Recently we have shown that maltoporin channels reconstituted into black lipid membranes have pronounced asymmetric properties in both ion conduction and sugar binding. This asymmetry revealed also that maltoporin insertion is directional. However, the orientation in the lipid bilayer remained an open question. To elucidate the orientation, we performed point mutations at each side of the channel and analyzed the ion current fluctuation caused by an asymmetric maltohexaose addition. In a second series we used a chemically modified maltohexaose sugar molecule with inhibited entry possibility from the periplasmic side. In contrast to the natural outer cell wall of bacteria, we found that the maltoporin inserts in artificial lipid bilayer in such a way that the long extracellular loops are exposed to the same side of the membrane than protein addition. Based on this orientation, the directional properties of sugar binding were correlated to physiological conditions. We found that nature has optimized maltoporin channels by lowering the activation barriers at each extremity of the pore to trap sugar molecules from the external medium and eject them most efficiently to the periplasmic side.

The outer membrane of Gram-negative bacteria consists of an asymmetrical bilayer with phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet. The lipopolysaccharide headgroups are cross-linked by divalent cations, thereby providing an impermeable network for hydrophilic solutes and protecting the cell from damaging agents such as bile salts, lipases, and proteases. Uptake of nutrients or secretion of end products at each extremity of the pore to trap sugar molecules from the external medium and eject them most efficiently to the periplasmic side.

The high resolution x-ray structure of maltoporin has been solved to 3.1 Å (5). The protein forms a homotrimer presenting three water-filled channels. A monomer consists of an 18-stranded antiparallel β-barrel embedded in the membrane, with large extracellular loops and short turns at the periplasmic side. The third loop, L3, folds inside the pore, contributing to a considerable constriction at the middle of the channel formed by the residue Tyr118. Interestingly, a line of six aromatic residues composed by Trp74* of an adjacent subunit, Tyr41*, Tyr6*, Trp420*, Trp358*, and Phe227*, called the “greasy slide,” extends from the entrance vestibule to the periplasmic outlet (see Fig. 1A) and follows the left-handed helical shape of the longer maltodextrins, conferring a screw-like character to the translocation process. Crystal structures of maltodextrins bound to maltoporin reveal that the sugar molecule is oriented with its nonreducing end pointing toward the periplasmic exit of the pore (6, 7). The complex also reveals three glucosyl-binding subsites where the apolar pyranose rings are in van der Waals’ interaction with the residues Tyr41*, Tyr6*, and Trp420* at the central part of the greasy slide, whereas the moieties at either end of the sugar curl away from the residues at the periphery (Trp74* at the vestibule and Trp358* and Phe227* at the periplasmic end). Simultaneously, sugar-hydroxyl groups are engaged in hydrogen bonds with two “polar tracks.” All of these interactions are supposed to provide a specific sugar translocation pathway. Specific binding of maltodextrins increases the local concentration inside the channel and thus facilitates translocation at low substrates concentrations. Maltporin has been extensively studied using the black lipid membrane technique. The maltoporin proteins are incorporated into planar lipid bilayers and characterized by their conductivity (8–11). Based on the observation that sugar addition reduces channel conductance in a concentration-dependent manner, conductance measurements can be used as a probe to reveal the thermodynamic parameter of the sugar binding. Information about the rate constants of sugar translocation can be obtained using the spectral analysis of the ion current fluctuations (10–13). In previous studies conducted on a large ensemble of porins, site-directed mutagenesis was employed to probe the functional role of the greasy slide (14–16). The mutations affecting the aromatic residues at the channel center considerably reduces the stability constants as suggested by the crystallographic data. The effect of the mutation is less pronounced for residues located at either end of the slide (W74*A, W358*A and F227*A) but demonstrates that they are also involved in the facilitated substrate transport through maltoporin channels.

