Sugar Transport through Maltoporin of *Escherichia coli*

ROLE OF POLAR TRACKS*

Received for publication, January 13, 2000, and in revised form, March 17, 2000
Published, JBC Papers in Press, March 27, 2000, DOI 10.1074/jbc.M00268200

Fabrice Dumais‡, Ralf Koebnik§, Mathias Winterhalter**, and Patrick Van Gelder‡

From the ‡Department of Biophysical Chemistry and the §Department of Microbiology, Biozentrum, University of Basel, 4056 Basel, Switzerland, and **Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, University of Toulouse, 31Q00 Toulouse, France

The three-dimensional structure of the maltooligosaccharide specific outer membrane channel LamB of *Escherichia coli* complexed with sugar molecules revealed a hypothetical transport pathway. Sugars are supposed to slide over a stretch of aromatic residues facilitated by continuous making/breaking of hydrogen bonds between the hydroxyl groups of the sugars and charged amino acids, the “polar tracks.” The effect of nine single and three multiple mutations in the polar track residues was investigated by current fluctuation analysis, swelling assays, and *in vivo* uptake of radiolabeled substrates. Additionally, sugar transport through wild-type LamB was investigated by current fluctuation analysis in water and deuterium. This way the effects on *k*<sub>on</sub> and *k*<sub>off</sub> could be investigated separately. Analyses of the various mutants revealed a strong effect on the *k*<sub>on</sub> values. Because steering to the binding site requires only a few interactions, consequently the loss of even one bond will have a strong effect. Deuterium experiments, which changed the characteristic of all hydrogen bonds, showed a strong effect on *k*<sub>off</sub> rates, because at this stage the sugar has numerous interactions with the channel. Furthermore, all the mutations induces a strong decrease of *in vivo* uptake of sugars. These results clearly demonstrate the importance of the polar track residues on both on and off rates in sugar transport and reveal a strong cooperative effect of hydrogen bond formation.

The outer membrane of Gram-negative bacteria contains passive diffusion channels, which are used for the uptake of nutrients and the exchange of ions through this protective barrier (1–3). Most abundant are the general porins, which allow the passage of small hydrophilic molecules with molecular masses of up to 500 Da (4, 5). Substrate-specific porins, like maltoporin, are moderately specific for a certain class of substances. The structure of the trimeric maltoporin (LamB protein) from *Escherichia coli* has been recently determined to a resolution of 3.1 Å (6). This protein is composed of three 18-stranded antiparallel β-barrels forming the framework of the channel. Three inwardly folded surface loops contribute to a constriction approximately halfway through the channel. Two structural features were observed along the sugar translocation path. First, six adjacent aromatic residues, dubbed the “greasy slide,” line the channel forming a path from the vestibule to the periplasmic outlet. Second, the remainder of the channel at the constriction zone is composed of ionizable residues, lined up in two “ionic tracks.” These ionizable residues are arranged pairwise but in most cases are too far apart from each other to form salt bridges, thus they constitute potential hydrogen bond donors/acceptors with substrates (7). Crystallographic studies (8) have shown that sugars are involved in van der Waals’ contacts with the greasy slide via the hydrophobic face of their glycosyl ring. Multiple H bonds are formed between the sugar hydroxyl groups and the charged residues of the two ionic tracks. Constant breaking and remaking of these hydrogen bonds has been suggested to allow movement of the substrate through the channel (8, 9). The role of the greasy slide in sugar translocation through the channel will be discussed in a future paper.

