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The Kinetics of Ampicillin Complexation by γ-Cyclodextrins. A Single Molecule Approach

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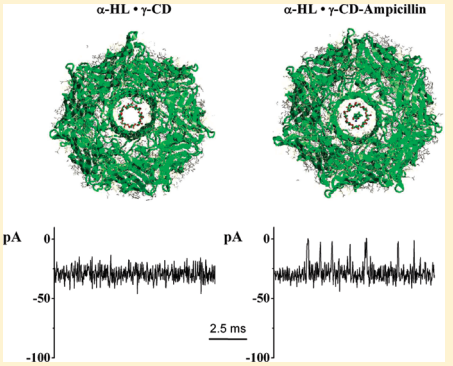
Supporting Information

ABSTRACT: Efficient measuring of binding thermodynamics of interactions between cyclodextrins (CDs) and antibiotics remains a challenge in the fields of physical chemistry and pharmaceutics. Here we report on a single-molecule investigation of pH- and voltage-dependent reversible interactions between ampicillin and γ-CDs, through monitoring of ionic current signatures across an α-hemolysin (α-HL) protein entrapping a γ-CD molecule. Our data reveal that electric and electro-osmotic driving forces alter the reversible reaction rates of ampicillin interaction with γ-CDs, as well as free energy changes accompanying the interaction. We found that close to neutral pH values facilitate more unstable γ-CD−ampicillin complexes, as well as a decreased affinity of the γ-CD−ampicillin reversible interaction, as compared to acidic pH. We posit that a pH-dependent partial electric charge on the ampicillin molecule and anionic selectivity of the α-HL·γCD complex account for the antibiotic and γ-CD intracavity manifestations. This approach may provide unique alternatives for the characterization of CD−guest interactions, useful for pharmaceutical formulations and tunable drug delivery systems.



INTRODUCTION

Single-molecule detection has attracted enormous interest in past years, mainly because it provides unique insights into the behavior and dynamics of molecular events that are buried under statistical averaging, and cannot be obtained from macroscopic measurements.[1−8] Previous seminal studies have established that either wild-type or engineered α-hemolysin (α-HL) pores, are among the most promising protein-based systems suitable for interrogating chemical reactions at the unimolecular level. To fully appreciate the usefulness of α-HL pores for such studies, one must consider α-HL's intrinsic structural sturdiness, which makes it stable over extreme experimental conditions,[3,9,10] and the lack of gating substates, which would complicate the analysis of single-protein current modulations in response to an external agonist. Therefore, since α-HL pore block is sensitive to the conformation, dimension, and orientation of molecules temporarily present within the pore, by recording changes of the current that flows through an individual pore and suitable interpretation of such traces, quantitative insights into single-molecule reactions confined in the pore can be obtained.[11−16] This paradigm proved to be particularly well-suited for stochastic sensing and nanobiotechnology applications.[17−22]

To increase the versatility of this approach, new stochastic sensing systems were devised by equipping the α-HL pores with an internal, noncovalently bound β-cyclodextrin molecule (β-CD),[23] belonging to a series of cyclic oligomers consisting of six or more α-l,4-linked D-glucopyranose units.[24,25] CDs' molecular shape and size helps them be lodged in the pore lumen, and their mostly hydrophobic nanocavity (having an average diameter of 5 Å (α-CD), 6.2 Å (β-CD), and 7.9 Å (γ-CD), respectively) can accommodate guest molecules, ranging from polar (e.g., alcohols, acids, and small inorganic anions) to hydrophobic molecules with surface exposed aliphatic and aromatic hydrocarbons.[26,27] The affinity of host compounds to interact with the interior of various CDs depends on the driving force for the formation of an inclusion complex, such as van der Waals and hydrophobic interactions, steric factors, as well as stereochemistry, polarity, electrostatic potentials, and the host propensity to form hydrogen bonds with the CD.[28−30] Since the inner size of the CD's nanocavity is comparable to that of relatively small molecules, the Brownian movement of various analytes within even nonfunctionalized CD's is greatly hindered. Therefore, the duration and frequency of ensuing fluctuating currents through the α-HL·CD complex are visible in single-molecule electrophysiology experiments, allowing the analyte to be identified and quantified. This technique has been successfully used for the sensing of organic molecules,[20,22] discriminating between structurally similar drugs and chiral enantiomers,[31,32] and distinguishing between antibiotic molecules of different size and charge, belonging to the β-lactam family.[33]

A considerable deal of effort is being devoted to understanding the thermodynamics of the complex formation between CDs and