Recent conductance measurements performed on single maltoporin trimers show that the temporary binding of a maltodextrin molecule inside the channel can be observed as ion current fluctuation as penetrating sugar molecules constrain the passage of ions (17–19). Reconstitution experiments at the single-channel level allowed unambiguous exploration of the directional properties of maltoporin channels. These studies clearly showed that the channel is highly asymmetric with respect to the sugar entrance side and applied voltages and revealed that the maltoporin insertion in artificial membranes is always unidirectional (17–19). However, the real orientation of maltoporin remained an open question.

In multichannel reconstitution experiments, the direction of maltoporin incorporation has been a matter of debate. Based on
the binding asymmetry of a pseudooligosaccharide modified at its nonreducing end (20), on mutants deleted in the large extracellular loops, and on asymmetrical pH-induced closure of the channel proteins, Benz and co-workers (21, 22) propose that maltoporin preferentially inserts with the short periplasmic turns moving through the membrane. In contrast, Van Gelder et al. (23) used bacteriophage-λ and observed the opposite orientation.
In the present study, we address the question of maltoporin orientation at the single channel level. First we investigate the influence of the mutations W74A at the extracellular vestibule and F227A at the periplasmic exit on the kinetics of maltohexaose binding. A second method to probe the orientation is based on the obvious asymmetric binding process of maltohexaose to maltoporin requiring an orientated penetration. We synthesized an ANDS-maltoheptaose derivative (note M6-ANDS in the following), a maltohexaose analogue with a bulky ANDS cap at its reducing end (see Fig. 1B). This modification prevents sugar molecules from penetrating the channel with the reducing end. The knowledge of maltoporin orientation associated with the functional role of the outermost residues of the greasy slide allowed us for the first time to provide a quantitative description of carbohydrate transport involving an asymmetric energy profile for a sugar molecule permeating the channel.

**Materials and Methods**

Preparation and Characterization of M6-ANDS—Maltoheptaose was purchased from Senn Chemicals (Basel, Switzerland), and ANDS was from Interchim (Paris, France). 60 mg of maltoheptaose (around 50 μmol) were labeled as a result of reductive amination by the addition of 250 μl of 0.2 M ANDS in 15% acetic acid and the same volume of NaBH₄/CN 1 m in tetrahydrofuran. The reaction was incubated for 3 h at 55 °C.

The choice of ANDS-maltodextrin cap to probe maltoporin orientation was motivated by the fact that synthesis and characterization of ANDS-maltodextrin derivatives have already been described (24). Maltoheptaose ANDS-derivative, called here M6-ANDS (see Fig. 1B), was first analyzed by capillary electrophoresis, and the major peak of M6-ANDS was identified. Characterization of this peak was performed by capillary electrophoresis coupled with electrospray ionization mass spectrometry under the conditions described below. The negative mass spectrum obtained was dominated by peaks at m/z 1438 and 718, assigned to single and doubly charged deprotonated molecular ions (M−H)− and (M−2H)2− of M6-ANDS.

Capillary electrophoresis-mass spectroscopy analyses were carried out on a CE system PACET™ MDQ (Beckman Coulter, Inc.) with a 75-μm × 80-cm fused silica capillary. The outlet of the capillary was integrated into the ESI spray needle that was directly coupled to an ion trap mass spectrometry system (LCQ™ DUO; ThermoFinnigan, Inc.). The separations were monitored with a Beckman laser-induced fluorescence detection system using a 4 mW argon ion laser with an excitation wavelength of 488 nm and an emission filter of 520 nm. During analysis, the temperature was maintained constant (25 °C) along the capillary, and a voltage of 4 kV was applied at the outlet end of the capillary. The sheath liquid (water/isopropanol, 20/80, v/v) at the rate of 5 μl/min and the sheath gas (nitrogen, 20 units) were infused coaxially to the CE capillary. For measurements, the negative mode was used, and all of the data were collected on X Calibur software (see Ref. 25 for details).

