On Translocation through a Membrane Channel via an Internal Binding Site: Kinetics and Voltage Dependence

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ABSTRACT Here we present a model for maltodextrin translocation through maltoporin channels. In a first step, our theoretical analysis does consider the case of a single binding site for a given substrate in a structurally unaffected channel with a possibly different entrance barrier on either side. It is shown how by means of conventional electrical conductance measurements (including current noise analysis) the basic equilibrium and rate constants can be determined as functions of the applied voltage. Then also the net translocation rate of the substrate becomes accessible quantitatively. This most simple model mechanism has been extended to include a voltage-dependent fast conformational change of the channel that prevents the binding process. The so developed approach has been tested with experimental data for a single maltoporin trimer being reconstituted in black lipid membranes when studied in the presence of maltohexaose as the substrate. The experimental results turned out to be clearly incompatible with binding alone. They are, however, very satisfactorily fitted by pertinent theoretical curves if also inhibition of binding by a conformational transition is taken into account. Accordingly, quantitative evaluations of the underlying parameters and eventually of the translocation rate have been carried out successfully. Our analysis reveals a set of parameters necessary for an optimal translocation that nicely corresponds to natural conditions.

INTRODUCTION

Bacteria require pore-forming proteins to mediate the exchange of substrate (e.g., nutrients or antibiotics) across their cell wall. This applies particularly to the porins of *Escherichia coli* whose structure and function have already been investigated experimentally in great detail (Schirmer et al., 1995; Boos and Shuman, 1998). In many of these studies the respective protein channels had been reconstituted into artificial black lipid membranes (BLM) (Benz et al., 1987; Nekolla et al., 1994; Winterhalter, 1999; van Gelder et al., 2000). The penetration of substrate could be measured by means of electrical conductance experiments owing to the inherent blockade of ionic current. More recently this was even extended to noise-analysis methods applied to current fluctuations observed with single protein molecules (Bezrukov et al., 2000; Kullman et al., 2002). In such a way, stationary as well as dynamic properties of the underlying molecular mechanism have been established. The results clearly revealed a very pronounced dependence on the applied voltage, i.e., the electric membrane potential.

The available evidence calls for a theoretical basis describing the thermodynamics and kinetics of the underlying substrate-specific binding that could then be utilized to derive the translocation rate from pertinent experimental data. Naturally, the binding site should be accessible from either side of the membrane. The pertinent kinetics of those two pathways must therefore be expected to feature (at least) one individual activation barrier for each of them. Such a two-barrier kinetic model was proposed some time ago with regard to sodium channels in nerve, but was not worked out in detail for general use (Woodhall, 1973). Also, the application of a one binding site/two barrier model was briefly discussed in the case of maltoporin translocation data (Benz et al., 1987). However, this did neither actually consider different barrier heights nor the nature and quantification of the voltage dependence.

In the present article we develop a comprehensive theoretical analysis of a fundamentally general model. It gives full quantitative account of experimentally observable parameters. Then the real net translocation rate can be evaluated on the basis of measured numerical results. The voltage effect is attributed to specific reaction dipole moments occurring in the extremely large electric field of the membrane (Schwarz, 1978). How this may be utilized in practice will be tested and demonstrated with available data for maltohexaose permeation through maltoporin (i.e., LamB) channels of *E. coli* being reconstituted in BLM.

THEORETICAL

The Model

The channels are assumed to be incorporated parallel to each other from one side (called *cis*). There is one internal binding site that, at any rate, is accessible from either side (*cis* as well as *trans*). Each of these access routes is envisaged to be subject to possibly quite different activation barriers (owing to an asymmetric channel structure). Parameters related to *cis* are marked by a prime superscript, *trans* by a double prime one. A bound substrate molecule closes the channel to ionic current.

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The adequate reaction scheme with the respective ''on'' and ''off'' rate constants is then written

$$S^{cis} + O \underset{k'_{on}}{\overset{k'_{off}}{\leftrightarrows}} B \underset{k''_{off}}{\overset{k''_{on}}{\leftrightarrows}} O^{trans} + S.$$

The symbols O and B stand for channels being open for binding or else are closed by a bound substrate molecule, respectively. The off-reactions are of the first order; the actual second order on-reactions will, however, be pseudo first order since the substrate concentrations on either side (denoted c' and c'') remain practically constant because of the small number of channels (in BLM experiments) that keeps the transport of substrate between *cis* and *trans* sides to an extremely low level. There is only one thermodynamic binding constant, K, related to the various rate constants according to

$$K = k'_{on}/k'_{off} = k''_{on}/k''_{off} = k_{on}/k_{off} \quad (1a)$$

$$\text{with } k_{on} = k'_{on} + k''_{on}, \quad k_{off} = k'_{off} + k''_{off}. \quad (1b)$$

Since we are going to consider directional properties (translocation flux, voltage, dipole moments), a positive direction is generally defined as *trans* toward *cis*.

