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The dynamic side of the Hofmeister effect: a single-molecule nanopore study of specific complex formation

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A crucial factor regulating the specific association of all molecules in solution is their desolvation and the modulation of this desolvation by cosolutes such as salts. Since the pioneering experiments of Hofmeister on the “salting out” of aqueous solutions of proteins^[1], a deluge of research has allowed us to think of changes in preferential solvation as the main cause of shifted equilibria^[2]. Whether preferentially excluded from or attracted to the surfaces of the associating molecules, these cosolutes can increase or decrease complex stability. However, information needed to determine the origin of Hofmeister effects involves more than equilibrium measurements. For this reason our newfound ability, reported here, to measure distinct changes in association (“on-rate”) versus dissociation (“off-rate”) kinetics, whose ratios are equilibrium constants, opens the possibility of instructive inquiry into the molecular basis of ionic action in all Hofmeister systems.

More than a decade ago it was demonstrated that a single protein nanopore could be used to detect exchange of neutral molecules between the pore and the bath^[3]. Here we combine this “molecular Coulter counting” with osmotic stress to study the involvement of water restructuring in molecular complexation. In particular, we use an alpha-hemolysin (α HL) nanopore^[4] as a sensor^[5] to follow the effect of cosolutes, focusing on the effect of anions on a simple bimolecular complexation reaction at the single-molecule level. As a model of hydrophobic association, we examine the specific guest-host complexation of the cyclic carbohydrate γ -cyclodextrin (CD) with adamantane carboxylate (AD) in the presence of different salts (Figure 1). By monitoring transient obstructions of current through the nanopore by the CD-AD complex, we are able to resolve the complexation kinetics and their modification in the presence of the various salts. We find that the salts used here act to stabilize CD-AD complexes and that the binding free energy varies linearly with solution osmotic pressure. This tendency is sensitive to the type of salt; it tracks the Hofmeister ranking that typically reflects the exclusion tendency of different ions from non-polar surfaces.

Remarkably, both on-rates and off-rates are affected. While chloride and bromide salts mainly impact the off-rates, sulfate dominates the equilibrium shift by changing the on-rate. This is a first indication that the mechanisms by which different salts modulate macromolecular association are quite distinct, providing valuable clues to the role of solvation as it modifies macromolecular interactions in crowded cellular environments^[6].

Cyclodextrin molecules are shaped as truncated hollow cones with a non-polar hydrophobic inner cavity. The ability to incorporate hydrophobic “guest” molecules^[7,8] not only makes cyclodextrins important in the pharmaceutical and food industries^[8] but also provides a convenient model for specific hydrophobic interactions^[9,10].

Recent thermodynamic studies of CD-AD complexation in bulk aqueous environments have shown that association involves the release of water from the interacting surfaces^[10,11]. Watching complexation as a dynamic process now allows us to assess the effect of salt on the kinetics of complex formation. To probe the association reaction of γ -cyclodextrin (eight glucose units) with AD, we first reconstitute a single α HL channel^[4] into a planar lipid bilayer^[12]. Using the method described by Gu and colleagues,^[5] we then monitor the changes in the channel ion current in the presence of CD and AD. Figures 1a and 1b illustrate the CD-AD complexation as detected with the wild-type α HL channel.

We determine the equilibrium binding constant of γ CD with the channel as $\sim 1.5 \times 10^4 \text{ M}^{-1}$, about 100 times greater than that reported for β -cyclodextrin (composed of seven glucose units)^[5]. Because γ CD is bound to the channel for longer periods, this molecule allows us to easily track multiple events of CD-AD complexation. The residual channel conductance (L1 in Figure 1b) is close to 0.4 nS, and is sensitive to the presence of AD which produces additional blockages to almost total current occlusion (L2 level). The unblocked state of the channel (level L0) is insensitive to the presence of AD. The on-rate for blockages, observed at level L1, scales linearly with AD concentration in the bulk, suggesting bimolecular reaction kinetics (Figure 1c).

