

Kinetics and thermodynamics of binding reactions as exemplified by anthrax toxin channel blockage with a cationic cyclodextrin derivative

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The thermodynamics of binding reactions is usually studied in the framework of the linear van't Hoff analysis of the temperature dependence of the equilibrium constant. The logarithm of the equilibrium constant is plotted versus inverse temperature to discriminate between two terms: an enthalpic contribution that is linear in the inverse temperature, and a temperature-independent entropic contribution. When we apply this approach to a particular case—blockage of the anthrax PA₆₃ channel by a multicharged cyclodextrin derivative—we obtain a nearly linear behavior with a slope that is characterized by enthalpy of about 1 kcal/mol. In contrast, from blocker partitioning between the channel and the bulk, we estimate the depth of the potential well for the blocker in the channel to be at least 8 kcal/mol. To understand this apparent discrepancy, we use a simple model of particle interaction with the channel and show that this significant difference between the two estimates is due to the temperature dependence of the physical forces between the blocker and the channel. In particular, we demonstrate that if the major component of blocker–channel interaction is van der Waals interactions and/or Coulomb forces in water, the van't Hoff enthalpy of the binding reaction may be close to zero or even negative, including cases of relatively strong binding. The results are quite general and, therefore, of importance for studies of enzymatic reactions, rational drug design, small-molecule binding to proteins, protein–protein interactions, and protein folding, among others.

protein-ligand binding | molecular docking

Motivated by the search for efficient small-molecule blockers of “virulent” transmembrane channels (1–3), we investigated the temperature behavior of the blockage reaction. The temperature dependence of the equilibrium constant of blocker association with the pore, that is, the reaction blocker(bulk) + channel(empty) \rightleftharpoons channel – blocker, was found to be surprisingly weak, with the temperature coefficient $|Q_{10}| \simeq 1.08$. Applying the standard linear van't Hoff analysis to evaluate the enthalpy of an association reaction (4) to that of channel blockage (5), we arrive at an estimated enthalpy for the channel–blocker interaction of about 1 kcal/mol. However, examination of blocker partitioning between the bulk and the channel interior gives an estimate for the depth of the potential well for the blocker in the pore of at least 8 kcal/mol. Although these two estimates generally do not have to coincide, this impressive difference compelled us to take a detailed look at the assumptions that are often used in the thermodynamic analysis of blocking reactions as well as at the possible physical forces involved in the channel–blocker interaction. Our analysis is based on consideration of a simple model of particle interaction with the channel (6–8), which allows an explicit calculation of the involved thermodynamics. As might be expected, in the case of the temperature-independent flat potential well, the enthalpy of the binding reaction is equal to the depth of the potential well. However, in the realistic

case of temperature-dependent interactions, the situation may be drastically different. In particular, we demonstrate that accounting for the temperature dependence of van der Waals and/or Coulomb forces may lead to very small or even negative enthalpy values. These conclusions go beyond the particular case of channel blockage to being helpful in the analysis of other problems such as protein–ligand binding, protein–protein interaction, and molecular docking for drug design, to mention just a few.

Results

The blockage of the anthrax PA₆₃ channel by per-6-*S*-(3-amino)propylthio- β -cyclodextrin (AmPr β CD), a derivative of β -cyclodextrin carrying seven positive charges (1–3), is temperature-dependent. Fig. 1 gives three representative records of the ion current through a single channel at 15, 23, and 40 °C (*Top* to *Bottom*, respectively) in the presence of 0.3 μ M AmPr β CD. It is seen that higher temperatures (*i*) speed up the kinetics of the blockage process and (*ii*) increase channel conductance. The increase in the on- and off-rates of the reaction manifests itself in the decrease of the average times spent by the channel in both open and blocked states. As temperature is elevated from 15 to 40 °C the conductance of the channel grows by a factor of 2, which is close to the effect of the temperature on ionic conductance of other large channels (9).