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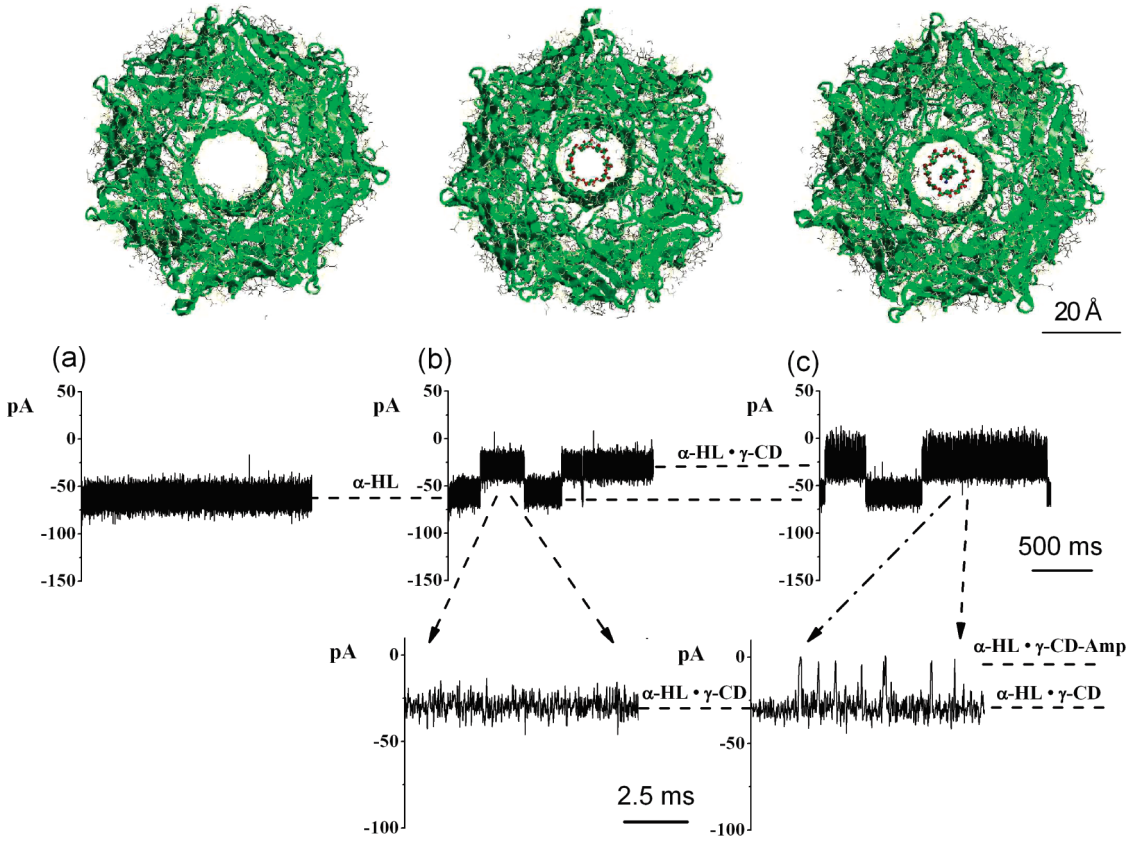

Figure 1. Representative traces showing (a) the current mediated by a single α-HL protein inserted in a reconstituted lipid membrane, (b) reversible partial blockages induced by trans-side-added γ-CD, at a concentration of 50 μM, in interaction with a single α-HL protein (denoted by α-HL·γ-CD), and (c) additional block events of the current mediated by a single α-HL·γ-CD complex, engaged in reversible interaction with 400 μM, trans-added ampicillin (denoted by the α-HL·γ-CD−Amp), seen better in the expanded trace from the corresponding, lower panel. In the absence of ampicillin, no additional bock events are present on a single α-HL·γ-CD complex (panel b, the lower, expanded trace). These experiments were performed at −90 mV, in symmetrical 1 M NaCl solutions buffered at pH 7.14. The upper panels display at scale the axial view from the trans side of (a) the free α-HL protein (the pore lumen diameter is ∼20 Å) (b) a γ-CD molecule temporarily trapped within the pore β-barrel, and (c) an ampicillin molecule residing within the α-HL·γ-CD complex. If assumed to possess a spherical topology, an ampicillin molecule measures ∼4.2 Å in diameter. The primary and secondary inner diameters of a γ-CD molecule are ∼7.7 and 9.5 Å.

various guests.[28,34,35] It is noteworthy that electrostatic interactions manifested between engineered β-CDs with neutral or charged guests revealed a counterbalancing effect between electrostatic and noncovalent interactions (e.g., van der Waals, hydrogen bonding, and hydrophobic interactions), and it was established that the cationic aminated β-CDs alter the molecular-binding ability and selectivity of the parent β-CDs toward anionic molecules.[36,37]