General kinetics

An open channel can be observed through its ionic conductance. Since bound substrate is assumed to result in zero conductance of the channel concerned, the measured ionic current I would be proportional to the number of open channels, $N_o = (I/I_{max})N_P$, where I_{max} denotes the current in the case that all N_P channels are open. Accordingly this N_o may be analyzed regarding its reaction kinetics. Following standard procedures, the appropriate rate equation is formulated as

$$dN_o/dt = -(k'_{on} \cdot c' + k''_{on} \cdot c'') \cdot N_o + k_{off} \cdot (N_P - N_o). \quad (2)$$

It involves two specific rate processes: i), a comparatively fast one that results from establishing a practically stationary state of N_o where the on-reactions are balanced out by the off-reactions, and ii), a translocation of substrate through the channels that runs extremely slow if only a few channels are available. The final thermodynamic equilibrium with $c' = c''$ will therefore not be attained within the timescale of BLM experiments if initially one has started with $c' \neq c''$. Thus dN_o/dt can be taken to be virtually zero to calculate the quantitative conditions in the prevailing intermediate stationary state (known in classical kinetics as the Lindemann-Hinshelwood approach, in biochemistry being applied to enzyme catalysis by Michaelis-Menten). This leads to a flow-equilibrium where

$$I_{max}/I - 1 = (N_P - N_o)/N_o = X \equiv K' \cdot c' + K'' \cdot c'', \quad (3a)$$

with the two *cis*, *trans* ''pre-equilibrium'' constants

$$K' = k'_{on}/k_{off} = K/(1 + k''_{off}/k'_{off}) \quad \text{and}$$
$$K'' = k''_{on}/k_{off} = K/(1 + k'_{off}/k''_{off}), \quad (3b, c)$$

respectively. Note that

$$K' + K'' = K \quad \text{and} \quad K'/K'' = k'_{on}/k''_{on} = k'_{off}/k''_{off}. \quad (4a, b)$$

A plot of experimental $(I_{max}/I) - 1$ versus respective concentrations according to Eq. 3 *a* can be used to determine the various K values (stationary approach).

The stationary state probability (i.e., fraction) of open channels accordingly turns out to be

$$p_o = 1/(1 + X). \quad (5)$$

Any deviations from this level tend to be canceled out in a linear relaxation process derived from the above Eq. 2. It involves a single relaxation time τ subject to the relation

$$1/\tau = k'_{on} \cdot c' + k''_{on} \cdot c'' + k_{off} = k_{off} \cdot (1 + X). \quad (6)$$

This can be used to determine the rate constants by means of a linear plot of the reciprocal relaxation times versus concentrations (dynamic approach).

The translocation rate

Under stationary-state conditions and $c' > c''$, a net transport of substrate can be expected to proceed in the negative direction, i.e., from *cis* to *trans* (due to a negative gradient of the chemical potential). It apparently becomes equal to the *trans*-off rate minus the reverse *trans*-on rate. Thus the resulting increase in the number of substrate molecules N''_S on the *trans* side when calculated per channel becomes

$$dN''_s/dt = k''_{off} \cdot (1 - p_o) - k''_{on} \cdot c'' \cdot p_o. \quad (7)$$

In the case of $c' < c''$, net transport naturally occurs in the opposite direction, which is described with prime instead of double prime superscripts in Eq. 7. Only for $c' = c''$ can no net flow of substrate take place—that is, of course, the state of the actual thermodynamic equilibrium.

Taking into account Eqs. 3–5, a general relation for the translocation rate can be formulated in terms of a conventional law of permeation with a permeability coefficient P. One obtains a flux J (substrate molecules per time) through one channel in the given positive direction

$$J = -P \cdot \Delta c \quad \text{with } P = K \cdot k^*_{off}/(1 + X), \; k^*_{off} = k'_{off}k''_{off}/k_{off} \quad (8a, b, c)$$

and $\Delta c = c' - c''$. Thus a negative Δc causes a positive flux whereas a positive Δc results in a negative flux, i.e., one that runs in the reverse direction (i.e., *cis* → t*rans*).

Voltage dependence

According to basic principles of thermodynamics, an electric field strength E is a variable of state, just like temperature and

external pressure. Equilibrium constants therefore depend on E as described by an appropriate van't Hoff equation

$$\partial \ln K/\partial E = \Delta M/RT \tag{9}$$

involving the molar reaction dipole moment ΔM, i.e., the change of the overall dipole moment parallel to the field for a molar turnover of the underlying chemical reaction. Actually the effect is absolutely negligible in ordinary non-membrane systems since ΔM and especially E are usually much too small. The situation changes into larger orders of magnitude, however, when it comes to reactions of oriented molecules in a biological membrane (Schwarz, 1978). Here one has $E = -V/d$, with V being the applied voltage (positive if the electric potential is higher on *cis*) whereas d is the effective channel length regarding the voltage drop in the membrane. This field would readily become extremely large. Also ΔM may take on rather substantial values. Thus a voltage effect could certainly be a significant factor in the present binding reaction. By integration of Eq. 9, one obtains an exponential dependence of K on V according to

$$K = K^{\circ} \cdot \exp(a \cdot V)(K^{\circ}: \text{ zero voltage equilibrium constant}),$$
$$\text{where } a = -\Delta m/(dkT) \tag{10a, b}$$

is determined by the molecular reaction dipole moment $\Delta m = m_B - m_O$, i.e., the change of the channel dipole moment in the positive direction upon binding one substrate molecule.