*Planar Lipid Bilayer Experiments*—Planar lipid bilayers have been prepared of diphytanoyl phosphatidylcholine (Avanti Polar Lipids Inc.) according to the technique of Montal and Mueller (26). They are formed across a 60-μm-diameter hole in a 25-μm-thick Teflon film (Goodfellow, Cambridge, UK) being sandwiched between two Delrine chambers, each containing 2 ml of an aqueous solution (1 mM KCl, 1 mM CaCl₂, 10 mM Tris buffered to pH 7.4). The whole setup was shielded from external electromagnetic fields as well as from vibrations to minimize extra noise from other sources. The Delrine cell was enclosed in a double isolated Faraday cage connected with the signal ground and also with a homemade acoustically isolating closet placed on a piezo-electric vibration isolating table (model "Elite 3", Newport Corp., Irvine, CA). The quality of the bilayer membranes was checked by capacitance and residual conductance measurements. The capacitance of the whole system proved to be about 150 picofarads. The residual membrane conductance (less than 7 picosiemens) was subtracted from the overall conductance.

The apparatus had been connected with the external circuit through a pair of homemade Ag/AgCl electrodes encased in 200-μl pipette tips filled with 5% agarose soaked with 1 mM KCl during the fabrication process. The electrode on the so called cis-side of the measuring cell was grounded, whereas the other (on trans) was connected with the head stage of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage clamp mode.

The mutagenesis and purification of maltoporin have previously been described in detail (15). Small amounts of wild type maltoporin from a 0.1 μg/ml buffer solution with 1% octyl-POE detergent (Alessis, Lauchringen, Switzerland) were injected into the cis-side compartment. Incorporation of maltoporin into the bilayer was promoted by applying a transmembrane voltage of 100–200 mV to tentatively disturb the membrane and by stirring for a few seconds after addition. We have optimized the protocol to observe single porin insertion in less than 10 min and to inhibit further insertion. A single maltoporin molecule in the bilayer could be kept stable for several hours without any significant change of its physical properties. The concentration of maltohexaose (Senn Chemicals) was adjusted by adding appropriate small amounts of a concentrated stock solution. The exact sugar concentration was then determined by means of optical polarization measurements (Perkin-Elmer 241). After sugar addition the aqueous solution was homogenized by stirring during a few seconds. Then signals were recorded 20 min later. Titration for the whole range of applied voltages were carried out with the same maltoporin molecule to avoid possible divergences between different individual single protein molecules. All of the measurements were performed at room temperature. The applied transmembrane voltage refers to the potential on the cis-side relative to the trans-side.

The data were filtered with the low pass Bessel filter of the amplifier at 2–5 kHz and then monitored with a Lecroy (Geneva, Switzerland) LT342 digital storage oscilloscope. The entire experiment was recorded on video tape with a digital type recorder (DTR-1204; Biologic, Claix, France). The average power spectrum of the current noise was recorded using the fast Fourier transform module of the oscilloscope. To overcome the additional noise sources mentioned above, the background spectrum of the membrane without sugar was subtracted from each individual spectrum. The curve fitting was carried out using the Marquard-Levenberg method. The total current recording was transferred to a personal computer via a GPIB card using the graphical program LabVIEW 4.01 from National Instruments. Statistical analysis of the blockade events was performed by means of the BioPatch Analysis (Science Instruments) and homemade software.

**Derivation of the Individual Rate Constants of Sugar Binding**—The binding of carbohydrates to the maltoporin channels was assumed for a long time to be symmetric (9, 12), so that once a sugar molecule is bound inside the pore it would have equal probability to translocate or to exit to the same side. Only recent measurements performed on individual maltoporin trimers demonstrated that the association rate is 3–5-fold different depending on the sugar entrance side (18, 19). The reaction for maltodextrin (M) binding has been proposed to be of first order. The internal binding site, P, is accessible from either side of the membrane (cis as well as trans). A bound substrate molecule, PM, closes the
channel to ion current. The related reaction scheme is written as follows,

\[
P \xrightarrow{k_{on}^{cis}} M_{cis} \xrightarrow{k_{off}^{cis}} P \quad \text{and} \quad P \xrightarrow{k_{on}^{trans}} M_{trans} \xrightarrow{k_{off}^{trans}} P
\]