In this study the role of the polar track residues was investigated in detail. We used a site-directed mutagenesis strategy for introducing mutations into the polar tracks. The ability of the various mutants to transport different sugars was checked by liposome swelling assays and *in vivo* uptake experiments. The on and off rates of maltoolhexaose to the binding site of the LamB protein mutant channels was determined by current fluctuation analysis and compared with those of wild-type LamB. The implications of the polar track residues for sugar uptake will be discussed, and a possible mechanism of translocation will be presented.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis, Expression, and Purification—Site-directed mutagenesis of a plasmid-encoded lamB gene was performed as described (10). Most polar track residues were substituted for alanines resulting in the mutant maltoporins, LamBR8A, LamBR33A, LamBR82A, LamBR109A, LamBD111A, LamBH113A, and LamBD116A. Also two isosteric replacements were made giving mutant proteins LamBD116N and LamBE43Q. Additionally two double mutant porins, LamBR109A-D111A and LamBE43Q-R109A, and a quintuple mutant, LamBR33A-E43A-R109A-H113A-D116A, were constructed. All mutations were confirmed by sequence analysis (11).*

*Mutant maltoporins were expressed in *E. coli* strain BL21(DE3)-omp85Δ(lamB ompR) (10) grown in 600 ml of LB under selection of the antibiotic ampicillin. Expression was enhanced by addition of 100 μM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after overnight growth, and proteins were isolated essentially as described previously (10).*

*Sugar Transport Assays—Four ml of minimal M9 medium, supplemented with ampicillin (100 μg/ml) and 0.2% maltose, was inoculated with plasmid containing derivatives of *E. coli* strain BL21omp5 (10) expressing the various maltoporin mutants. Gene transcription depended on the leakage of the promoter (no isopropyl-1-thio-β-D-galac-
topranoside was added) to avoid overexpression of the proteins. Furthermore, the use of maltose as sole carbon source induced the ABC transporter (12), resulting in a situation where the maltose concentration in the periplasm approaches zero. The bacteria were harvested at late log phase, collected by a quick spin, extensively washed in M9 medium, and resuspended in M9 medium to an A_{600} of 0.2. Two ml of the suspension were used to prepare cell envelope fractions as described previously (13), the rest was used for the \textit{in vivo} transport assay. The isolated cell envelopes were analyzed on SDS-polyacrylamide gel electrophoresis together with a series of increasing amounts of purified LamB of known concentration. After Coomassie Blue staining, the protein bands were quantified using a Computing densitometer model 300A (Molecular Dynamics).

For the transport assay, 10 \mu l of [\textsuperscript{14}C]maltose (ARC Chemical Inc.; specific activity, 600 mCi/mmol\textsuperscript{-1}) was added to 1.5 ml of the cell suspension, and the final sugar concentration was adjusted to 1 \mu M with cold maltose. At different time points after the addition of the sugar, 150 \mu l of the suspension were filtered through a glass microfiber filter (Whatman GF/C) and washed with 5 ml of M9 medium. The filters were dried 10 min at 60 °C and counted in a scintillation counter.

\textbf{Current Fluctuation Analysis and Liposome Swelling Experiments—}

Lipid bilayers were made as described (14) with some minor modifications. Bilayers (Soybean lipid, asolectin type II S, Sigma) were formed across a hole (0.1 mm diameter) in a 0.025-mm-thick polytetrafluoroethylene film (Goodfellow, Cambridge, GB dividing two half-cells containing 5 \% of buffer (1 X KCl, 1 mM CaCl\textsubscript{2}, 10 mM MgCl\textsubscript{2}, and 10 mM Tris, pH 7.4) each. Highly purified protein in detergent (1% octyl-polyoxyethylene) was added to the electrolyte, and its insertion into the bilayer was favored by applying 120–170 mV across the membrane. Subsequently, the buffer in one of the half-cells was exchanged by approximately 20 half-cell volumes of fresh buffer. Ag/AgCl electrodes (Biologic, Claix, France) were used to measure membrane current \textit{via} an amplifier (10\textsuperscript{9} \Omega, BLM-120 from Biologic) allowing the application of adjustable potentials (typically 20 mV) across the membrane. The output voltage was recorded on a storage oscilloscope (LeCroy) equipped with a Fast Fourier Transform module. The Fast Fourier Transform was performed with a rectangular filter on the oscilloscope. Power spectrum densities were recorded with a resolution of 1–5000 Hz and averaged 100–200 times. Analyzing the ion current fluctuations with respect to their frequency yields a background 1/f power spectrum, which was subtracted from the Lorentz power spectrum obtained in presence of sugar (15). For experiments in a deuterated buffer, the pH was adjusted to 7.8, which corresponds to a pH of 7.4 (16). Sugar permeation rates were determined by liposome swelling assays as described by Luckey and Nikaido (17).