An analogous relation applies to any elementary rate constant (i.e., one related to a single activation barrier) (Schwarz, 1978), namely

$$k = k^{\circ} \cdot \exp(\alpha \cdot V)(k^{\circ}: \text{ zero voltage rate constant}),$$
$$\text{where } \alpha = -\Delta m^{\ddagger}/(dkT) \tag{11a, b}$$

involving the reaction dipole moment $\Delta m^{\ddagger}$ in the transition state (i.e., on top of the activation barrier).

Our approach is simply empirical, being based on the Arrhenius equation with i), some preexponential factor k° subject to experimental evaluation as an adjustable parameter, and ii), an activation energy that changes proportional to the membrane potential in analogy to the thermodynamically derived equation for the equilibrium constant (Eq. 10) It stands to reason that in any case there must be an activated state to be crossed. We do not propose any structurally founded theoretical relation for the two relevant parameters. They are to be determined from the measured data. These obviously confirm quite well our predicted relationships, particularly as far as the voltage dependence is concerned.

Conformational change

So far it has been assumed that the channel itself is not affected by the applied voltage. However, the effective field could possibly induce a conformational change that alters the binding affinity. In the simplest case this may be a one step transition from a basic open state (o) to another one (•) whose ionic conductance as well as binding affinity is different. The equilibrium constant $K_{\bullet} = N_{\bullet}/N_o$ would naturally be subject to an analogous field effect as expressed by Eqs. 9 and 10 (then ΔM is the change of the molar dipole moment in the course of the structural transition). This phenomenon does of course complicate the general kinetics of the open channels if binding of substrate and structural change proceed at comparable rates. For the sake of simplicity, we do now only consider the case that the transition goes much faster than the binding reaction. Then the former process has always established an instantaneous equilibrium with $N_{\bullet} = K_{\bullet} \cdot N_o$ in the time range of binding. Also the converted state • is assumed to have completely lost its binding affinity. However, it may nevertheless still have an ionic conductance that is by a factor γ different from the basic state. Under these circumstances, the number of apparently open channels, being actually measured using the ionic conductance method, becomes equal to $N_\gamma = N_o \cdot (1 + \gamma K_{\bullet})$. This must be taken into account to derive the properly extended rate equation for N_γ. As is easily seen, Eq. 2 may simply be multiplied by the factor $(1 + \gamma K_{\bullet})$ while the number of channels without bound substrate, namely $N_o \cdot (1 + K_{\bullet})$, is substituted for N_o in the k_{off} term. So one arrives at

$$dN_\gamma/dt = k_{off} \cdot (1 + K_{\bullet}) \cdot N^*_\gamma - [k'_{on} \cdot c' + k''_{on} \cdot c'' + k_{off} \cdot (1 + K_{\bullet})] \cdot N_\gamma,$$
$$\text{where } N^*_\gamma = N_P \cdot (1 + \gamma K_{\bullet})/(1 + K_{\bullet}) \tag{12}$$

stands for the apparently total number of channels when taking into account a change of ionic conductance upon the structural transition under consideration.

Now the conductance approach including noise analysis results in an apparent equilibrium constant of binding

$$K^{(app)} = K/(1 + K_{\bullet}). \tag{13a}$$

This reduction of the pure binding constant by a change of the channel structure naturally also applies analogously to K' and K''. Regarding rate constants in the reciprocal relaxation time versus concentration plot, it turns out that the k'_{on}, k''_{on} (slope) remain unaffected whereas the intercept changes to

$$k^{(app)}_{off} = k_{off} \cdot (1 + K_{\bullet}). \tag{13b}$$

Furthermore, the permeability coefficient of Eq. 8 *a* also has to be modified. Instead of Eq. 8 *b*, one must now use the adjusted formulation

$$P = K \cdot k^*_{off}/(1 + K_{\bullet} + X). \tag{14}$$

EXPERIMENTAL

The present model theory has been tested in the light of experimental data obtained in the case of maltohexaose translocation through maltoporin (LamB) channels in planar lipid membranes. To this end, theoretical predictions are compared with experimental findings of our own and more pertinent ones that had been reported in the literature.

Materials and Methods

BLM experiments

Planar lipid bilayers have been prepared of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) according to the technique of Montal and Mueller (1972). 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 a freshly purified aqueous solution (1 M KCl, 1 mM $CaCl_2$, 10 mM Tris buffered to pH = 7.4). A small magnetic stirrer was placed in each compartment to quickly homogenize the solution after adding a new substance. The whole setup was shielded from external electromagnetic fields as well as from vibrations to minimize irrelevant membrane current fluctuations. 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 Corporation, 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 ~150 pF. The residual membrane conductance (<7 pS) 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 M KCl during the fabrication process. The electrode on the *cis* side of the measuring cell was grounded whereas the other (on *trans*) was connected with the headstage of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage clamp mode.