Fig. 2 demonstrates the temperature dependence of the characteristic on- and off-times of the blockage reaction. Both the average time between successive blockages, τ_{on} , measured at the fixed blocker concentration of 0.3 μ M, and the average time the blocker molecule spends in the channel, τ_{off} , decrease with increasing temperature. The temperature-induced drop in τ_{off} only slightly exceeds that in τ_{on} . The times were calculated from the characteristic corner frequencies of Lorentzian spectra and the independently measured probability of finding the channel in the blocked state, as described earlier (3).

The plot of the natural logarithm of the equilibrium constant of the blocking reaction as a function of the inverse temperature, $1,000/T$, the linear van't Hoff plot (4), is given in Fig. 3. The equilibrium constant is calculated from the probability of finding the channel in the blocked state at a given blocker concentration, as described earlier (3). It is seen that within the accuracy of our measurements the dependence is linear and very weak. To illustrate this point, the figure also shows the conductivity of salt solution in the same temperature range, with $Q_{10} \simeq 1.18$.

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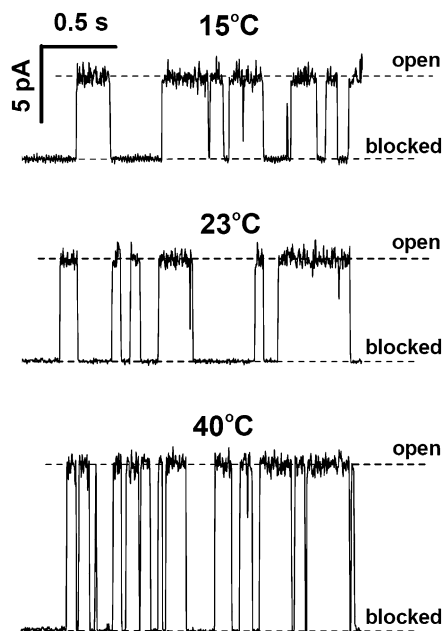


Fig. 1. Typical recordings of an ionic current through a single PA₆₃ channel in the presence of 0.3 μM AmPrβCD on the *cis* side of the membrane demonstrate temperature-dependent amplitudes and kinetics. Measurements were performed in 1 M KCl (pH 6.6) bathing solution at 50-mV transmembrane voltage and are presented at 10-ms time resolution. It can be seen that the current through the open channel and the frequency of blockage events increase with solution temperature, whereas the AmPrβCD binding time decreases.

Discussion

Temperature Dependence of the Equilibrium Binding Constant. Our analysis of the data in Fig. 3 gives $K_{eq}(10^\circ\text{C})/K_{eq}(50^\circ\text{C}) \approx 1.38$ for the change of the equilibrium constant when the temperature of the system is varied from 10 to 50°C. This leads to a $|Q_{10}|$ value of about $(1.38)^{1/4} \approx 1.08$ that is significantly smaller than even the Q_{10} for solution conductivity. This weak dependence is usually interpreted as an anomalously low binding energy, which would correspond to a very shallow potential well for the blocker–channel attraction.

Indeed, if we fit the equilibrium constant data in Fig. 3 with the linear van't Hoff equation widely used for the thermodynamic analysis of binding reactions (4, 10, 11),

$$\ln K_{eq} = \Delta H/RT - \Delta S/R, \quad [1]$$

where T and R are the absolute temperature and universal gas constant, respectively, and, if both enthalpy ΔH and entropy ΔS are assumed to be temperature-independent, we obtain $\Delta H \approx 1$ kcal/mol or about $2 k_B T$ per molecule, where k_B is the Boltzmann constant. The enthalpy term is supposed to characterize the strength of the blocker–channel interaction and be equal to the depth ΔU of the temperature-independent flat potential well for the blocking particle inside the channel (see model considerations below).

This energy estimate is drastically smaller, by more than $10 k_B T$, than the depth of the potential well estimated from blocker partitioning between the channel lumen and the bulk. For the depth of the potential well describing blocker partitioning under the simplifying assumptions detailed below, we have

$$\Delta U \approx k_B T \ln \left(\frac{C_{ch}}{C_b} \right), \quad [2]$$

where C_{ch} is the blocker concentration in the channel and C_b is the blocker concentration in the bulk.