As a result of their broad spectrum of antibacterial activity, antibiotic molecules belonging to the β-lactam family are widely used in the treatment of a variety of infectious diseases, which makes them relevant for pharmacology and the dairy industry.[38] Nevertheless, β-lactam antibiotics degrade easily by hydrolysis and β-lactamase-producing bacteria. Previous data have established that the degradation of such antibiotics is inhibited by complexation with CDs.[39,40] By using isothermal microcalorimetry, NMR spectrometry, and molecular dynamic simulation, it was established that the overall charge of ampicillin and amoxicillin is a determinant factor for the formation and stability of inclusion complexes with CDs.[41] Since CDs are considered drug solubilizers and stabilizers of yet untapped potential, knowledge of the binding constants and the thermodynamic parameters of the interaction still remain of central importance for understanding the molecular interaction of β-lactam antibiotics with CDs.[42]

In this paper, we report on a quantitative, single-molecule investigation of the interaction between ampicillin and a γ-CD molecule trapped within the wild-type α-HL pore. Our data reveal the pH and voltage dependence of the kinetic constants and free energy changes, which characterize the reversible interaction between the α-HL·γCD complex and ampicillin. In our view, this reflects contributions from distinct electrostatic, antibiotic, and γ-CD intracavity manifestations, including the pH-dependent alterations of the ionization state of ampicillin, and anion selectivity of the α-HL·γ-CD complex as well.

EXPERIMENTAL METHODS

The planar lipid membranes were obtained from L-α-phosphatidylcholine (Fluka, Germany) by using the technique and protocols previously described.[33,43] The cis (grounded) and trans bilayer chambers contained 1 M NaCl buffered in 5 mM MES (Sigma−Aldrich, Germany) (pH = 2.8) or 10 mM HEPES (Sigma−Aldrich, Germany) (pH = 7.14). Wild-type α-HL (Sigma−Aldrich, Germany) was added to the cis chamber only in its monomeric form, from a stock solution made in distilled

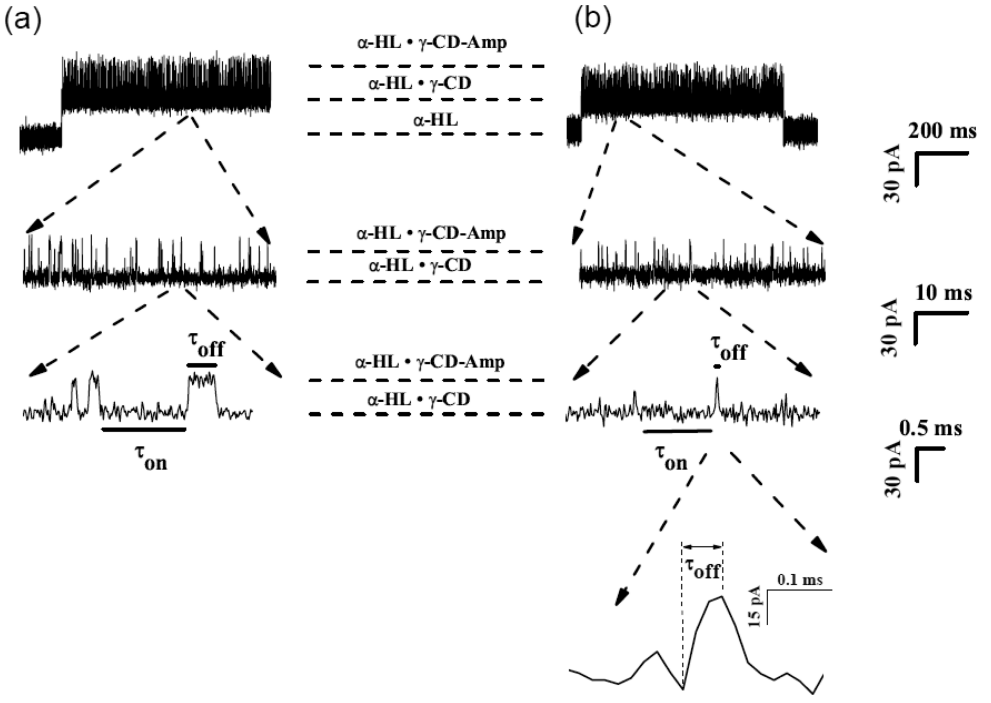

Figure 2. Representative current traces showing the response of the α-HL·γ-CD complex to 400 μM ampicillin, recorded at −90 mV, at pH = 2.84 (a) and 7.14 (b). The dashed line indicates the current through the ligand-free, α-HL single protein (denoted by α-HL), and the residual current through the α-HL protein temporarily occupied by a γ-CD molecule (α-HL·γ-CD). The magnified, lower traces display at higher time resolution reversible blockages induced by an ampicillin molecule interacting with the α-HL·γ-CD complex at the indicated pH values, which are seen to separate two distinct conductive states denoted by "1" (α-HL·γ-CD) and "2" (α-HL·γ-CD−ampicillin). The additional inset shown at pH 7.14 illustrates the detrimental electronic filtering prone to affect the pulse width analysis of ampicillin-induced reversible blockages. The start and stop points for the τ_{off} data associated with the reversible α-HL·γ-CD−ampicillin interaction are indicated by vertical lines (see text).