\textbf{RESULTS}

\textbf{In Vitro Transport through the Mutant Channels—}

Purified protein was reconstituted in liposomes. The rate of diffusion of sugars into vesicles was monitored by light scattering measurements of swelling of the liposome induced by the transport of sugars. For each mutant the uptake of maltose was normalized to 100\%. Wild-type protein showed relative permeation rates of 222 and 57\% for glucose and maltotriose, respectively, whereas sucrose transport was below the detection limit (data not shown). All mutants showed similar relative permeation rates with marginal variations. Noticeable exceptions were R3A, R109A, E43Q/R109A, and the quintuple mutant, which had acquired the ability to transport sucrose (with a rate of about 10\% of that of maltose).

\textbf{In Vivo Uptake of [\textsuperscript{14}C]Maltose—}

All the mutants showed a strong decrease of sugar transport compared with wild type (Fig. 1), revealing the physiological importance of residues belonging to the polar tracks in the transport process.

\textbf{Influence of Polar Track Mutations on Kinetic Constants—}

Current fluctuation analysis reveals the time-dependent closing and opening of the channel caused by the sugar binding, thus yielding the on and off rates of the sugar to the binding site in the channel. To avoid the problem of asymmetrical channel energy barriers (14), sugar was added to both sides of the membrane. The resulting kinetic rates are thus an average of the dissociation/association rates from both sides of the channels.

The maltotetraose on and off rates and the corresponding affinity constants (K) were determined for each mutant, and the corresponding transport rates were estimated for high or low sugar concentration (Table I). Compared with wild-type LamB protein, all mutants showed a strongly decreased affinity (53–99.7\%) for maltotetraose. These results clearly show that all the charged amino acids constituting the polar tracks play an important role in sugar translocation as predicted by structure analysis of protein-sugar complexes (8). Altogether, the association constants for maltotetraose have considerably decreased in all mutant proteins, even by 2 orders of magnitude for D116N and the multiple mutants. Interestingly, mutant D116A, which is not only unable to form H bonds but additionally also decreases the channel constriction, shows less affected k_{on} values than the isosteric substitution mutant D116N, which displayed a dramatic decrease of the k_{on} constant. Dissociation constants, however, showed little change; in most cases, the change was an increase. Only the single mutants R33A and D116N and the multiple mutants show a slightly decreased k_{off} rate. Again, drastic differences were observed with D116A and D116N.

\textbf{Effect of Deuterium Bonds on Sugar Transport—}

Ion current fluctuation experiments were performed in deuterated water, which is known to give slightly stronger bonds as compared with hydrogen (18, 19). A decreased k_{off} of 50\% for maltotetraose and of 30\% for maltotriose (Table II) was observed. Thus, tighter H bonds in the sugar-protein complex results in a longer residence at the binding site. Remarkably, no effect on the on rates was detected.

\textbf{DISCUSSION}

Previously, structure determination of LamB-maltooligosaccharide complexes revealed the presence of several hydrogen bonds between the hydroxyl groups of the sugar and charged amino acids at the channel lining, the polar tracks (Fig. 2). A see-saw mechanism of hydrogen bond formation was supposed to aid sugar translocation through the porin. This study aimed to justify this hypothesis and to determine the role in transport of each of these polar track residues. The polar track residues were changed by one or in combinations substituted by other amino acids (see "Experimental Procedures"). All these mutants could be isolated as trimers from the bacterial cell envelope as visualized by SDS-polyacrylamide gel electrophoresis (results not shown), and they formed functional channels as demonstrated in liposome swelling assays and planar lipid bilayers.