**REACTION 1**

where \(k_{on}^{cis}\) and \(k_{on}^{trans}\) refer to the association rate constants for a sugar molecule entering the channel from the cis- and the trans-sides, respectively; and \(k_{off}^{cis}\) and \(k_{off}^{trans}\) stand for the dissociation rate constants of a sugar molecule exiting from the binding site, respectively, to the cis- and the trans-sides. The two respective pairs of on-off rate constants associated with the cis- and trans-sides are related to the single thermodynamic equilibrium constant, \(K\), according to the detailed balance principle.

\[
K = \frac{k_{on}^{cis}}{k_{off}^{cis}} = \frac{k_{on}^{trans}}{k_{off}^{trans}} \quad \text{(Eq. 1)}
\]

The on rate constants \(k_{on}^{cis}\) and \(k_{on}^{trans}\) can individually be found out from the respective simple cases where sugar is only added to the cis- or the trans-side of the channel. We cannot directly access the off rate constants \(k_{off}^{cis}\) and \(k_{off}^{trans}\) from the current fluctuation measurements because they are not distinguishable. Nevertheless, they can easily be derived from the apparent \(k_{off}\) by \(k_{off} = k_{off}^{cis} + k_{off}^{trans}\) and Equation 1. The relaxation time constant of the binding process, \(\tau\), that modulates the ion current depends on the rate constants and sugar concentration, \([M]\), as follows.

\[
\tau^{-1} = 2\pi f_c = \frac{k_{on}^{cis}[M]^{cis} + k_{on}^{trans}[M]^{trans}}{k_{off}^{cis} + k_{off}^{trans}} \quad \text{(Eq. 2)}
\]

The parameter \(f_c\) can be determined by fitting the power spectral density of the sugar-induced ion fluctuations with the Lorentzian form (12),

\[
S(f) = \frac{S_0}{(1 + (f/f_c)^2)} \quad \text{(Eq. 3)}
\]

where \(S_0\) is the plateau power density at frequencies \(f \ll f_c\), and \(f_c\) is the corner frequency at \(S(f) = S_0/2\).

**RESULTS**

**Effect of the Mutations W74A and F227A on Ion Conduction**—As seen in Fig. 1A, the residue Trp74 is located at the entrance, and Phe227 is located at the periplasmic exit, and both were changed into alanine. To study the influence of these mutations on the directional insertion of maltoporin channels, we first recorded the ion current through single mutant W74A at different applied voltages and calculated the corresponding conductance values. In a second series of measurements, we repeated the recording with the F227A mutant. The results of both recordings are shown in Fig. 2. We observe that the conductance of the wild type and mutated channels increases with the applied voltage and depends on the sign of the external potential. Repeated recordings on porin insertion revealed in all the cases (more than 200 attempts for the wild type...
maltoporin and more than 50 attempts for each mutated proteins) an orientation having the high conductance under smaller potentials on the side of channel addition, i.e. at negative voltages according to our sign convention. At ±190 mV, the asymmetry is 14% for both mutant channels. It is noteworthy that larger conductance at negative voltages has been obtained for all insertions independently on the sign of the potential applied during channel incorporation. However, it depends on the side of protein addition. Conductance of the mutated channels is slightly higher than the wild type maltoporin. Apparently the mutagenesis did not modify the asymmetry in ion conduction, suggesting that the mutations W74A and F227A do not affect the unidirectional insertion of the maltoporin channels into artificial membranes.