water. The monomers insertion and heptamer oligomerization was usually obtained within 5 min of continuous stirring. After a single α-HL channel formation in the membrane, 50 μM from γ-CD (Sigma−Aldrich, Germany) was added on the trans side of the membrane, in which ampicillin (Sigma−Aldrich, Germany) was subsequently injected at concentrations ranging between 100 and 500 μM. The rationale of using a relatively high ionic strength (1 M NaCl) was twofold: (i) at low ionic strength, the HL channel obeys frequent current transitions, presumably due to conformational fluctuations of the β-turns,[44] and this had to be avoided, and (ii) the relatively high ionic strength used throughout ensured an optimal signal-to-noise ratio of current fluctuations stemming from ampicillin interaction with the α-HL·γ-CD complex.

Current fluctuations reflecting CD reversible interaction with the single α-HL heptamer, or ampicillin complexation with the α-HL·γ-CD complex, were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, USA) set in the voltage-clamp (whole-cell mode), and filtered at 10 kHz with the built-in low-pass Bessel filter. Measurements were carried out at a room temperature of ∼23 °C, and data acquisition was performed with a NI PCI 6221, 16-bit acquisition board (National Instruments, USA) at a sampling frequency of 50 kHz, within LabVIEW 8.20 (National Instruments, USA).

Given the relatively small number of dwell-time events available in the distinct set of experiments involving the interaction between α-HL·γ-CD complex and ampicillin, the statistical inference of rate constants was implemented using an alternative procedure to dwell-time histograms, as done previously.[16,45] Numerical analysis and graphing were done with the help of the Origin 6 (OriginLab, USA) and pClamp 6.03 (Axon Instruments, USA) software. When the ion selectivity of the α-HL·γ-CD complex was studied, we used a salt gradient of 0.1 M (cis) KCl/3 M (trans) KCl, buffered in 5 mM MES at pH values of 2.84, whereas 10 mM HEPES was used to buffer the solutions at pH 7.14. During such experiments, Ag−AgCl electrodes were connected to the bilayer chamber via salt bridges made of agarose (∼1% w/v) dissolved in 3 M KCl. From the corresponding current−voltage diagram drawn on the α-HL·γ-CD complex, the reversal potential (Ψ_{rev}) was determined, and the charge selectivity (P_{K^+}/P^{Cl^-}) was assessed by a formula derived from the Goldman−Hodgkin−Katz equation:

$$\frac{P_{K^+}}{P_{Cl^-}} = \frac{[Cl^-]_{trans} - [Cl^-]_{cis}\exp\left(\frac{\psi_{rev}F}{RT}\right)}{[K^+]_{trans}\exp\left(\frac{\psi_{rev}F}{RT}\right) - [K^+]_{cis}}$$

In this relation, $[K^+]_{cis}$ and $[Cl^-]_{cis}$ refer to the activities of potassium and chloride ions on the grounded cis side of the membrane, whereas $[K^+]_{trans}$ and $[Cl^-]_{trans}$ denote the activities of potassium and chloride ions on the trans side, Ψ_{rev} is the measured reversal potential expressed in volt, and F, R, and T have their usual thermodynamic meanings.