Most mutants showed a significant decrease of the on rates (from 31\% to 0.31\% of wild-type values; Table I), indicating that polar track residues are clearly involved in the fixation of the

\textbf{FIG. 1. Comparison of \textit{in vivo} transport properties of the mutants.} The diagram compares the maltose transport capacities of the mutants to that of the wild type.
Role of Polar Tracks in Sugar Transport through Maltoporin

On and off rates and resulting binding constant $K$ as determined by current fluctuation analysis in the presence of maltohexaose

<table>
<thead>
<tr>
<th>Amino acid change in LamB</th>
<th>$k_{on}$ values</th>
<th>$k_{off}$ values</th>
<th>Affinity constant ($K$)</th>
<th>$V_{max}$a</th>
<th>$V_{b&gt;10^{-5}}$a</th>
</tr>
</thead>
<tbody>
<tr>
<td>D116N</td>
<td>0.25 ± 0.04</td>
<td>450 ± 50</td>
<td>556 ± 150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R109A/D111A</td>
<td>0.2 ± 0.03</td>
<td>750 ± 65</td>
<td>267 ± 63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>19 ± 3</td>
<td>1000 ± 85</td>
<td>9000 ± 4500</td>
<td>500 (5 mM)</td>
<td>161</td>
</tr>
<tr>
<td>R8A</td>
<td>2.5 ± 0.31</td>
<td>2900 ± 250</td>
<td>862 ± 170</td>
<td>1450 (116 mM)</td>
<td>30</td>
</tr>
<tr>
<td>R33A</td>
<td>0.9 ± 0.13</td>
<td>720 ± 90</td>
<td>1250 ± 340</td>
<td>360 (80 mM)</td>
<td>11</td>
</tr>
<tr>
<td>E43Q</td>
<td>2.5 ± 0.21</td>
<td>2200 ± 120</td>
<td>1136 ± 160</td>
<td>500 (11 mM)</td>
<td>91</td>
</tr>
<tr>
<td>R82A</td>
<td>9 ± 0.9</td>
<td>1000 ± 60</td>
<td>9000 ± 1440</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E43Q/R109A</td>
<td>0.1 ± 0.01</td>
<td>750 ± 30</td>
<td>133 ± 18</td>
<td>375 (750 mM)</td>
<td>1.2</td>
</tr>
<tr>
<td>R109A</td>
<td>0.06 ± 0.01</td>
<td>600 ± 70</td>
<td>100 ± 14</td>
<td>300 (1000 mM)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Each value is the mean of at least three independent measurements. Confidence intervals are computed for a 90% confidence level.

a Calculated number of sugar molecules transported per monomer and per second in saturating conditions ($V_{max}$) or with a sugar concentration of $5 \times 10^{-3}$ M ($V_{b>10^{-5}}$). The saturating concentration of sugar needed to reach the $V_{max}$ is indicated in parentheses.

![Fig. 2. Side (a) and top views (b) of maltoporin pore. Amino acids of the polar tracks located around the constriction site are shown in yellow and green, and sugar is presented with black and red. All colored amino acids are involved in sugar translocation. Green residues (Arg8 and Arg109) play a role in specificity, their conversion to alanine enlarge the channel allowing sucrose passage.](http://www.jbc.org/)

sugar to the binding site of the protein. The $k_{off}$ values, however, showed more variability with, unexpectedly, increasing dissociation rates for most single mutants (Table I). Intuitively, a decreased $k_{off}$ is expected when H bonds, which facilitate sugar movement through the channel, are removed. Replacement of charged residues by the less bulky alanine side chain, however, may not only remove H bonding partners but at the same time may also enlarge the channel and thereby lower the steric hindrance, thus giving a possible explanation for the increase in off rates. Interestingly, all multiple mutants show strongly decreased $k_{off}$ values. Here, the loss of multiple hydrogen bonds can probably not compensate for the decrease in steric hindrance, and thus these residues must be involved in facilitating movement of the sugar molecule. It also reveals a high cooperative effect of hydrogen bond formation with the sugar. Moreover, a similar effect is observed with mutant D116N, which showed a decreased $k_{off}$, whereas mutant D116A showed an increase of the dissociation rate, thus supporting the idea that decreased steric hindrance is compensating for the loss of H bonds.