From the very beginning it is necessary to point out that Eq. 2 provides only the lower limit for the depth of the potential well describing the blocker–channel interaction. The main reason, as explained below, is that the notion of “blocker concentration in the channel” is a simplification based on the assumption of a homogeneous, square-well interaction potential for the blocker within the whole length of the blocker-accessible part of the channel. This concentration could be estimated by combining the data in Fig. 1, showing that channel occupancy by the blocker molecule is of the order of 1, with the existing predictions for the channel structure (12) illustrated in Fig. 4. However, even with the accepted simplifications, the estimate in Eq. 2 is inaccurate in the case of high occupancy, because of the strong repulsion between blocker molecules. To escape this additional complication, we make use of the data obtained at an order of magnitude lower blocker concentration in the bulk, $C_b = 3 \times 10^{-8}$ M (3). In this case the channel occupancy by the blocker, $P_b(C_b)$, which is equal to the average number of blocker molecules in the channel, $\langle N(C_b) \rangle = P_b(C_b) = \tau_{off}/(\tau_{off} + \tau_{on})$, is much smaller than 1. Taking the values of $\tau_{on} \approx 1.2$ s and $\tau_{off} \approx 0.11$ s from figure 3 b and c of ref. 3, for this blocker concentration we have $\langle N(C_b) \rangle \approx 0.084$. To calculate the blocker concentration in the channel, $\langle N(C_b) \rangle/v_{ch}$, we need an estimate for the volume v_{ch} available for the center of the blocker molecule inside the channel lumen, $v_{ch} = \pi(R_{pore} - R_{blocker})^2 L$, where R_{pore} is the pore radius, $R_{blocker}$ is the blocker radius, and L is the blocker-accessible channel length; we assume that this part of the channel has a cylindrical shape. Based on the structure of the blocker (2) and structural predictions of ref. 12 for the PA₆₃ pore reproduced in Fig. 4, we accept the following values for the parameters: $R_{pore} \approx 2$ nm, $R_{blocker} \approx 1$ nm, $L \approx 4$ nm. This leads to $\langle N(C_b) \rangle/v_{ch} \approx 0.7 \times 10^{25}$ m⁻³, which translates into the molar blocker concentration in the channel $C_{ch} \approx 1.2 \times 10^{-2}$ M at $C_b = 3 \times 10^{-8}$ M in the bulk. Substituting these concentrations into Eq. 2, we obtain $\Delta U \approx 13 k_B T$ or about 8 kcal/mol. It has to be noted here that this value gives only a lower estimate for the depth of the potential well, because the effective “binding volume” (13) of the blocker molecule can indeed be much smaller than the volume anticipated on the basis of the structure shown in Fig. 4.

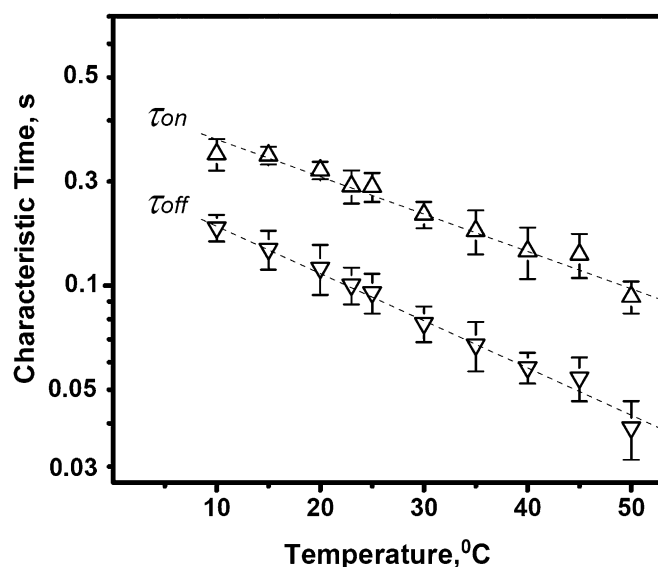


Fig. 2. Kinetic parameters of AmPrβCD-induced current blockage depend on the temperature of the bathing solutions. Both τ_{on} (time between successful blockages) and τ_{off} (binding time) decrease exponentially when the temperature increases. Error bars show SDs.