RESULTS

1. The Reversible Interaction between the α-HL·γ-CD Complex and Ampicillin. To probe the interaction of ampicillin

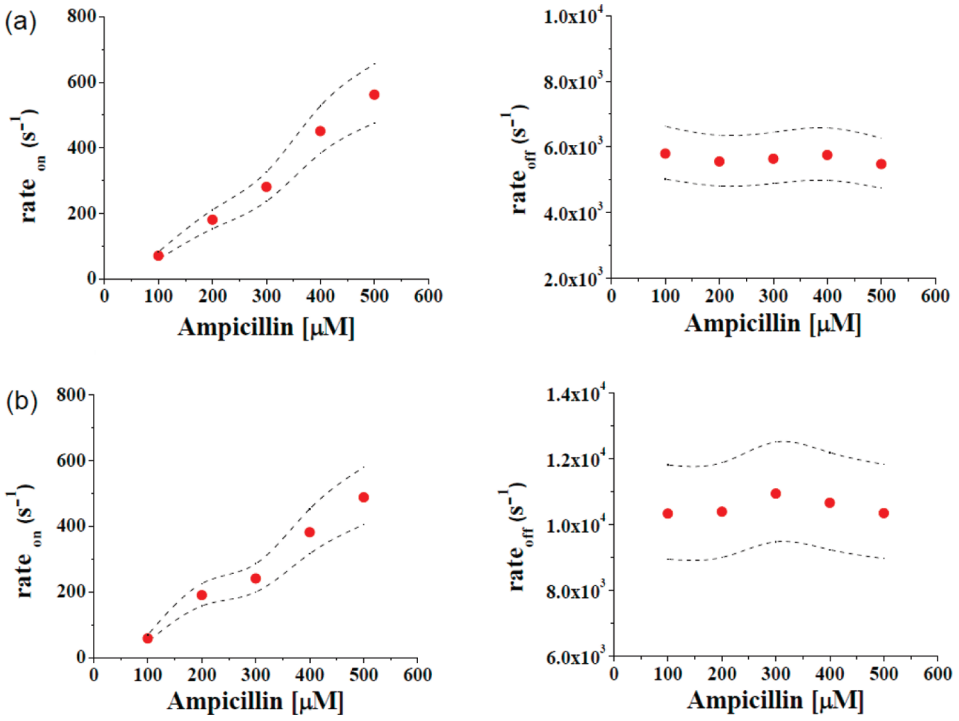

Figure 3. Typical data recorded at −90 mV, showing the pH-dependent effect of ampicillin concentration on reversible current blocking events of the α-HL·γ-CD complex, at pH = 2.84 (a) and 7.14 (b). The frequency and duration of the ampicillin-induced current blockades of the α-HL·γ-CD complex were analyzed within the statistics of exponentially distributed events.[45] The inverse values of time constants corresponding to the free and occupied pore levels provided quantitative estimations of the dissociation ($rate_{off}$) and association ($rate_{on}$) reaction rates that characterize the α-HL·γ-CD−ampicillin reversible interaction. At both pH values, the ampicillin dissociation rate from the α-HL·γ-CD complex was unchanged with the increasing concentration of added antibiotic, whereas the association rate was linearly related to the concentration of ampicillin. Dotted lines reflect the 95% confidence intervals for the estimated values of the dissociation ($rate_{off}$) and association rates ($rate_{on}$), at all corresponding ampicillin concentration values.

with γ-CD at the single-molecule level, and derive kinetic and thermodynamic parameters that characterize their complexation, we first reconstituted a single wild-type α-HL protein into a planar lipid bilayer. The addition of γ-CD on the trans side of the bilayer containing an inserted α-HL protein led to a reversible reduction in the proteins's conductance (from 0.67 ± 0.02 nS of the free α-HL pore to 0.32 ± 0.04 nS of the formed α-HL·γ-CD complex, in 1 M NaCl, 10 mM HEPES, pH = 7.14) (Figure 1a,b).

To a first approximation, one may account for this conductance drop by the decrease in pore's lumen conductivity, caused by the displacement of a volume of electrolyte from the pore as a γ-CD molecule diffuses into the lumen of the α-HL and get stabilized by seven Met 113 and seven Lys147 α-HL residues, close to the narrowest region in the channel. Structural studies support the model in which hydrogen-bonds from Met 113 and Lys-147 residues to the secondary 2- and 3-hydroxyls of the CD contribute to stabilizing the γ-CD into the pore lumen. A thorough work in this regard has been published recently, whereby the nature of the binding interactions between β-CD's and the α-HL pore has been studied by protein engineering and high-resolution X-ray crystallography.[46] Subsequent binding of trans-added ampicillin (400 μM) to the transiently formed α-HL·γ-CD complex leads to additional reversible reductions of the current, to almost total current occlusion (remaining residual current through the α-HL·γ-CD−ampicillin complex equals −3.3 ± 0.2 pA); see Figure 1c, whereby transient upward current steps correspond to time-resolved ampicillin binding events within the α-HL-trapped γ-CD.

2. The pH- and Voltage-Dependent Interaction of Ampicillin with the α-HL·γ-CD Complex. The reversible partial blockades of the single-channel currents through the α-HL·γ-CD complex, ensued by the interaction with trans-added ampicillin, were examined further at acidic and neutral pH values. While the amplitude of the ampicillin-induced additional block remains insensitive to pH ($I_{residual}^{pH\ =\ 2.84}$ = −3.45 ± 0.05 pA, $I_{residual}^{pH\ =\ 7.14}$ = −3.3 ± 0.2 pA), the on- and off-times of α-HL·γ-CD−ampicillin interactions vary across the pH values used during experiments (Figure 2).