The second isosteric replacement, E43Q, results in an opposite effect on the off rate, a finding that this time cannot be explained by steric influences. Tentatively, two opposite effects may result from hydrogen bonding. First, H bonds can facili-
tate transport (Arg^{33}, Arg^{109}, Asp^{111}, and Asp^{116}), consequently after (isosteric) replacement, this will lead to a decrease in $k_{off}$. Second, H bonds may stabilize the sugar-protein complex (Glu^{43}); thus removal of an H bond will result in increased $k_{off}$ values.

Another line of evidence for steric effects is provided by osmotic swelling experiments. In agreement with earlier studies (17), liposome swelling clearly shows that short sugars diffuse faster than longer ones. The most significant change observed is that some of them (R8A, R109A, E43Q/R109A, and the quintuple mutant) gained the ability to transport sucrose. This may again be explained by a decrease in steric hindrance because the substitutions will lead to an enlargement of the channel. Indeed, it has been observed that most of the polar track residues are conserved in the sucrose porin ScrY (20), except that the corresponding residue of Arg^{109} has a considerably shorter aspartyl group.

Measurements in D_{2}O have the advantage that protein-sugar interactions can be modified without changing the protein structure; the deuterium bonds are known to be slightly stronger than hydrogen bonds (18, 19). The results show that increasing the strength of noncovalent bonds between the protein and the sugar increases the off rate (Table II). Surprisingly, the $k_{on}$ values are not affected.

Although mutagenesis mainly affects the $k_{on}$ values, experiments with D_{2}O affect the $k_{off}$ rates. This is easily understood considering that both kinetic constants represent two different states of the sugar at the constriction site (Fig. 3).

The $k_{on}$ correspond to the association rate of the sugar. During this step only the first (and maybe also part of the second) glucosyl moiety of the oligosaccharide interact with amino acids via a few hydrogen bonds (Fig. 3a). For this reason, the $k_{on}$ is very sensitive to the loss of bonds (i.e. mutations) and weakly sensitive to a slight increase of the strength of these bonds (i.e. D_{2}O experiments).

The $k_{off}$ correspond to the dissociation rate of the sugar. At this point the sugar is deeply embedded in the protein, all the glucosyl moieties can (and must) interact with the protein, and thus many more hydrogen bonds are formed (only a limited number of all possible interactions are shown in Fig. 3b). Loss of a few H bonds by amino acid substitution will only weakly affect the dissociation rate (i.e. mutations), whereas increasing strength of the sum of H bonds will maintain the sugar longer at the binding site (i.e. D_{2}O experiments).

In agreement with our previous observations, in vivo experiments demonstrated that all the mutants showed a strongly decreased sugar transport as compared with that of wild-type maltoporin (Fig. 1). For the multiple mutants, the variations observed here with maltose in vivo are less pronounced than those observed in vitro with maltohexaose. Possibly the effect of these mutations might be stronger on the transport of longer sugars.

The calculated $V_{max}$ value for wild-type maltoporin is rather low as compared with the $V_{max}$ of many of the mutant channels. However, the mutant channels need much higher concentrations, far beyond physiological conditions, to reach their maximal flow (Table I). Indeed, at very low sugar concentrations wild-type channel is performing optimally (Table I), which is in harmony with the in vivo experimental results.

Finally, the channel can be considered as an enzyme that catalyzes the transport of substrates from one compartment to another (21). In response to the external low sugar concentra-
tion, this system appears to have evolved in a way to optimize the $k_{on}$ rather than the $k_{off}$ to transport-specific substrates with a high efficiency.

Acknowledgments—We thank the anonymous referee for invaluable suggestions and comments on the in vivo assays. We thank Professor Gerhard Schwarz for support.

REFERENCES
Sugar Transport through Maltoporin of *Escherichia coli*: ROLE OF POLAR TRACKS

Fabrice Dumas, Ralf Koebnik, Mathias Winterhalter and Patrick Van Gelder

*J. Biol. Chem.* 2000, 275:19747-19751. doi: 10.1074/jbc.M000268200 originally published online March 27, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000268200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 18 references, 5 of which can be accessed free at http://www.jbc.org/content/275/26/19747.full.html#ref-list-1