The purification of maltoporin has previously been described in detail (Dumas et al., 2000). Small amounts of wild-type maltoporin from a 0.1 μg/ml buffer solution with 1% octyl-POE detergent (Alexis, 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 and by stirring for a few seconds after addition. 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, Basel, Switzerland) 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 (PerkinElmer 241) (PerkinElmer, Wellesley, MA). This revealed a nonnegligible surplus of the commercially quoted amount (up to 25%). After sugar addition, the aqueous solution was homogenized by stirring during a few seconds. Then signals were recorded 20 min later. Titrations 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 the measurements were performed at room temperature. The applied transmembrane voltage refers to the potential on the *cis* side relative to the *trans* side.

Data acquisition and analysis

The data were filtered with the low pass Bessel filter of the amplifier at 2–5 kHz and then monitored with a LeCroy LT342 digital storage oscilloscope (LeCroy, Geneva, Switzerland). The resulting standard deviation of noise after amplification was 1 pA. The entire experiment was recorded on videotape with a digital-type recorder (DTR-1204, Bio-Logic, Claix, France). The averaged power spectrum of the current noise was recorded using the fast Fourier transform module of the oscilloscope. The data were sampled at a rate reflecting a Nyquist frequency of 12.5 kHz. With these parameters, the frequency interval between two successive points in the power spectrum becomes 2 Hz. 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 Marquardt-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 (Austin, TX). Statistical analysis of the blockade events was performed by means of Bio-Patch analysis (Bio-Logic, Grenoble, France) and further homemade software.

Sugar-induced current fluctuation analysis

Earlier studies have shown that sugar binding reduces channel conductance in a concentration-dependent manner (Benz et al.,1986, 1987). Based on this observation, the pore conductance can be taken as a probe for the apparent equilibrium constants of sugar binding to maltoporin by means of linear regression analysis according to Eq. 3 *a*. Since we know that the three channels of a maltoporin molecule are equivalent and independent of each other (see below), we can so determine the apparent equilibrium binding constant based on an established simple binomial distribution of open and closed channels (Kullman et al., 2002) (stationary approach). More recently the power spectral densities of the sugar-induced current fluctuations were fitted to Lorentzian frequency functions (Nekolla et al., 1994; Bezrukov et al., 2000), suggesting a simple one-step model where the channels are either fully open (i.e., conducting) or completely blocked for ion current (i.e., nonconducting) when a sugar molecule happens to be inside and assuming that the three channels of a single protein are independent of each other (Verveen and De Felice, 1974; Lindemann, 1980; De Felice, 1981). A Lorentzian curve as a function of frequency f reads

$$S(f) = S_o/(1 + (f/f_c)^2), \quad (15)$$

where S_o is the zero frequency spectral density, and the corner frequency f_c (relaxation frequency) can be determined at $S(f) = S_o/2$. The proposed linear dependence of $2\pi f_c = 1/\tau$ on sugar concentration can thus be taken advantage of to determine the appropriate on- and off-rate constants according to Eqs. 6 and 12 (dynamic approach).

RESULTS

Statistical analysis of time-resolved blockade events

The mechanism of maltohexaose transport through a maltoporin channel was investigated at the single molecule level by reconstituting one maltoporin trimer (three channels) into a lipid bilayer. The addition of maltohexaose in the aqueous solution gives rise to time-resolved conductance steps as illustrated in Fig. 1. The transient blockade events are related to a reversible sugar binding on the affinity site of a channel. These transitions were observed whatever the side of sugar addition had been. It suggests that maltohexaose can reach its binding site from either *cis* or *trans* side. The conversion from one state to the other is governed by the rate constants of the binding process. Those reactions can be envisaged as random block-unblock sequences experienced by individual maltoporin channels that generate fluctuations in the course of time. Within the accuracy of our measurements, no evidence of different monomer channel conductances could be observed that would reflect three distinct modes of sugar-induced single channel blockades.

Fig. 1 also illustrates the amplitudes histogram of the conductance measurements for sugar added only on the *cis* side (Fig. 1, *A* and *C*), and only on the *trans* side (Fig. 1, *B* and *D*), both taken at ±150 mV. Any channel subunit of the trimer can be occupied independently revealing four different conductance states being distinguishable (namely fully open,