**Effect of the Mutations W74A and F227A on Maltodextrin Binding**—After showing the directional incorporation through the asymmetry in conductance, we titrated maltohexaose molecules on one side only to probe the orientation of reconstituted maltoporin channels. Typical current recordings obtained from single wild type, W74A, and F227A maltoporin channels in the presence of one-side addition of maltohexaose are shown in A, B, and C of Fig. 3, respectively. We observed transient blockade events whatever the side of sugar addition was. Kinetic analysis shows that the power spectral density of the fluctuations in the current through individual maltoporin mutants have a Lorentzian form (Fig. 4). Similar behavior has already been reported for the wild type maltoporin channels (17). The rate constants governing the open-close sequences experienced by a given channel can then be determined using Equation 2 by means of a linear plot of the reciprocal relaxation times versus maltohexaose concentrations. The on and off rate constants are reported in Table I. The first observation is that the apparent dissociation rate constant does not depend on the side of sugar addition. The mutation W74A leads to an increase of the off rate comparing with the wild type channels, whereas a significant decrease is found for the modification F227A. This result is directly related to the longer average sugar residence time in F227A channels as observed in Fig. 3. At +150 mV, maltohexaose stays 1.1, 0.7, and 1.7 ms on average inside wild type, W74A, and F227A maltoporin channels, respectively. The second observation is that the mutation W74A introduces a pronounced on rate asymmetry with the side of sugar addition, whereas the asymmetry tends to be reduced in F227A. The replacement F227A displays a small reduction of the on rate at the trans-side, whereas a small increase is observed at the cis-side. Mutant W74A shows a major reduction of the on rate constant at the cis-side compared with wild type by a factor of 3.9 at +150 mV.

The kinetics of maltohexaose binding through wild type maltoporin channels has been shown to be sensitive to the external voltage with higher influence on the apparent dissociation rate of bound substrates (17–19). This binding property has been also used to detect the directional insertion of wild type maltoporin. We studied whether the mutations W74A and F227A influence this voltage dependence by determining the individual off rate constants, \( k_{cis}^{off} \) and \( k_{trans}^{off} \), at ±75 and ±150 mV from one-sided sugar addition experiments (see “Materials and Methods”). The results are presented in Fig. 5. Elementary dissociation rate constants of maltohexaose binding to wild type and mutated channels exhibit the same voltage dependence. Off rates are always higher at negative voltages. This voltage-induced asymmetry is more pronounced at high applied potentials and predominantly concerns \( k_{trans}^{off} \). About 50–60% difference in \( k_{trans}^{off} \) is seen at −150 mV versus +150 mV for all three channels studied.

**Asymmetrical Addition of M6-ANDS**—In a second series of measurements we probe the orientation using an asymmetric substrate. Covalent linkage of a bulky ANDS substituent at the reducing end of maltodextrides strongly increases the inherent asymmetry of substrates. The mechanism of M6-ANDS transport through maltoporin channels was investigated at the single protein level by reconstituting one wild type maltoporin trimer into lipid bilayers. Further evidence of the asymmetry of the channel is observed after one-sided sugar addition (18, 19). Typical recordings of maltohexaose-mediated current fluctuations are shown in Fig. 3A. It is interesting to note that the natural substrate closes the channels independently of which side it enters. In clear contrast to maltohexaose, Fig. 6A shows that one-sided addition of M6-ANDS generates conductance interruptions by multiples of one-third of the initial value only for substrate molecules entering from the cis-side, i.e. the side of maltoporin addition. On the other hand, no modification occurs in the current through maltoporin channels when M6-ANDS is added to the trans-side of the membrane. When maltoporin is injected to the trans-side, open channel conductance asymmetry is inverted, and the side of M6-ANDS addition that generates blockade events becomes
we prepared a strongly asymmetrical maltodextrin analogue modified at its reducing end with an ANDS group (Fig. 1B).

We have chosen a mutation on the two outermost aromatic residues of the greasy slide, Trp74 at the extracellular vestibule and Phe227 at the periplasmic outlet (Fig. 1A), involved in maltodextrin translocation (14, 15). One would expect that the on rate is more affected for sugar molecules entering the protein channels from the side of the modification. This assumption is supported by the recent crystal structure of W74A that revealed only minor structural changes induced by the mutation (15). The mutation W74A shows a strong asymmetrical influence on the on rate because reductions by factors of 3.9 and 1.4 are observed at the cis- and trans-sides, respectively, at +150 mV (Fig. 3B and Table I). The maltoporin orientation with the extracellular exposed surface at the cis-side provides a good explanation for the major contribution of Trp74 to the association rate for sugars penetrating the channel from the cis-compartment. The minor on rate decrease associated with sugar entry from the periplasmic vestibule may derive from small mutation-induced rearrangements inside the pore that in turn modify the subtle position of the affinity determinants. It is noteworthy that the increase of channel conductance generated by the mutations W74A and F227A (Fig. 2) may come from larger vestibule diameters induced by the replacement of the bulky aromatic residues that permits higher ions flow.