Figure 2a gives current traces of the CD-blocked state of the channel without AD and in its presence at two different KCl concentrations. Rate constants for CD-AD complexation are derived from the mean unblocked time (τ_o) and mean residence time (τ_r) obtained by noise analysis^[13] (Figure 2b). The equilibrium binding constant (K_{eq}) was directly determined from the AD-induced reduction of the current of the CD-blocked state by $K_{eq} = (1 - p)/p[AD]$, where $p = \langle I \rangle / I_{max}$ is the probability of finding the α HL-CD complex in the AD-unblocked state, $\langle I \rangle$ is the average (over all points) current through the CD-blocked state of the is the current α HL channel in the presence of AD, and I_{max} through the CD-blocked state in the AD-free solution. Power spectra were well fit by a Lorentzian function (Figure 2b) suggesting bimolecular reaction kinetics of CD-AD complexation for all salt concentrations.

To eliminate the strong effect of electroosmotic water flow through the channel when voltage is applied^[14], we considered the binding in the limit of $V \rightarrow 0$ by interpolating values of τ_o , τ_r , and K_{eq} , measured over a wide voltage range (Figure 3). We find that the CD-AD binding constant K_{eq} (in 1.0 M KCl) is close to $2 \times 10^2 \text{ M}^{-1}$, with a characteristic on-rate of $\sim 8.5 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$ and residence AD time inside CD of $\sim 2 \times 10^{-5} \text{ s}$. In contrast, in bulk aqueous solutions K_{eq} as measured by Isothermal Titration Calorimetry (ITC) is $\sim 3.5 \times 10^2 \text{ M}^{-1}$ (present work and ref. [10a]). This difference is likely due to altered molecular solvation in the bulk vs. that in the pore. In addition, CD binding to the channel may impose specific steric constraints on the complexation geometry as well as possible distortion of CD conformation.

To assess the impact of salt on association, we follow CD-AD binding as we vary the concentration of salts in the bath solution (Figure 3). Upon increase of the KCl concentration from 1.00 M to 2.25 M we observe changes in both unblocked and residence time.

The influence of KBr and Na₂SO₄ salts on the CD-AD interaction was similarly evaluated. For these measurements, we reconstituted the α HL channel in the membrane at 1.0 M KCl (or NaCl) concentration and injected aliquots of concentrated KBr or Na₂SO₄ salts keeping AD and CD concentrations fixed.

For all three salts we observed an increase in binding constant, while the relative changes in τ_0 and τ_r were different. Thermodynamically, the effect of salt on binding constants can be translated into a difference in the number of solute-excluding water molecules that are associated with complex formation. In all cases studied here, the changes in binding free energy of CD-AD complex formation ($\delta\Delta G = \Delta G_0 - RT \Delta \ln K_{eq}$) scale linearly with solution osmolality, indicating a constant number of solute-excluding water molecules released from the interacting surfaces of CD and AD (or, equivalently, indicating a number of preferentially excluded solutes that varies linearly with solute concentration). This linearity allows us to characterize the CD-AD complexation reaction with a constant preferential hydration coefficient Δn_{water} , defined by:

$$\Delta n_{water} = - \frac{d\Delta G}{d\mu_{water}} = \frac{55.6}{RT} \frac{d\Delta G}{dm_s^{osm}}, \quad (1)$$

where μ_{water} is water's chemical potential, T the absolute temperature, R the ideal gas constant, 55.6 the number of moles of water in 1 Kg at normal conditions, and m_s^{osm} the solute m_s osmolal concentration^[10]. To compare complexation in the bulk with complexation inside the α HL channel, we measured $\delta\Delta G$ in aqueous solutions using ITC in the presence of the same set of salts. Changes in $\delta\Delta G$ in the bulk also show a linear dependence in solution osmolality. Figure 4 compares $\delta\Delta G$ derived from ITC and channel experiments. For both channel (Figure 4a) and ITC (Figure 4b) measurements, CD-AD complexation is most affected by sulfate. Chloride has an intermediate effect, while bromide has the smallest. In contrast, substitution of potassium with sodium did not measurably influence CD-AD association; with both KCl and NaCl we obtained similar values of $\delta\Delta G$ (data not shown). Using Eq. (1) we estimate the corresponding value of Δn_{water} in the channel and bulk (tables in Figure 4). For all the probed salts, the number of excluding water molecules follows the Hofmeister ranking for the anions ($SO_4^{2-} > Cl^- > Br^-$)^[1, 10b, 15]. We note that the preferential hydration is often, but not always, similar for complexation in bulk solution.