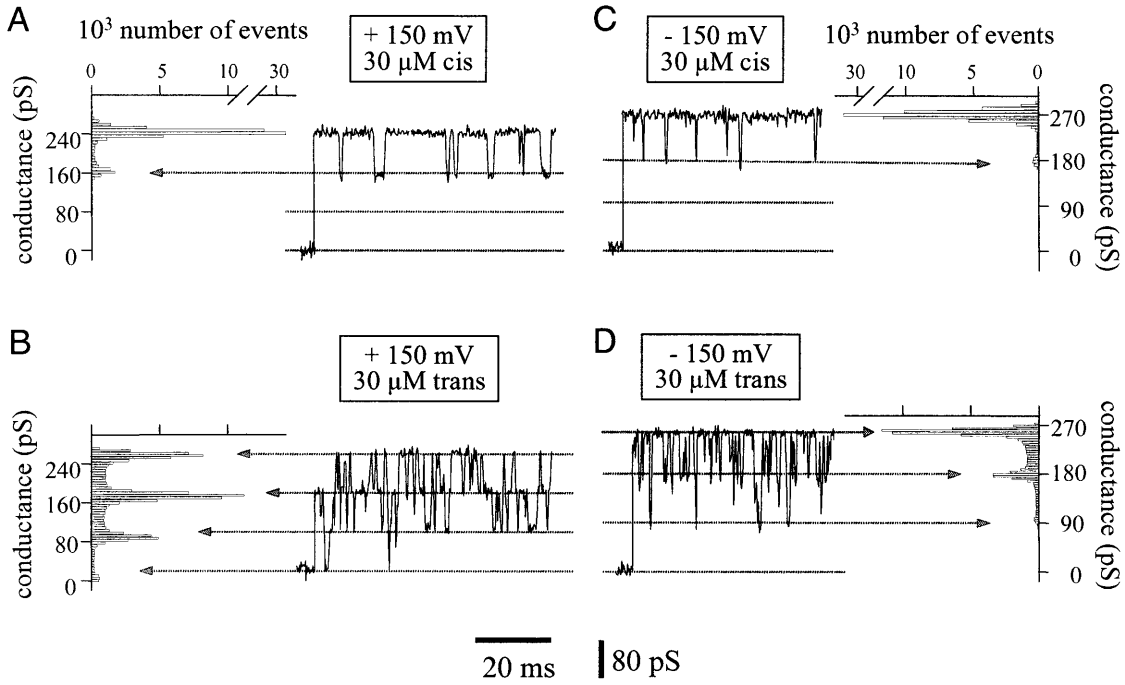

FIGURE 1 One-sided addition of maltohexaose in compartment *cis* (panels *A* and *C*) and *trans* (panels *B* and *D*) produces transient blockades of the single maltoporin current showing that the sugar molecule can reach its binding site from both channel openings. The associated amplitude histograms of fluctuations between different conductance levels are shown near the traces. They correspond to a total recording of 1 min for each of the experimental conditions. Single-channel events with a well-defined amplitude can be observed demonstrating the homotrimeric form of maltoporin. Zero conductance reflects complete blocking of all three monomeric channels. The mean lifetime of the fully open state is smaller when sugar is added on the *trans* side due to $k''_{on} > k'_{on}$. Note that the mean duration of blocking events is longer at +150 mV than at −150 mV owing to the larger apparent binding constant.

single closed, double closed, and fully closed). The difference between two consecutive peaks corresponds to the mean of the single channel conductance. At negative voltages the open channel conductance is higher than at the positive side (Kullman et al., 2002). In the case of −150 mV and +150 mV, we determined 91 pS and 84 pS, respectively, for one channel. For each transition this demonstrates the homotrimeric nature of the inserted maltoporin molecule as well as the asymmetry of the conductive state of the channel. The larger conductance at positive voltages has been observed for all insertions (more than 200) and was taken to probe the unidirectional insertion of maltoporin into the bilayer.

Stationary state

The apparent equilibrium constant of maltohexaose binding as it varies with the applied voltage is exhibited in Fig. 2. This includes measurements of stationary conductance changes versus concentration (Eq. 3 *a*) as well as findings obtained in the dynamic way based on a ratio of pertinent rate constants. Obviously both approaches yield results that agree rather well in view of a possible error margin that may extend up to ~20–25%. Also we note a good conformity with relevant reports in the literature.

The chosen semilogarithmic presentation clearly demonstrates the absence of a single exponential voltage dependence as predicted for a one site equilibrium binding constant according to Eq. 10 *a*. Necessarily the simple binding model must therefore be improved to be in line with the experimental facts. It would, however, not be useful to introduce further potential binding sites since this will only lead to a sum of exponential terms. As can be easily verified, such

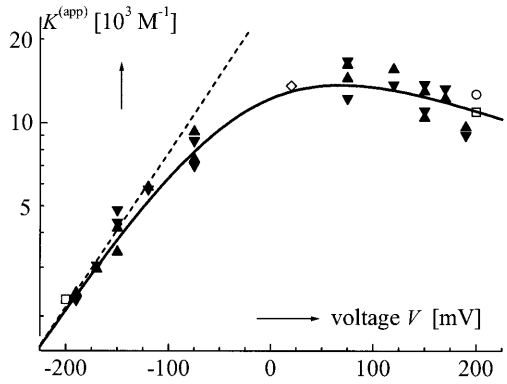

FIGURE 2 Voltage dependence of the apparent binding constant of maltohexaose determined by the stationary (▲) and dynamic approaches (▼), respectively (see text). Some data taken from the literature are shown for comparison: (◯) (Bezrukov et al., 2000), (◇) (Hilty and Winterhalter, 2001), and (□) (Kullman et al., 2002). The straight line (*dashed*) reflects the actual binding constant (according to Eq. 10 *a*) whereas the solid curve depicts a fit taking into account the modification caused by the proposed structural change of the channel in terms of Eq. 13 *a*.

a functional relationship does at any rate imply an upward curvature but in no case would fit the given negative curvature that over and above displays a maximum.