integral for a particular system must include the effects of the changes in all internal degrees of freedom, including those of the water molecules that are pushed out by the blocker molecule, the change in blocker and channel hydration, and so forth. However, for the purpose of the present analytical consideration, all these effects can be packed into the cross-sectional area $A(x, \beta)$ and the potential of mean force $U(x, \beta)$, whose temperature dependences would then account for the above-mentioned effects. From Eqs. 5–7 it follows that

$$K_{eq} = Q_{ch}, \quad [9]$$

and the van't Hoff equation is

$$\frac{d \ln K_{eq}}{d\beta} = \frac{\Delta H}{N_A} = \left\langle \frac{\partial}{\partial \beta} [\ln A(x, \beta) - \beta U(x, \beta)] \right\rangle_B, \quad [10]$$

where N_A is the Avogadro number and the angular brackets denote Boltzmann averaging over the channel,

$$\langle f(x, \beta) \rangle_B = \frac{\int_0^L f(x, \beta) A(x, \beta) \exp(-\beta U(x, \beta)) dx}{\int_0^L A(x, \beta) \exp(-\beta U(x, \beta)) dx}. \quad [11]$$

Temperature-Independent Rectangular Potential. We first consider the case of a cylindrical channel of radius a and length L , assuming that the interaction with the blocker particle is described by a square-well potential of depth ΔU , which is temperature-independent (Fig. 4A). For such a channel, Eq. 10 takes the form $d \ln K_{eq} / d\beta = \Delta U$, leading to

$$\ln \frac{K_{eq}(T)}{K_{eq}(T_0)} = \left(\frac{1}{T} - \frac{1}{T_0} \right) \frac{\Delta U}{k_B}, \quad [12]$$

where T_0 is a reference temperature. It is seen that Eq. 12 yields a linear dependence of $\ln K_{eq}$ on the inverse temperature with the slope equal to the well depth that characterizes the strength of blocker–channel interaction, measured in k_B units. Importantly, in this case, $\Delta H / N_A = \Delta U$, that is, the depth of the potential well equals the enthalpy of the binding reaction.

Arbitrary Temperature-Independent Potential. The square-well potential of mean force occupying the entire blocker-accessible part of the channel represents a grossly simplified situation. We will now consider a cylindrical channel with an arbitrary coordinate-dependent potential. In this case, it is easy to show that Eq. 1 does not hold true even for temperature-independent $U(x)$ and a constant cross-sectional area. Indeed, from Eq. 10 it follows that the derivative $d \ln K_{eq} / d\beta$ is

$$\frac{d \ln K_{eq}}{d\beta} = -\langle U(x) \rangle = -\frac{\int_0^L U(x) e^{-\beta U(x)} dx}{\int_0^L e^{-\beta U(x)} dx}, \quad [13]$$

where the Boltzmann-averaged potential $\langle U(x) \rangle$ is a function of temperature. This temperature dependence comes from the fact that at different temperatures the blocker molecule explores different parts of the potential well with different probabilities.

As an example, consider the case when the bottom of the potential well occupying the entire channel is a step with the parameters L_1 and L_2 and the corresponding depths ΔU_1 and ΔU_2 , as shown in Fig. 4B. In such a case, Eq. 10 leads to

$$\frac{d \ln K_{eq}}{d\beta} = \frac{\Delta H}{N_A} = \frac{L_1 \Delta U_1 \exp(\beta \Delta U_1) + L_2 \Delta U_2 \exp(\beta \Delta U_2)}{L_1 \exp(\beta \Delta U_1) + L_2 \exp(\beta \Delta U_2)}. \quad [14]$$

This shows that $\Delta H / N_A$ is a function of temperature unless $\Delta U_1 = \Delta U_2$ and/or L_1 or L_2 is zero. As a consequence, Eq. 1 is inapplicable in this case.