To deduce the underlying thermodynamic and kinetic parameters of the observed pH-dependent interaction, rate constants of α-HL·γ-CD−ampicillin interactions were derived from the mean unblocked times (τ_{on}) and mean residence times (τ_{off}), obtained as previously described.[45] Due to the limited rise time (τ_{rise}) of the Bessel filter employed by the recording setup, which at the corner frequency used in our experiments (f_c = 10 kHz) equals $\tau_{rise} \sim 0.33 f_c = 33\ \mu s$, the pulse edges corresponding to the α-HL·γ-CD−ampicillin association−dissociation events differ from idealized square shapes. This is particularly visible at pH = 7.14 (Figure 2b, lower inset), making the nominal pulse width underestimated. Therefore, to more reliably quantify mean residence times, values corresponding to the ampicillin-induced blocked events of the α-HL·γ-CD complex were hand-picked as previously reported,[16,45] using the start point of a blocked state, the value before the current starts dropping, and the last point the one corresponding to the time before the signal starts to return to its initial, unblocked value.[47] The reciprocal of average τ_{on}

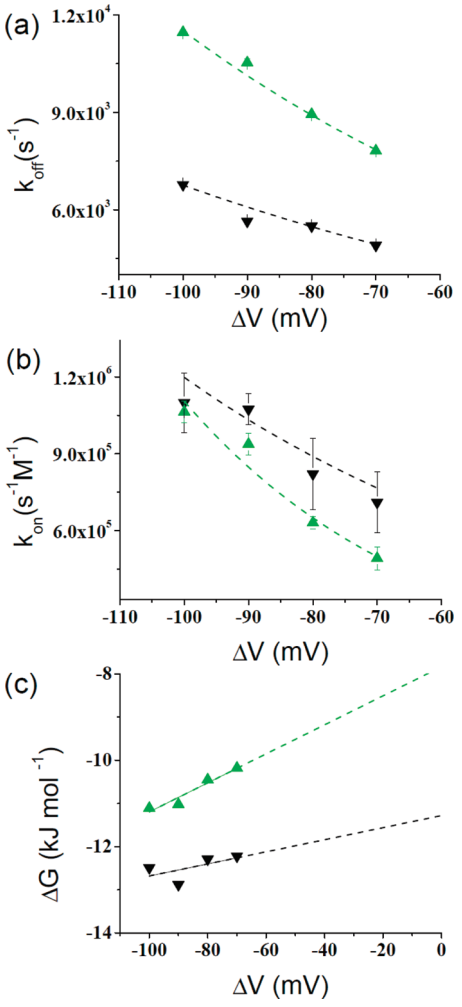

Figure 4. Plots of the off (a) and on (b) rate constants characterizing the α-HL·γ-CD−ampicillin reversible interaction versus potential difference (ΔV), at pH = 2.84 (▼) and 7.14 (▲). These values yield the potential difference dependence of the standard free energy of interaction between ampicillin and the α-HL·γ-CD complex, estimated at the two pH values (c). By invoking Eyring's transition state theory, and considering that within a qualitative kinetic model for the ampicillin−α-HL·γ-CD interaction the effects of the transmembrane potential (either electric or electro-osmotic) can be envisioned as alterations of the association and dissociation activation free energies, the rate constants can be fitted with single decaying exponentials. By extrapolation to the limit of $\Delta V = 0$, we deduced the equilibrium standard free energy of the α-HL·γ-CD−ampicillin interaction when perturbing effects of the electroosmotic flow of water through the protein and electric interaction between the antibiotic and the electric field across the pore are being eliminated.

provides an estimate of the association rate (rate_{on}) of ampicillin to α-HL·γ-CD, whereas the reciprocal of τ_{off} yields an estimate of the ampicillin dissociation rate (rate_{off}). This analysis of ampicillin-induced current fluctuations revealed that the dissociation rate of ampicillin was independent of its concentration, whereas the association rate was linearly dependent on ampicillin concentration (Figure 3).

Thus, a simple bimolecular interaction between ampicillin and the α-HL·γ-CD complex can be assumed at both pH values, and the k_{on} rate constant of ampicillin association to single α-HL·γ-CD complexes was derived from the slope of the linear fit through zero of the rate_{on} versus ampicillin concentration plot (at pH = 2.84, k_{on} = 1073710 ± 61000 s^{-1} M^{-1}; at pH = 7.14, k_{on} = 937370 ± 43540 s^{-1} M^{-1}). By virtue of the simple bimolecular model taken into account between ampicillin and the α-HL·γ-CD complex, rate_{off} equals the dissociation constant k_{off} of ampicillin from the α-HL·γ-CD complex, and was derived from a zero-slope fit of rate_{off} versus ampicillin concentration, resulting in k_{off} = 5648.97 ± 59.82 s^{-1} (at pH = 2.84) and k_{off} = 10533.11 ± 116.11 s^{-1} (at pH = 7.14).