The peculiar course of the data indicates a weakening of the effective binding affinity. Such a situation actually occurs when a fast voltage gating of the channel interferes with the binding process so that an apparent binding constant according to Eq. 13 *a* would be measured using the present experimental approaches. Indeed this extended model does solve the problem at issue. The solid curve in Fig. 2 has been calculated accordingly with

$$K = 3.0 \cdot 10^4 \cdot \exp(0.013 \cdot V/\mathrm{mV})\mathrm{M}^{-1};$$
$$K_\bullet = 1.45 \cdot \exp(0.016 \cdot V/\mathrm{mV}).$$

Each of these equilibrium constants does feature the single exponential voltage dependence required by Eq. 10 *a*. The actual occurrence of such a structural transition is corroborated by the observation that the apparent ionic channel conductance changes when going from the negative to the positive voltage domains.

In the present context it should be noted that the curve fit under consideration involves the choice of two adjustable parameters (K^o, a in Eq. 10 *a*) for each of the two equilibrium constants. This implies a larger range of uncertainty affecting their quantitative evaluation. Actually the smaller values $K = 1.7 \cdot 10^4 \cdot \exp(0.01 \cdot V/\mathrm{mV})\,\mathrm{M}^{-1}$ and $K_\bullet = 0.55 \cdot \exp(0.015 \cdot V/\mathrm{mV})$ would also allow a still fairly satisfactory curve fit within the error margin of the measured data. However, there can be no doubt that the proposed basic approach does well describe the observed course of the apparent binding constants.

Kinetics

Rate constants have been determined based on our power spectral density data (see Eq. 15) They were evaluated applying linear regression analysis of $2\pi f_c = 1/\tau$ versus sugar concentration. Examples are presented in Fig. 3.

The apparent off-rate constants derived are depicted in Fig. 4. Our results are found to be practically the same no matter whether symmetric or asymmetric allotment of concentrations had been chosen just as expected for the proposed model. In addition, these results are compared with a number of others in the literature. All of those data are evidently in good agreement as far as the voltage effect is concerned. In view of the applicable change of channel conformation, this should be considered as the apparent off-rate constant expressed by Eq. 13 *b*. The actual k_{off} for the pure binding is thus obtained as $k_{off}^{(app)}/(1 + K_\bullet)$. Once all the data have been treated in this way, the resulting points fall into a rather well-pronounced straight line represented by

$$k_{off} = 340 \cdot \exp(-0.0123 \cdot V/\mathrm{mV})\ \mathrm{s}^{-1}$$

or alternatively $k_{off} = 580 \cdot \exp(-0.009 \cdot V/\mathrm{mV})\ \mathrm{s}^{-1}$ when adopting the smaller equilibrium constants mentioned above.

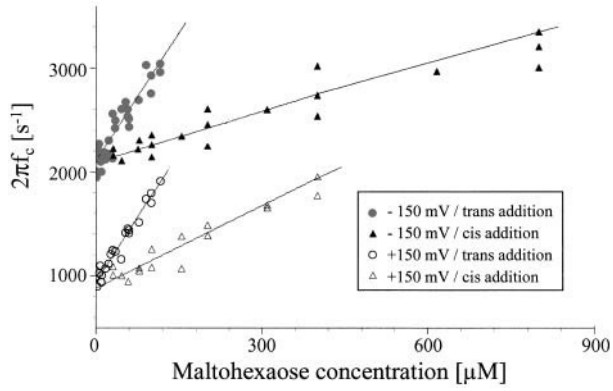

FIGURE 3 Plots of $2\pi f_c = 1/\tau$ versus sugar concentration calculated from the measured power spectral densities. The linear regression analysis was performed over three to five independent titration experiments of the same one-sided sugar addition type. Note that for each specific condition, linearity is ensured for sugar concentrations lower than the approximate apparent dissociation constant ($= 1/K^{(app)}$). This implies that less than half of the channels are closed. The tendency of a downward curvature at comparatively high sugar concentration is presumably caused by an average blockade frequency beyond the filter cut-off frequency. It can be clearly seen that at a given voltage, the intercept for *cis* as well as *trans* addition is the same ($= k_{off}$) whereas the slope is substantially smaller in the former case ($k'_{on} < k''_{on}$!).

Reapplying Eq. 13 *b* with this k_{off} and the present $K_\bullet$ does then indeed turn out to be an excellent fit of the originally measured $k_{off}^{(app)}$. Since k_{off} is anticipated to be the sum of the appropriate *cis* and *trans* rate constants, a merely single exponential course proves to be a bit of a surprise. After all, it simplifies the outcome in the present special case. The *cis* as well as *trans* on- and off-rate constants should accordingly have the same exponential factor (see Eq. 1, *a* and *b*). This also implies the same constant value for their ratios.

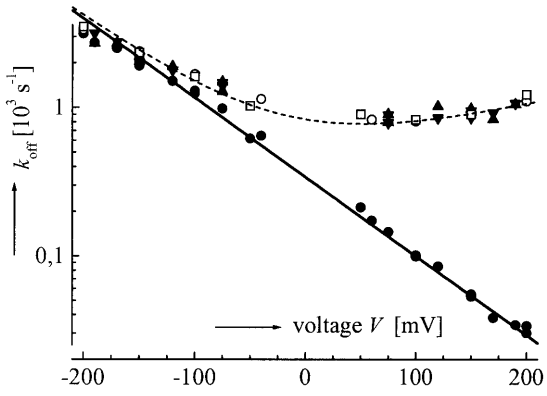

FIGURE 4 Voltage dependence of the apparent off-rate constant in the case of having maltohexaose added only on the *cis* (▲) or *trans* (▼) sides, respectively. Further relevant data have been taken from the literature: (○) (Bezrukov et al., 2000) and (□) (Kullman et al., 2002). The actual off-rate constants (●) have been calculated in view of Eq. 13 *b* (see text). They can evidently be fitted by a very pronounced straight line (*solid*). This relationship for k_{off} being inserted vice versa in Eq. 13 *b* together with the present $K_\bullet$ values results in the dashed curve that does indeed quite well fit the original experimental data.