Nevertheless, in many important cases of temperature-independent $U(x)$, the plot of $\ln K_{eq}$ versus $1/T$ in the narrow temperature range corresponding to functional protein states is remarkably linear. One of them corresponds to $\Delta U_1 \gg \Delta U_2$ or $\Delta U_1 \ll \Delta U_2$. Another one is the case of a localized potential well—a narrow deep binding site, illustrated in Fig. 4C. Similar close-to-linear behavior is found for a tilted potential (Fig. 4D) when the slope of the tilt is large enough. Moreover, as we demonstrate below, even in the case of a temperature-dependent potential, which leads to $\Delta H \neq \Delta U$ for the square-well potential shown in Fig. 4A, the van't Hoff plot may be nearly linear.

Temperature-Dependent Strength of the Interaction: Electrostatic, van der Waals, and Hydrogen-Bonding Forces. In the considerations presented above, we assumed that the physical interactions, described by the potential of mean force, are temperature-independent. However, for the water-filled channel pore under consideration, this is hardly the case, and potential $U(x)$ is a function of temperature. Then, in the simplest case of a cylindrical channel with the square-well potential of depth $\Delta U(\beta)$ (Fig. 4A), Eq. 10 leads to

$$\frac{d \ln K_{eq}}{d\beta} = \Delta U(\beta) + \beta \frac{d \Delta U(\beta)}{d\beta}. \quad [15]$$

As we show below, the temperature dependence of the interactions determining the depth $\Delta U(\beta)$ of the potential well is able to significantly change the slope $d \ln K_{eq} / d\beta$ and even invert its sign.

Experimental evidence, especially the strong reduction in binding strength with the increase in bathing solution salt concentration (3), suggests that the channel–blocker interaction involves Coulomb forces. If so, the depth of the potential is expected to be temperature-dependent. This is due to the temperature dependence of the water dielectric constant $d \ln \epsilon(T) / d \ln T \simeq -1.35$ at room temperature (15).

Let us consider a hypothetical case wherein the depth of the potential well is defined by Coulomb interactions between the “fixed” charges on the channel lumen and the charge on the blocker molecule. The interaction energy of two charges $Q_1 e$ and $Q_2 e$ positioned at distance r in 1:1 electrolyte with number concentration $c = C_{salt} N_A 10^3 \text{ m}^{-3}$, where C_{salt} is the molar electrolyte concentration and e is the elementary charge, is given by

$$\Delta U = \frac{1}{4\pi \epsilon(T) \epsilon_0} \frac{Q_1 Q_2 e^2}{r} \exp\left(-\frac{r}{\lambda_D(T)}\right), \quad [16]$$

where ϵ_0 is the dielectric constant of vacuum, $\epsilon(T)$ is the temperature-dependent ratio of the dielectric constant of the medium to that of vacuum, and $\lambda_D(T)$ is the temperature-dependent Debye length,

$$\lambda_D(T) = \sqrt{\frac{\epsilon(T) \epsilon_0 k_B T}{2e^2 c}}. \quad [17]$$