We find that $\delta\Delta G$ in the channel differs somewhat from that in the bulk. Interestingly, however, for all salts, the off-rates decreased with a slope that correlates well with change in $\delta\Delta G$ in the bulk (table in Figure 4a, panel (2)). In fact, the ratio of slopes calculated from Figure 4a, panel (2) give 1 : 1.3 : 3 while ITC in the bulk gives 1 : 1.5 : 4.5 for the Br⁻, Cl⁻, and SO₄²⁻ (Figure 4b). This may come as no surprise, because intermediate states involved in complex dissociation must include steps that require the entry of water between the interacting hydrophobic surfaces. Salt, then, changes the cost of water penetration, making it more unfavorable for a water molecule to leave the bulk. Similar correlations were found in experiments with endonuclease and DNA^[16].

Much less intuitive is our finding that the reaction on-rates can also depend on the presence of cosolute (Figure 4a, panel (1)). The slight change in the on-rate induced by KBr might be well within the measurement error, but the data for KCl and, especially, for Na₂SO₄

demonstrate a clear increase in the on-rate with the increase of salt concentration. In the case of Na_2SO_4 , this increase is so pronounced that it dominates the calculated Δn_{water} . Thus, based on our findings, we conclude that “salting out” is a result of either accelerated on-rates or slower off-rates, depending on the salt, and that either of these may, surprisingly, dominate the Hofmeister effect. The sulfate anion measurements demonstrate that not only *the stability of the complex*, as measured by the life time of the inclusion complex, but also *the accessibility of the complexation site* can be critically increased by preferentially excluded cosolutes.

Though the present study deals with a simple complexation reaction, our results are clearly relevant for more complicated processes, such as enzyme catalysis, where the role of solvation in the reaction rates is only recently starting to be recognized^[6].

Experimental Section

Single-channel measurements

Bilayer lipid membranes were formed by the lipid monolayer opposition technique^[12], from diphtanoylphosphatidylcholine (Avanti) on an aperture in a Teflon partition, dividing two (*cis* and *trans*) compartments of the chamber^[13b]. Wild-type α -hemolysin (Calbiochem) from a diluted stock solution of 25 $\mu\text{g/ml}$ containing 8 M urea solution was added to the *cis*-side of the chamber; γCD (100 μM) and AD (200 μM) were added to the *trans*-side. The sign of the applied voltage was given with respect to the *trans*-chamber. All the probing salt solutions were buffered with 10 mM Na phosphate buffer to pH 7. We reconstituted the αHL channel in the membrane at 1.0 M KCl (or NaCl) concentration and injected aliquots of concentrated KCl, KBr, or Na_2SO_4 salts from stock solutions that also contained identical molar fractions of γCD and AD. This enabled us to follow events of CD-AD complexation at the best possible resolution while still observing linearity in $\delta\Delta G$ over a wide range of probing anion concentrations. All salts and were from Sigma; components of the Na phosphate buffer from Burdick and Jackson, CD and AD were from Fluka. All experiments were done at room temperature, $T = 23.0(\pm 1.0)^\circ\text{C}$.

Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments). The output signal was filtered by either a 100 kHz amplifier filter or by an in-line low-pass 8-pole Butterworth filter (Model 9002, Frequency Devices) at 15 kHz and saved with a sampling frequency of 50 kHz. Amplitude, life-time, and fluctuation analysis were performed using Clampfit 9.2 (Axon Instruments) and the in-house software.