In Fig. 5 *A*, the two on-rate constants are shown as determined with *cis* or *trans* side added substrate. The latter quantity proved to be less susceptible of experimental error ($\approx \pm 10\%$). A linear regression analysis results in rather well-fitting straight line described by

$$k''_{on} = 8.6 \cdot 10^6 \cdot \exp(0.0006 \cdot V/\text{mV})\ \text{M}^{-1}\,\text{s}^{-1}.$$

The error margin for the k'_{on} is markedly enhanced ($\approx \pm 20$—25%) owing to the substantially lower absolute values. Within such given limits they can nevertheless rather well be fitted using the same exponential factor as

$$k'_{on} = 2.2 \cdot 10^6 \cdot \exp(0.0006 \cdot V/\text{mV})\ \text{M}^{-1}\,\text{s}^{-1}.$$

Accordingly one obtains a ratio $k''_{on}/k'_{on} = 3.9 = k''_{off}/k'_{off}$ so that

$$k'_{off} = 0.204 \cdot k_{off},\ k''_{off} = 0.796 \cdot k_{off}.$$

Finally also the apparent *cis*, *trans* flow-equilibrium constants can so be fitted quite satisfactorily by $0.204 \cdot K^{(app)}$ and $0.796 \cdot K^{(app)}$, respectively, as is demonstrated in Fig. 5 *B*. Thus all the results are very well consistent with each other as far as our proposed model is concerned.

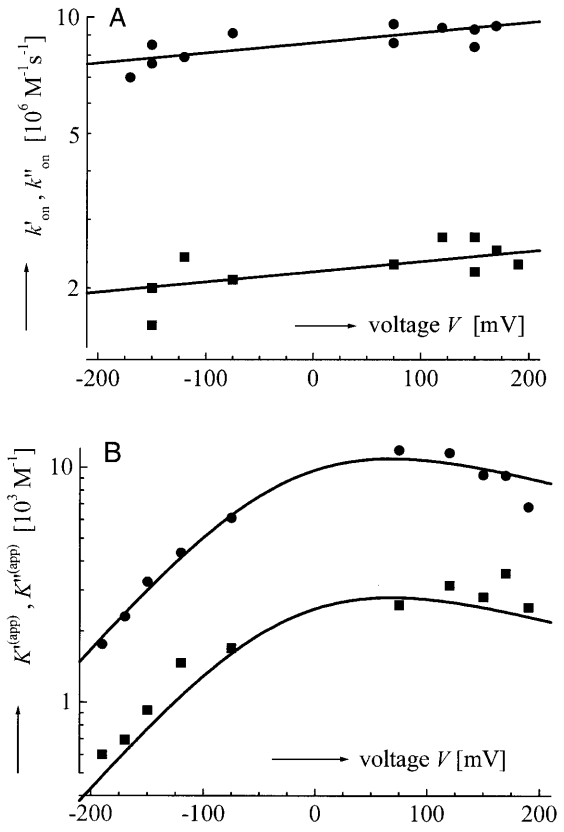

FIGURE 5 (*A*) Voltage dependence of the on-rate constants with maltohexaose only on the *cis* (■) or *trans* (●) sides, respectively. Within the given error margins, either set can be well fitted by a single exponential relationship (see text). (*B*) Voltage dependence of the apparent flow-equilibrium constants. Averaged *cis* (■) and *trans* (●) side results are shown together with theoretical fit curves based on the present evaluation of relevant rate constants (see text).

DISCUSSION

The available data for maltohexaose transport through maltoporin channels do evidently rule out a mechanism that considers only binding of the substrate but leaves the channel structure unchanged when the applied voltage is varied. We argue that in such a case the apparent binding constant must at any rate display either a linear course or a positive curvature. Actually just the opposite has been observed. This indicates a voltage-induced structural transition of the channel impairing its binding affinity. Such a process would also be in line with our finding that the channel conductance is markedly altered upon the application of positive voltages in comparison with negative ones.

In view of the experimental facts reflected in the observed voltage dependence, it appears to be opportune to propose a model mechanism involving one binding site that gets lost upon a fast structural transition. This did indeed provide a successful means to describe the measured results. We note that the involved structural gating apparently does not very much affect the ionic channel current conductance. Thus only a minor change of the channel aperture is indicated that still allows a largely unrestricted passage of small ions. Nevertheless it may certainly abolish the binding and permeation of the bigger sugar molecules just by some minor but crucial alterations of the atomic structure.