Thus, the temperature behavior of the equilibrium constant, Eq. 15, depends on both the changing dielectric constant and the

distance between charges. The temperature dependence of the dielectric constant for macroscopic samples of water is well-studied. If the values of $\epsilon(T)$ are taken for bulk water, which are 84.1 at 10 °C and 69.9 at 50 °C (15), then the expected temperature dependence of $\ln K_{eq}$ not only gets weaker but is reversed. We plotted this dependence in Fig. 5 (solid line) in comparison with the dependence expected for the temperature-independent rectangular potential well of depth $\Delta U = 13 k_B T$ at 20 °C (dashed-dotted line). The solid line is calculated by substituting Eqs. 16 and 17 into 15 and taking the temperature-dependent $\epsilon(T)$ from ref. 15. Other parameters are $Q_1 = Q_2 = 7e$, $c = 6.02 \times 10^{26} \text{ m}^{-3}$ (1 M KCl), and $r = 0.51 \text{ nm}$ (to match $13 k_B T$ at 20 °C for the dashed-dotted line). For both curves, we use the same reference temperature, $T_0 = 20 \text{ °C}$, to facilitate their comparison. It is clear that the formal application of Eq. 1 would give a negative enthalpic contribution even in the case of very deep potential wells, corresponding to the strong attractive interactions between the channel and the blocker.

In the case of van der Waals forces at room temperature, the corresponding ΔU is supposed to grow nearly proportionally to the temperature (16). Then, Eq. 15 leads to $d \ln K_{eq} / d\beta \approx 0$, and the enthalpy calculated from the linear van't Hoff plot is close to zero. On the contrary, the forces due to hydrogen bonding may decrease linearly with temperature (17). Thus, according to Eq. 15, the slope of the temperature dependence of $\ln K_{eq}$ will increase, as illustrated by the counterclockwise arrow in Fig. 5. It should be noted that quantitative treatment of these two cases is complicated. The point is that the depth of the potential well for the blocker in the channel is the difference between the temperature-dependent van der Waals and hydrogen-bonding interactions of the blocker molecule with water in the bulk and with water and protein in the channel. Therefore,

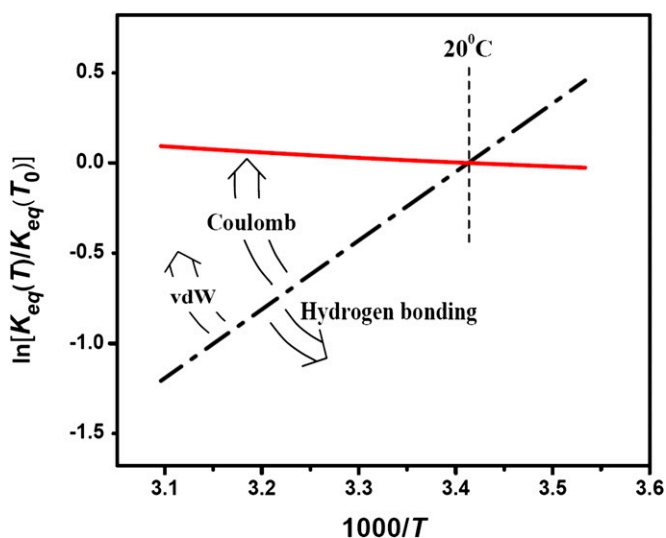


Fig. 5. The van't Hoff plots of the temperature dependence of the equilibrium constant are very sensitive to a particular interaction responsible for the depth of potential wells shown in Fig. 4. The dash-dotted line shows $\ln[K_{eq}(T)/K_{eq}(T_0)]$ calculated according to Eq. 12 with the temperature-independent potential well depth $\Delta U = 13 k_B T$ using $T_0 = 20 \text{ °C}$ as the reference temperature, indicated by the vertical dashed line. In the case of Coulomb interactions, the temperature dependence of the dielectric constant may significantly compensate the van't Hoff slope and even lead to its inversion, staying approximately linear in this temperature range. This is illustrated by the solid curve calculated according to Eqs. 15–17, as explained in the text. Temperature dependence of van der Waals (vdW) forces may induce slope corrections in the same direction, whereas in the case of hydrogen bonding the slope of the temperature dependence is expected to increase (arrows around the dash-dotted line).

the explicit calculation of the slopes is not as straightforward as for Coulomb interactions in bulk water, and only tentative directions of slope changes, assuming predominance of interactions with the channel, are given in Fig. 5. The crucial importance of hydration effects is commonly recognized in the wide spectrum of biologically relevant processes from simple binding reactions (18) to macromolecular association (19) and protein folding (20).