In a previous study (19), we attributed voltage effects to changes in the overall dipole moment of the protein in the course of field-induced structural transitions that, in turn, alter the binding affinity. The neutral amino acids Trp74 and Phe227 are expected not to contribute significantly to the dipole moment of the maltoporin channel. The unaffected features of

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**TABLE I**

| Side of sugar addition | Wild type | W74A | F227A |
|------------------------|-----------|------|-------|
|                        | $k_{on}$  | $k_{off}$ | $K$  | $k_{on}$  | $k_{off}$ | $K$  | $k_{on}$  | $k_{off}$ | $K$  |
| **cis**                | $10^6$ M$^{-1}$s$^{-1}$ | 2.7 | 935 | 2890 | 0.7 | 1300 | 540 | 3.2 | 550 | 5820 |
| **trans**              | $10^6$ M$^{-1}$s$^{-1}$ | 9.3 | 855 | 10880 | 6.6 | 1680 | 3930 | 7.2 | 600 | 12000 |

---

**Fig. 5. Effect of the applied voltage on the elementary dissociation rate constants.** Voltage dependence of the individual off rate constants for wild type (circles), W74A (squares), and F227A (triangles) channels with maltohexaose only on the cis-side (open symbols) or the trans-side (filled symbols). The lines are a third order regression through experimental points.
voltage-induced asymmetry in sugar transport through the W74A and F227A mutant channels compared with the wild type (Fig. 5) are in agreement with our previous model. We studied the binding properties of the maltodextrin analogue M6-ANDS to confirm the orientation of maltoporin channels proposed by the asymmetric influence of the mutations W74A and F227A on sugar translocation. The drops in the conductance to multiples of one-third of the initial value can clearly be assigned to reversible and complete occlusions of the individual maltoporin channels among the trimeric protein by M6-ANDS molecules (Fig. 6A). In contrast to natural maltohexaose that reach the central binding site from both sides of the membrane (Fig. 3A), the highly asymmetric maltohexaose derivative, M6-ANDS, binds to the pore only when added to the same compartment as maltoporin.

The energy-minimized structure of M6-ANDS illustrated in Fig. 8 shows that the six glucopyranosyl rings keep a left-handed helical shape close to the natural substrate maltohexaose conformation. This finding shows that M6-ANDS molecules entering maltoporin channels with the nonreducing end first can be recognized as natural maltodextrins despite the modification at the reducing extremity. This is in agreement with the crystallization of maltoporin ligated with nitrophenyl-maltotrioside (7). The helical arrangement defined by the aromatic lateral chains lining the pore allows the translocation of the left-handed sugar helix in a screw-like manner (6, 7). The orientation of sugar molecules entering into the pore is dictated by its helical conformation and the asymmetry of the molecules (28). Only sugars with the good configuration are translocated. To be in agreement with the orientation of bound sugars obtained by crystal structures, maltodextrins have to enter with the nonreducing end first from the extracellular side, whereas they have to present the reducing end when coming from the periplasmic side (Fig. 1). Penetration of sugars (natural substrates as well as M6-ANDS analogue) presenting the nonreducing end from the periplasmic extremity of the pore, thus resulting in bound molecules oriented in the opposite direction.
than observed in the crystallized complex, is improbable because it would strongly modify the network of hydrogen bonds between the sugar hydroxyl groups and the residues of the polar tracks. Therefore, one would expect that M6-ANDS binds to the channel only when coming from the side of the membrane corresponding to the extracellular side of the bacterial outer membrane. Stepwise transitions of the current through one maltoporin trimer resulting from reversible bindings of M6-ANDS entering the channels with the nonreducing end first appear after it has been added to the same side of the membrane than maltoporin proteins (Fig. 6A). In agreement with the orientation of maltoporin channels suggested by the kinetics of maltohexaose transport through the mutants W74A and F227A, we conclude that maltoporin inserts with the periplasmic turns leaving the extracellular loops facing the extracellular medium, which is the cis-side in our experimental procedures.