Microcalorimetry

Isothermal titration calorimetric measurements were made at 25 $^\circ\text{C}$ using a VP-ITC microcalorimeter (MicroCal) as described in^[10a]. In each experiment, 40 successive 10 μl injections of 40 mM γCD solutions were mixed into the thermostated cell containing 2 mM AD solution; solutions were stirred at 300 rpm. Injections lasted 10 s and were spaced at 3.5 min intervals. The heat release associated with AD and CD dilution was measured separately and did not substantially change the calculated thermodynamic properties. Solutions were in 10 mM Na phosphate buffer at pH 7. Both AD and CD solutions contained an additional salt, both with the same osmolality. Equilibrium constants were evaluated using standard MicroCal Origin software procedures. All fits to the data were consistent to with a 1:1 molar complexation ratio. Attempted fits to other possible complex ratios did not improve the overall fit, supporting the simplest assumption of 1:1 binding under all studied conditions. Presented results are averages of 2–4 repeats.

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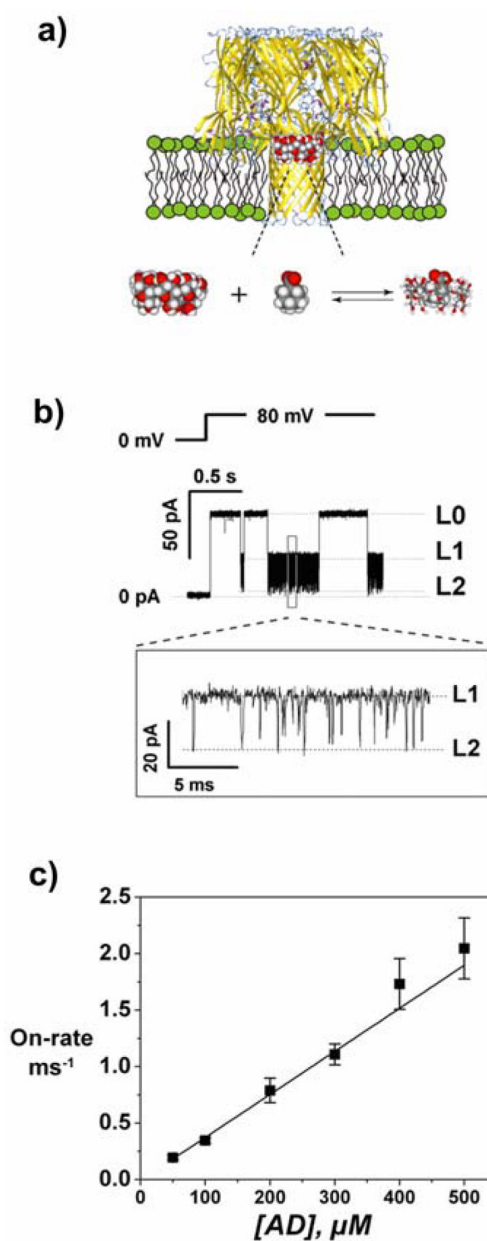


Figure 1.

Using an alpha-hemolysin (α HL) pore as a sensor^[5] to probe cyclodextrin/adamantane carboxylate (CD-AD) complexation. a) Cartoon of the complexation process. b) Corresponding recording of single pore current. The lipid bilayer was bathed in 1.0 M NaCl solution; α HL was added to the *cis*-side of the bilayer, and CD (100 μM) and AD (200 μM) to the *trans*-side. In the absence of CD and AD, the single α HL pore is fully open (level L0); CD produces transient partial blockages of the pore from level L0 to level L1; AD does not affect the current through the fully open channel, but additionally blocks its CD-blocked state to level L2. c) On-rate of AD binding to the α HL-CD complex scales linearly with AD concentration in the bulk, suggesting bimolecular reaction kinetics of the CD-AD complexation within the α HL pore.