Conclusions

Evaluating the depth of the potential well describing the blocker interaction with the anthrax channel, we have shown that the linear van't Hoff analysis of the temperature dependence of $\ln K_{eq}$ may be grossly misleading (21, 22) because of the temperature dependence of the actual physical forces involved in the blocker-channel interaction. The enthalpy of the reaction is equal to the potential well depth only for temperature-independent interactions and a simple shape of the well. Importantly, in the narrow temperature range corresponding to the fully functional conformations of many proteins, $\ln K_{eq}$ can be nearly linear in the inverse temperature even if the interactions are temperature-dependent. This is illustrated by a hypothetical case of purely Coulombic interaction shown by the solid line in Fig. 5. We conclude that in the case of van der Waals or Coulomb interactions, one should generally expect a decrease or even a change in the sign of the slope of the van't Hoff plot. At the same time, for interactions whose strength decreases with temperature, such as hydrogen bonding, the slope of the plot characterizing the apparent enthalpy increases.

Although the analysis given above deals with a relatively narrow subclass of binding reactions, namely channel blockage by a low-molecular-weight compound, the results obtained clearly go well beyond this example. For example, in many instances of theoretical analysis of experimental data on ion channels, the temperature dependence of channel conductance is used to estimate the number and structure of barriers and wells for ion translocation. Our results clearly demonstrate that a straightforward interpretation of the temperature effects in terms of the rate theory may be misleading if the temperature dependence of barriers and wells is ignored. In many cases of practical interest, the energy landscape seen by an ion in the channel is a sum of contributions due to interactions of different natures, as exemplified by the analysis of selectivity of potassium channels (23). As many of these interactions, similar to Coulomb forces in water, include an entropic component (24), caution is needed in interpreting experimental results. Only highly detailed approaches, such as all-atom molecular dynamics simulations, where temperature effects on all of the elements of the system are explicitly taken into account, can be free of this defect. However, this is not necessarily true for methods of multiscale modeling, which inevitably include certain simplifications of interaction potentials.

For the AmPr β CD interaction with the PA $_{63}$ pore studied here, the involvement of electrostatics is made clear by the strong decrease in blocker binding at increasing salt concentration in the membrane-bathing solution (3). According to the arguments presented above, the temperature dependence of $\ln K_{eq}$ could be expected not only to be reduced but inverted. In reality, the temperature dependence of the forces creating the potential well for the blocker molecule in the channel is much more complex. Even if long-range electrostatics were the major driving force of the binding reaction, calculating the total electrostatic energy is not an easy task (25). Several effects should be taken into account. First, the Debye screening in the channel must be different from that in the bulk due to limitation of space and, therefore, of the number of available counterions in the channel cavity. Second, the dielectric behavior of water in the strong fields around a 3-nm-diameter particle carrying seven positive charges and in the predominantly negatively charged channel pore is significantly

different from that of bulk water (26), including its dependence on temperature. Third, as the binding reaction takes place within the strong confinement of the channel-forming protein, protein dielectric properties and its conformational dynamics are of importance. Except for field-induced dielectric response saturation, these effects are able to factor in both directions, either increasing temperature sensitivity or decreasing it. Nevertheless, we hope that our thermodynamic analysis of this particular case—blockage of the anthrax PA₆₃ channel by a cationic blocker—within the framework of a simple model will provide valuable insights for the broad community of researchers who work on similar problems, including those of structure-inspired design of new efficient drugs.

Materials and Methods

Reagents, lipid bilayer technique (after ref. 27), and methods of channel reconstitution were identical to those described earlier (3). Different temperatures were maintained with a thermostatic bath connected to a water jacket with the bilayer chamber (3) and controlled by a Cole-Parmer (model 92000-00) Digi-Sense scanning thermometer. AmPrbCD was custom synthesized at CycloLab (Budapest, Hungary) with the details of the synthesis given earlier (2).

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