The net translocation of M6-ANDS through the channel is clearly inhibited for sterical reasons generated by the bulky ANDS substituent. Structural accommodations associated to the passage of the ANDS cap through the pore would be energetically too expensive. We propose that all the time-resolved transitions in the ion current induced by M6-ANDS originate from penetrating M6-ANDS molecules that are forced to exit to the same side. Examination of the kinetics of M6-ANDS binding also provides an elegant way to probe the elementary dissociation rate constant of maltohexaose at the cis-side; the activation energy to exit to the trans-side is infinite, which simplifies, $k_{off} = \frac{k_{off}}{\tau_i} = -200 \text{ s}^{-1}$. This is in excellent agreement with the experimental dissociation rate constant of M6-ANDS at the cis-side.
agreement with the value of $k_{\text{off}}^{\text{cis}}$ for maltohexaose binding to wild type channels at $+150$ mV reported in Fig. 5. In comparison with M6-ANDS, the shorter residence time for maltohexaose is due to another issue to escape with a lower energy barrier.

Physiological Implication of Trp$^{74}$ and Phe$^{227}$—Now that we have solid evidence of maltoporin channel orientation in our reconstitution protocol, it is interesting to link the directional properties of maltohexaose binding with the asymmetric topology of the maltoporin channels. Even if the aromatic residues Trp$^{74}$ and Phe$^{227}$ have symmetrical position into the pore, our single maltoporin study revealed that they play totally different roles in the kinetics of sugar binding. The strong decrease of the on rate introduced at the extracellular side by the mutation W74A demonstrates that this residue helps the association step probably by properly positioning maltodextrins before they diffuse deeper into the channel. At the other end of the slide, Phe$^{227}$ facilitates the sugar release to the periplasm because its mutation in alanine slows down the dissociation rate.

According to the one-site two-barrier model associated with sugar transport through wild type, W74A, and F227A mutant channels, the corresponding free enthalpy energy diagram is illustrated in Fig. 9. The kinetic association constants depend on the free enthalpy of activation, $E_a$, according to Eyring equation: $k_{\text{on}} = k_{\text{max}} \exp(-E_a/RT)$, where $R$ is the gas constant, $T$ is the temperature, and $k_{\text{max}}$ is a temperature-dependent factor. In classical transition state theory, $k_{\text{max}} = 6 \times 10^{12}$ s$^{-1}$ at 20 °C. In the present system, where the sugar transport involves motions in solution, this value is inappropriate. The pre-exponential factor, which reflects the diffusion-limited rate of encounter of sugar molecules and channel extremities in aqueous buffer, has been approximated for sugar binding to periplasmic proteins as 10$^9$ m$^{-1}$s$^{-1}$ (29). We chose this value as an estimate for the pre-exponential factor of the association rate constants $k_{\text{on}}^{\text{cis}}$ and $k_{\text{on}}^{\text{trans}}$ that permits evaluation of the corresponding $E_a^{\text{cis}}$ and $E_a^{\text{trans}}$ reported in Fig. 9. Importantly, the relative onset activation energies between the cis- and trans-sides or between mutated and wild type channels can directly be calculated from the individual rate constants for sugar binding without any estimation of $k_{\text{max}}$ . Trp$^{74}$ participates in the reduction of the extracellular activation barrier, $E_a^{\text{cis}}$, because the mutation W74A induces an increase of 3.3 kJ/mol at $+150$ mV. The activation barrier that has to overcome a bound substrate molecule to exit the pore by the periplasmic outlet equals $\Delta G_0 + E_{\text{trans}}$. In the wild type channel, Phe$^{227}$ helps the sugar release to the periplasmic side by decreasing this activation barrier by 1.3 kJ/mol compared with the mutant F227A.