By applying our pertinent theoretical relations, all the measured data could be very well fitted quantitatively with no inconsistency. So the voltage dependence of the various equilibrium and rate constants has been evaluated. Eventually the net translocation flux could then be calculated by means of Eq. 14. The actual flux for concentrations added only on

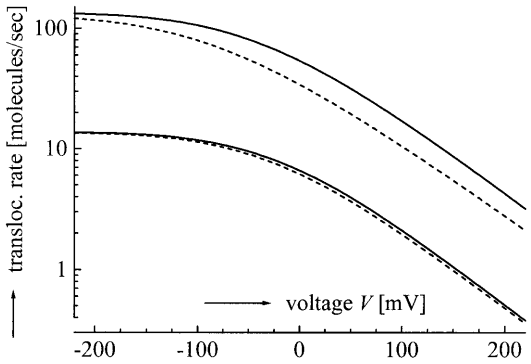

FIGURE 6 Voltage dependence of the translocation rate (molecules per channel per second) from the *cis* to the *trans* side (*solid*) and from the *trans* to the *cis* side (*dashed*), respectively, calculated by means of Eqs. 8 *a* and 14 using the pertinent parameters as they have been evaluated from the experimental results. The upper curves refer to a concentration of 100 μM, the lower ones to 10 μM (with no substrate added to the other side).

one side is displayed for illustration in Fig. 6. We note that (due to $K' < K''$!) the *cis* to *trans* flux is generally somewhat larger than the reverse *trans* to *cis* flux, although this can hardly be distinguished any more at the lower concentration of 10 μM. Furthermore it turns out that a comparatively high rate of translocation occurs in the negative voltage range (about an order of magnitude below free diffusion through an unrestricted water-filled channel). It does, however, level down substantially at increasing positive voltages.

Another point of interest concerns the reaction dipole moments that give rise to the voltage effects. According to Eq. 10 *b*, this amounts to −63.5 D (Debye units) in the case of the present binding constant K (or only −49 D with the above mentioned alternative choice of a smaller K). It implies that upon binding a substrate molecule, the dipole moment of the channel (including the bound material) decreases by said amount if measured in the positive direction (*trans* to *cis*). This is a quite reasonable order of magnitude in view of the involved macromolecular structure. Actually the present evaluation does assume an effective $d = 4$ nm (Eq. 10 *b*). A possible smaller d would accordingly reduce the appropriate reaction dipole moment. This also applies to the structural change where −78 D is obtained in the case of our first choices of $K_\bullet$ and d.

Turning to the rate constants, it proves to be somewhat unexpected to find the same activation dipole moments of +60 D (or alternatively +44 D) for each k_{off} and −3 D for each k_{on}, respectively. In other words, the electrical charge distribution changes do not differ for either one of the activation barriers. These barriers do nevertheless have different overall energy levels since the *trans* rate is about four times higher than the respective *cis* rate.

Looking at the sugar translocation process in terms of a basic one site/two barrier model appears to be a great simplification of a problem that comprises a whole sequence of interactions of the sugar molecule with various different parts of the channel (Dutzler et al., 2002). Crystal structures of maltodextrins bound to maltoporin reveal three glycosyl-binding subsites at the channel constriction (Dutzler et al., 1996). The results suggest the existence of a specific sugar translocation pathway with an aromatic amino acid ''greasy slide'' being aligned by ''polar track'' residues. Indeed it has recently been shown that aromatic residues at each extremities of the channel facilitate sugar transport (Hilty and Winterhalter, 2001). Nevertheless, the resulting ''smooth'' energy profile determined by Meyer and Schulz (1997) showed that no large energy barriers have to be overcome during the sugar movement from one subsite to the next. The sugar binding can then be described by a merge energy minimum, in agreement with our simplified reaction model that can quite well describe the data as they reflect all successive steps of sugar translocation.

It stands to reason that the apparent reaction dipole moments must arise from a difference in the structure of the involved molecular states. The crucial point is the concomitant change of the electrical charge distribution. So far the atomic structure of the open and ligated maltoporin channel, respectively, was seen to be the same at a resolution of 3.1 Å (Schirmer et al., 1995) and 2.4 Å (Meyer et al., 1997). Nevertheless, it is quite conceivable that a sufficient number of charge displacements below 2 Å may occur, resulting in dipolar changes of the present order of magnitude. In addition we emphasize the possible role of polar water domains. The constriction zone of unligated maltoporin was indeed found to be filled with strongly bound water molecules (Schirmer et al., 1995). For steric reasons the sugar transport implies the expulsion of bound water both from the sugar molecules as well as from the channel lumen. Also the ionic residues of the ''polar tracks'' provide a specific polar environment designed as solvation substitute and for favorable electrostatic interactions with the sugar. Reaction-induced changes of such structural factors may substantially contribute to the particular reaction dipole moments.

CONCLUSIONS

We have presented a comprehensive theoretical reaction model that describes the thermodynamic and kinetic parameters of substrate translocation through a membrane channel featuring one binding site and two access barriers. It could be very successfully tested with a maltoporin system, provided a certain voltage-induced conformational change of the channel is taken into account. By means of quantitative fits of the voltage dependence of pertinent experimental data, the basic parameters could be evaluated and be utilized to calculate the actual translocation rate as a function of the applied membrane potential. This suggests further applications to similar systems.

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