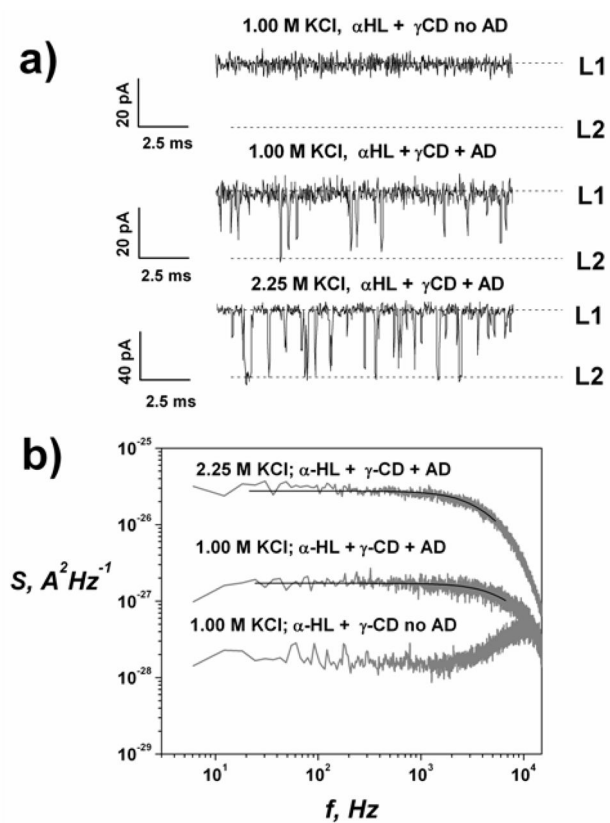


Figure 2.

a) Effect of KCl concentration on CD-AD complexation in the α HL pore at -80 mV applied voltage. b) Power spectral densities of the AD-induced fluctuations in the α HL-CD complex. Solid lines are Lorentzian fits to the spectra. In the absence of AD the Lorentzian component is not seen (lowest spectrum).