The net flux of substrate molecules, $\phi$, across asymmetrical one-site two-barrier model under one-sided sugar addition at concentration $[M]$ is given by the following equation,

$$\phi = \frac{K_{\text{off}}^{\text{cis}} k_{\text{off}}^{\text{trans}} [M]/(k_{\text{on}}^{\text{cis}} + k_{\text{on}}^{\text{trans}} + k_{\text{on}}^{\text{cis}})}{k_{\text{on}}^{\text{trans}} + k_{\text{off}}^{\text{trans}} + k_{\text{off}}^{\text{cis}} - [M]} \quad \text{(Eq. 4)}$$

under the assumption that the sugar concentration on the opposite side sets to 0, which is fulfilled in our single molecule study. $k_{\text{on}}^{\text{cis}} = k_{\text{on}}^{\text{cis}}$ for $[M] = [M]^{cis}$ and $k_{\text{on}}^{\text{trans}} = k_{\text{on}}^{\text{trans}}$ for $[M] = [M]^{trans}$. Fig. 10 shows the flux of maltohexaose through a single wild type, W74A, and F227A channel at $+150$ mV for translocation from cis to trans (thick lines) according to the free enthalpy diagram of Fig. 9 and from trans to cis (thin lines). Two main points have to be noted. First, the number of translocated substrate molecules through the three types of studied channels is higher in the cis to trans direction. The effect is more pronounced at concentrations higher than 100 $\mu$M. Interestingly, although the activation barrier is higher on the cis-side (resulting in a smaller number of binding events for sugar added to the cis-side; Fig. 3), the channel permeability is more efficient from the cis-side, because a substrate molecule will have a higher probability to translocate (>0.5) once it reached the binding site than to exit to the same side. The opposite is valid for sugars coming from the trans-compartment. This finding demonstrates that the orientation of maltoporin channels into the bacterial outer membrane is important to enhance sugar permeability from the extracellular medium (the cis-side in our protocol) to the periplasmic side. Second, the wild type channel is already optimized for long linear maltodextrins translocation because the mutations W74A and F227A reduce the maltohexaose flux.

Structural considerations showed that the periodic energy profiles of the hydrogen bonds and hydrophobic contacts are shown to be shifted resulting in a “smooth” total energy profile (30). This permeation type involves significant energy steps only on initial association and final dissociation of the channel. Furthermore, no significant rearrangements leading to energy reorganization had to occur along the translocation pathway of a sugar molecule (30, 31). This suggests that maltoporin can reduce the onset activation barrier by having its affinity site prearranged with an hydrophobic environment and a charge
distribution complementary to the permeant sugar. This mechanism of transition state stabilization by specific solvation substitution constitutes the basis of enzymatic catalysis process in a polar solvent (32). In this sense, maltoporin channels catalyze the flux of maltodextrins the same way that enzymes catalyze chemical reactions. Protein channels and enzymes seem to employ the identical molecular mechanism of transition state stabilization to ensure their functions. In the particular case of maltoporin channels, we showed here that residues Trp^{74} and Phe^{27} are crucial for directional transport by reducing the two encountered activation barriers.

Concluding Remarks—To our great surprise maltoporin channels insert in the opposite direction than in vivo. This observation remains to be investigated in further detail. The in vivo mechanism of porins folding and insertion is not well established, but the implication of several patterns like lipopolysaccharides, periplasmic chaperones, and catalysts has been proposed (33–37). Black lipid membrane experiments represent a highly simplified model system where folded porins spontaneously insert into symmetric and planar lipid bilayers. In a future experiment the effect of lipopolysaccharides should be investigated.

On the other hand, the orientation of maltoporin with the external loops facing to the side of protein addition corresponds nicely to the orientation with an optimal turnover number of sugar molecules. Based on the presented data, we may easily quantify the energy barrier and turnover number of maltodextrins across maltoporin.

We use the asymmetric voltage effects as a quick tool to demonstrate the directional insertion of maltoporin in an artificial membrane. At 100 mV applied voltage, the signal allows a clear distinction between the two orientations. It is interesting to note that the voltage across the outer membrane is much lower (38). Therefore, variations of the transmembrane voltage probably have a minor influence on the uptake of substrates in vivo (39).

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