GalP.

**Binding of antibiotics**

The binding of cytochalasin B and forskolin induces a quench in the protein fluorescence of both GLUT1 and GalP. This observation has allowed the kinetics of the binding of cytochalasin B to GLUT1 and GalP to be monitored by fluorescence spectroscopy. The overall affinities of these sugar transporters for cytochalasin B has been determined by titration of the protein fluorescence, while the association and dissociation rate constants for the binding of cytochalasin B have been determined by stopped-flow fluorescence spectroscopy (Table 1). The latter kinetic parameters were obtained from the gradient and intercept respectively of a linear fit of the rate of binding as a function of the cytochalasin B concentration. In the case of GalP, the dissociation constant \((K_d)\) calculated from the association and dissociation rate constants is lower than that obtained by titration, but the reverse situation holds for GLUT1.

The apparent discrepancies between these values can be explained by the following model:

\[
T_1 \xrightarrow{k_+} T_2 \xrightarrow{k_-} T(CH) \xrightarrow{k_+} T(CH) (I)
\]

with \(T_1\) and \(T_2\) are respectively the outward- and inward-facing forms of the unloaded transporters, and \(T(CH)\) are two different conformations of the transporter-cytochalasin B (CB) complex. For such a model the apparent dissociation constant of the transporter–CB complex is given by:

\[
K_d = \frac{K_2(1 + K_1)}{1 + K_1} (1)
\]

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

In the case of GLUT1, \(k_1\) and \(k_{-1}\) (and hence \(K_1\) are known) and so \(K_2\) can be calculated as 0.074 by substituting \(K_2 \approx 600 \text{ s}^{-1}\), \(k_{-1} = 148 \text{ s}^{-1}\) and \(K_3 = 0.247\) at 20° C.

**Table 1**

<table>
<thead>
<tr>
<th>Transporter (antibiotic)</th>
<th>(k_2) ((\mu\text{M}^{-1} \cdot \text{s}^{-1}))</th>
<th>(k_{-2}) (s(^{-1}))</th>
<th>(K_2) ((\mu\text{M}))</th>
<th>(K_d) ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalP (cytochalasin B)</td>
<td>6.0</td>
<td>2.5</td>
<td>0.41</td>
<td>1.90</td>
</tr>
<tr>
<td>GalP (forskolin)</td>
<td>6.0</td>
<td>8.6</td>
<td>1.45</td>
<td>1.83</td>
</tr>
<tr>
<td>GLUT1 (cytoskolin B)</td>
<td>2.4</td>
<td>3.9</td>
<td>1.63</td>
<td>0.14</td>
</tr>
</tbody>
</table>
values of $K_d$ and $K_2$ into eqn. 1. Thus at equilibrium (at 20°C) the GLUT1-CB complex is present as 8% $T_2$(CB) and 92% $T_2$(CB).

In the case of GalP there is no evidence for a secondary conformational change in the cytochalasin B complexes since $K_2$ is less than the $K_a$ for this transporter. As a first approximation we can therefore assume that $1/K_2$ is zero, and $K_1$ can then be calculated as 3.63 for GalP. This is in fact a minimum value for $K_1$, since if $1/K_2$ has a finite value $K_1$ will have a higher value than 3.63. In any event it is evident that at 20°C the outward-facing conformation predominates for GalP.

The rate of binding of forskolin was also linearly dependent upon the forskolin concentration, yielding the association and dissociation rate constants given in Table 1. The value calculated for the dissociation constant ($K_d$) was similar to that obtained by titration, indicating that $K_3$ must have a similar value to $K_1$ (Table 1 and [13,14]).

In contrast, a Trp395Phe mutant of GalP was characterized by an apparently hyperbolic increase in the rate of binding. These data provide clear evidence that the transporter-forskolin (F) complex undergoes a further conformational change, the $T_2$(F) to $T_3$(F) 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 were fitted to a hyperbolic function, yielding minimal and maximal values for $k_1$ and $k_3$ of 3.6 s$^{-1}$ and 28.4 s$^{-1}$ respectively and an apparent $K_d$ of 3.7 µM. These values will correspond to those for $k_3$, $k_1$ and $K_2$ respectively in scheme I. Using these values and the measured $K_3$ (0.58 µM), $K_1$ can be calculated as 0.41 from eqn. 1.

This mutation has caused a large shift in the equilibrium between $T_1$ and $T_3$, so that ~29% of the transporters are in the $T_1$ (or outward-facing) conformation in the absence of ligands, while stabilizing the $T_2$(F) complex ($K_1 = 7.7$). After equilibration with forskolin, the GalP-F complex is present as 11.3% $T_2$(F) and 88.7% $T_3$(F). Furthermore, the affinity of the $T_2$ conformation for forskolin was reduced 2.5-fold. The above data also provide minimal values for $k_3$ [$(k_3 > (k_1 + k_3)/K_3)$] and $k_1$ [$(k_3 > k_1)$] of 8.6 µM$^{-1}$ s$^{-1}$ and 28.4 s$^{-1}$, 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 replacement of Trp395 with phenylalanine causes a destabilization of the $T_1$ (or outward-facing) conformation ($K_1 > 3.63$ for the wild-type GalP protein [15], $K_1 = 0.41$ for Trp395Phe mutant GalP protein), while stabilizing the $T_3$(antibiotic) conformation.

On the other hand, the binding of cytochalasin B to the Trp395Phe mutant GalP was characterized by a linear increase in the rate of binding; yielding association and dissociation rate constants of 0.6 µM$^{-1}$ s$^{-1}$ and 10.5 s$^{-1}$ respectively. The $T_2$ conformation has a lower affinity ($K_2 = 17.5$ µM) than the overall affinity ($K_d = 6.7$ µM), again indicating that there must be a further isomerization of the $T_2$(antibiotic) complex.