To further explore the nature of the α-HL·γ-CD−ampicillin binding, the above-mentioned procedure has been extended to additional data, in which the voltage-dependence of the reciprocal of average τ_{on} and τ_{off} versus ampicillin concentration at pH values used were analyzed (Figures S1−S3, Supporting Information). This provided an in-depth look into the voltage-dependence of the association (rate_{on}) and dissociation (rate_{off}) rates of ampicillin to α-HL·γ-CD at pH = 2.84 and 7.14, at the unimolecular level. Single-molecule measurements indicate that the applied transmembrane potential alter significantly both association and dissociation constants of the interaction between ampicillin and the α-HL·γ-CD complex (Figure 4a,b).

This suggests that the binding interactions are largely dependent on the partial charge present on ampicillin, as well as on electro-osmotic flow of water through a slightly anion-selective α-HL·γ-CD complex (*vide infra*). The derived kinetic constants (Figure 4a,b) allowed us to quantify values of the standard free energy of interaction ($\Delta G_0 = -RT \ln((k_{on})/(k_{off}))$), and subsequent calculations revealed that the binding strength of ampicillin to the α-HL·γ-CD complex depends both upon the pH value and the applied transmembrane potential, as well (Figure 4c). To compensate for the perturbing effects of the water electro-osmotic flow through the α-HL·γ-CD complex and the electric interactions manifested between the antibiotic and the transmembrane electric field, we analyzed the binding in the limit of $\Delta V = 0$. The resulting standard free energy of the α-HL·γ-CD−ampicillin interaction was estimated to be −11.3 kJ mol^{-1} at pH = 2.84, and −7.8 kJ mol^{-1} at pH = 7.14.

3. The pH-Dependent Selectivity of the α-HL·γ-CD Complex. Previously, it was shown that the wild-type α-HL pore is weakly anion selective (P_{K^+}/P_{Cl^-} = 0.55−0.79) at pH's around 7.5, and a trapped γ-CD slightly increases the anion selectivity of the pore (P_{K^+}/P_{Cl^-} = 0.44).[48] By comparison, the effect of β-CD on the anion selectivity of wild-type α-HL was found to be more pronounced (P_{K^+}/P_{Cl^-} = 0.23−0.25). Recent molecular dynamics simulations and potential of mean force calculations indicate that the α-HL·CD ion selectivity appears as a result of the electrostatic interactions manifested between the anions exiting the CD toward the narrowest region of α-HL, and positive amino groups of the Lys147 ring.[49,50]

The charge distribution on the β-barrel lumen of the wild-type α-HL protein, i.e., the seven Lys147 and Glu111 residues toward the constriction region of the protein, and the total fourteen Asp127 and Asp128, as well as the seven Lys131 residues from the end of the β-barrel, allows for a continuous pH-tuning of the effective charge α-HL.[51] This in turn facilitates a pH-dependent selectivity of the wild-type α-HL protein, especially at low salt activity and increased Debye length.

To experimentally probe pH-dependent changes in the ion selectivity of the α-HL channel equipped with a γ-CD molecule, single-channel currents were recorded under asymmetric conditions (0.1 M (cis) KCl/3 M (trans) KCl) and I−V curves were plotted for the currents flowing through the α-HL·γ-CD

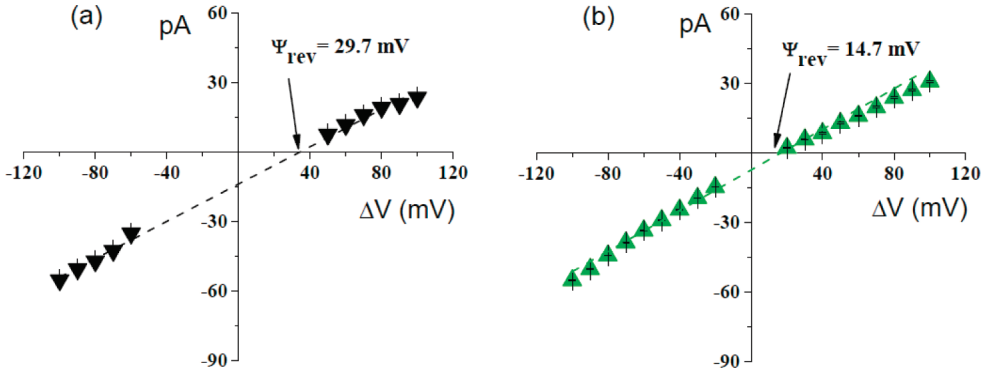

Figure 5. Representative I−V diagrams illustrating the ionic current at various transmembrane potentials, through a single α-HL·γ-CD complex, drawn to calculate the zero current potential (Ψ_{rev}) used to estimate the charge selectivity of the complex at pH =2.84 (a) and pH = 7.14 (b).

complex (Figure 5). The charge selectivity at various pH values were then calculated from reversible potential (Ψ_{rev}) and the Goldman−Hodgkin−Katz equation as follows: at pH = 2.84, P_{K^+}/P_{Cl^-} = 0.28, and at pH = 7.14, P_{K^+}/P_{Cl^-} = 0.54.