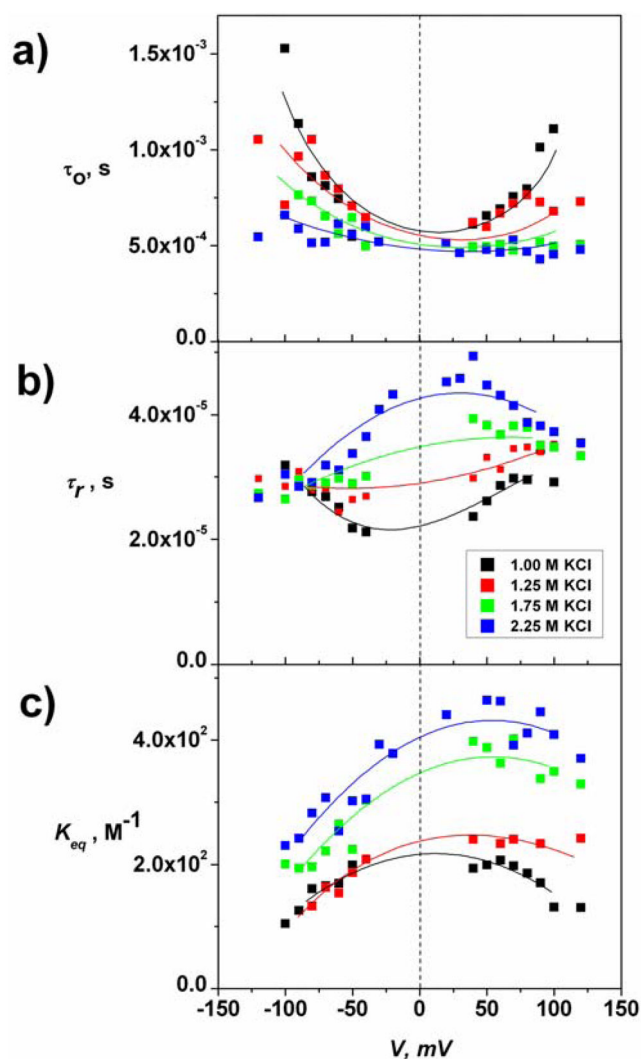


Figure 3. Kinetic and equilibrium parameters of CD-AD complexation at different KCl concentrations. (a) Unblocked time (τ_0) for α HL-CD, (b) AD residence time (τ_r) within CD-AD complexes in the α HL pore, (c) CD-AD association constant, K_{eq} . At zero voltage a 2.25 increase in KCl concentration produces an approximately two-fold increase in the stability of the CD-AD inclusion complex, as τ_r increases from $\sim 2 \times 10^{-5}$ s to $\sim 4.5 \times 10^{-5}$ s, while τ_0 , in the limit of small voltages stays almost unaffected, decreasing from $\sim 5.7 \times 10^{-4}$ s to $\sim 4.9 \times 10^{-4}$ s (Figure 2B). The change in residence time accounts for almost all the increase in the CD-AD equilibrium binding constant.

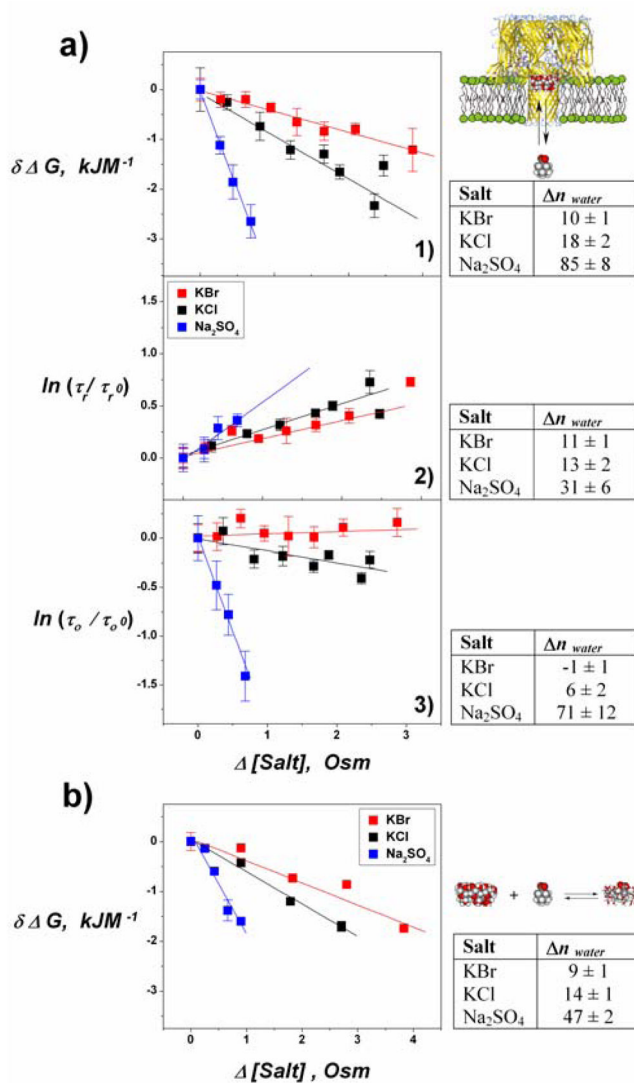


Figure 4. Equilibrium and kinetic parameters for CD-AD complexation measured with α HL pore (a) and with Isothermal Titration Calorimetry, ITC (b). Free energy of CD-AD complexation $\delta\Delta G$, vs. changes in salt concentration (1) and kinetic parameters for binding: AD residence time (2) and unblocked time (3) of the α HL-CD complex. Using Eq. (1), the slope of these plots translates into changes in preferential hydration Δn_{water} upon CD-AD association shown in the corresponding tables. In deriving Δn_{water} in the tables of panels 2 and 3, we have assumed that the shift in equilibrium is solely due to the change in a given kinetic parameter inside the α HL ion channel.