Hence, these studies suggest that the binding of antibiotics to both GLUT1 and GalP involve at least three steps: a transition from an outward- to an inward-facing conformation, binding of the antibiotics, and induction of a further conformational change in the transporter-antibiotic complex. The exact nature of the latter transition is not known, but one possibility is that it is a partial reorientation of the transporters.

The Trp371Phe mutation in GalP did not cause a substantial perturbation in the kinetics of either the binding of cytochalasin B or forskolin and the overall affinity of the mutant for these antibiotics was similar to the wild-type.

### Binding of sugars

The binding of sugars (and antibiotics) to GalP can be monitored from the changes in the fluorescence of 8-anilino-1-naphthalenesulphonic acid (ANS) equilibrated with inside-out vesicles [12]. Transported sugars, such as D-glucose and D-galactose, cause an enhancement in the ANS fluorescence, of up to 13%, and the overall affinity of GalP for these sugars can be obtained by fluorescence titration. Non-transported sugars that have little, if any, affinity for GalP, such as L-galactose and L-glucose, have no effect upon the ANS fluorescence. Cytochalasin B and forskolin, which are potent inhibitors of the transporter, produce little change in the fluorescence, but are capable of reversing the D-galactose/D-glucose enhancement in fluorescence. Sugars that bind to GalP but are not transported, such as methyl β-D-glucose, produce only a slight quench in the ANS fluorescence, but again reverse the enhancement in fluorescence induced by transported sugars. A simple interpretation of these findings is that 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 sugars and antibiotics, which are thought to bind at the inner membrane face of the transporter, are able to
reverse the fluorescence enhancement by binding to the inward-facing conformation.

Moreover, the conformational changes induced in GalP by the binding of transported sugars can be time-resolved by stopped-flow fluorescence spectroscopy, by monitoring the increase in ANS fluorescence [12]. For transported sugars the observed rate constant for this process was found to increase in a hyperbolic manner, providing a measure of the dissociation constant for sugar binding to the inward-facing transporter and of the rate constants for outward and inward reorientation. The dissociation constant for the binding of D-galactose to the inward-facing site ($K_{d,\text{in}}$) was 7.22 mM, and the rate constants for outward ($k_2$) and inward ($k_1$) reorientation of the transporter were 4.06 s$^{-1}$ and 1.36 s$^{-1}$ respectively. The overall affinity of GalP for D-galactose was determined from a hyperbolic plot of the increase in signal amplitude (ANS fluorescence) with the D-galactose concentration, yielding a $K_d$ of 0.66 mM. The overall $K_d$ is lower than that calculated from the rate data [$K_d = K_{d,\text{in}}/(1 + K_I) = 1.31$ mM$^{-1}$], suggesting that there may be an additional conformational change in which the binding of D-galactose (D-Gal) is tightened:

\[
\begin{align*}
\text{D-Gal} + T_{\text{in}} & \rightleftharpoons \text{D-Gal-T}_{\text{in}}; \\
\text{D-Gal-T}_{\text{in}} & \rightleftharpoons \text{D-Gal-T}_{\text{o}}; \\
\text{D-Gal-T}_{\text{o}} & \rightleftharpoons \text{D-Gal-T}^*_{\text{o}}
\end{align*}
\]

where $T_{\text{o}}$ and $T_{\text{in}}$ are the inward- and outward-facing conformations of the transporter. $T^*_{\text{o}}$ is an additional outward-facing conformation that must differ in structure to $T_{\text{o}}$, but in an unknown manner.

**A model for sugar and antibiotic binding**

The kinetic models derived from the binding of antibiotics and for the binding and translocation of sugars can be accommodated into a single kinetic model, which represents the minimal translocation cycle under non-energizing conditions:

\[
\begin{align*}
T_{\text{i}} + L & \rightleftharpoons T_{\text{i}}L; \\
T_{\text{i}}L & \rightleftharpoons T_{\text{o}} + L; \\
T_{\text{i}} + S & \rightleftharpoons T_{\text{o}}S; \\
T_{\text{i}}S & \rightleftharpoons T_{\text{o}} + S
\end{align*}
\]

where $T_{\text{i}}$ and $T_{\text{o}}$ are the inward- and outward-facing conformations of the transporter, $S$ represents transported sugars and $L$ represents non-transported sugars or antibiotics; $K_i$ and $K_o$ are the dissociation constants for transported and non-transported sugar (or antibiotic) binding to the inward-facing site; $K_d$ is the dissociation constant for the binding of a transported sugar to the outward-facing site; and $K_s$ is the equilibrium constant for reorientation of the unloaded transporter. $T_{\text{i/o}}$ is a putative conformation in which the transporter is intermediate between inward- and outward-facing and $K_s$ is the equilibrium constant for this transition. In the case of cytochalasin B and D-galactose the following kinetic parameters were determined:

\[
\begin{align*}
K_i &= (k_1/k_2) = 7.22 \text{mM}; \\
K_o &= (k_1/k_2) = 2.99; \\
K_d &= (k_1/k_2) = 2.32; \\
K_s &= (k_1/k_2) = (K_j, K', K'/K_3) = 13.8 \text{mM}; \\
K' &= 3.36; \\
K'' &= 0.41 \mu M; \\
K_i &= 0.
\end{align*}
\]

This kinetic model will be used as the benchmark against which the kinetics of mutants generated in GalP can be compared and hence will be of immense importance in elucidating structure–function relationships in this important class of transporters.

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14 Reference deleted

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