The buffer acidity proved to be a modulator of the anionic selectivity of the α-HL protein equipped with the γ-CD adaptor, so that acidic pH values lead to an increased anion selectivity of the α-HL·γ-CD complex. This may be rationalized by the practically permanent state of protonation of the seven Lys-147 residues (amino group pK_a in bulk solution ∼10.8) from the α-HL protein, manifested at pH values of 2.84, which in turn reflects in a more pronounced, electrostatically mediated disruption of the hydration shell of the anions exiting the temporarily immobilized γ-CD by the Lys-147 ring.

DISCUSSION

In a previous work,[33] we reported on a α-HL·γ-CD molecular system able to detect and distinguish various antibiotic molecules (ampicillin, amoxicillin, and azlocillin), based not only the antibiotic physical size, but also the polarity of the drug. In addition, we undertook a quantitative approach, and characterized the kinetics of the α-HL·γ-CD−ampicillin interaction, and evaluated the standard free energy of interaction at a fixed acidity.

The aim of this work was to extend the previous insights through investigating the pH- and voltage-dependent behavior of the inclusion complex between ampicillin and γ-CD, at the single-molecule level, using electrophysiology techniques. Our data confirmed a simple 1:1 bimolecular model for the interaction between the antibiotic and the transient α-HL·γ-CD complex at acidic and neutral pH values, which was employed to derive 'on' and 'off' reaction rates that characterize the ampicillin−γ-CD reversible association.

Phenyl and penam groups of ampicillin assume distinct ionization states, depending on the pH value of the buffer. By using the Henderson−Hasselbalch equation, it can be calculated that the penam's carboxyl group (pK_a = 2.5) will be found deprotonated with a probability of ∼0.7 at pH = 2.84, whereas at pH 7.14 it is practically deprotonated at all times. Similarly, the ampicillin's amino group (pK_a = 7.5) from the phenyl moiety is protonated at pH 2.84, and its chance of being protonated at pH = 7.14 is ∼0.7.

Taking into account that the most stable mode of binding between ampicillin and γ-CD would most likely involve the interaction of the less polar part of the ampicillin molecule with the CD cavity,[52,53] the realization of distinct γ-CD−ampicillin complexes as well as their stability depend on the pH value of aqueous solution. That is, since the uncharged carboxyl-group of the penam may show affinity to the hydrophobic cavity of the γ-CD, it is probable that at pH = 2.84 only, the ampicillin molecule may interact with the γ-CD via the penam's carboxyl group. However, because the charged amino-group in the phenyl side chain of ampicillin may contribute to the stability of γ-CD−ampicillin via hydrogen bonding formation with the primary hydroxyl groups of CD molecule, it is likely that at pH 2.84 an ampicillin molecule can form a distinct complex with the γ-CD, via its phenyl side chain. On the basis of a similar rationale (*vide supra*), it can be rationalized that at pH = 7.14, the most likely complex formed between the γ-CD and ampicillin involves the phenyl side chain only, whose stability into the γ-CD nanocavity is mediated by CH−π interaction between the C−H bonds and the inserted phenyl ring.

Our single-molecule analysis allowed us to further test the possible presence of two distinct routes for the formation of γ-CD−ampicillin inclusion complexes at pH = 2.84, mediated by formation mechanisms involving penicillin's either phenyl or penam groups (γ-CD−(penam) ampicillin and γ-CD−(phenyl) ampicillin). As it is shown in Figure 6, at both pH = 2.84 and 7.14, the histograms of transient times when a single ampicillin molecule resides on the α-HL·γ-CD complex (τ_{off}) (a,b), or of time-intervals in-between blocking events (τ_{on}) (c,d) were best fitted with only one decaying exponential. This in turn suggests that not more than one "open" (i.e., a free α-HL·γ-CD complex) or "closed" (i.e., a α-HL·γ-CD complex occupied by a single ampicillin molecule) state species is visible;[54] while both "closed" α-HL·γ-CD−ampicillin complexes (i.e., ampicillin's phenyl ring or its penam moiety included in the CD cavity) can coexist at pH = 2.84, our analysis precludes the identification of two distinct pathways via which ampicillin interacts with γ-CD. This may be also a reflection of the fact that the association and dissociation rate constants characteristic for both pathways are indistinguishable, thus making impossible a separation between them based on kinetic analysis only. We also note that based on current data, we have insufficient proof to pinpoint the particular type of α-HL·γ-CD−ampicillin complex that forms at acidic pH, whereby two distinct α-HL·γ-CD−ampicillin complexes are most likely to coexist.

As reflected above, pH and the transmembrane potential difference alter the γ-CD−ampicillin kinetics, as well as the value of the standard free energy of interaction.

How do we interpret these results?

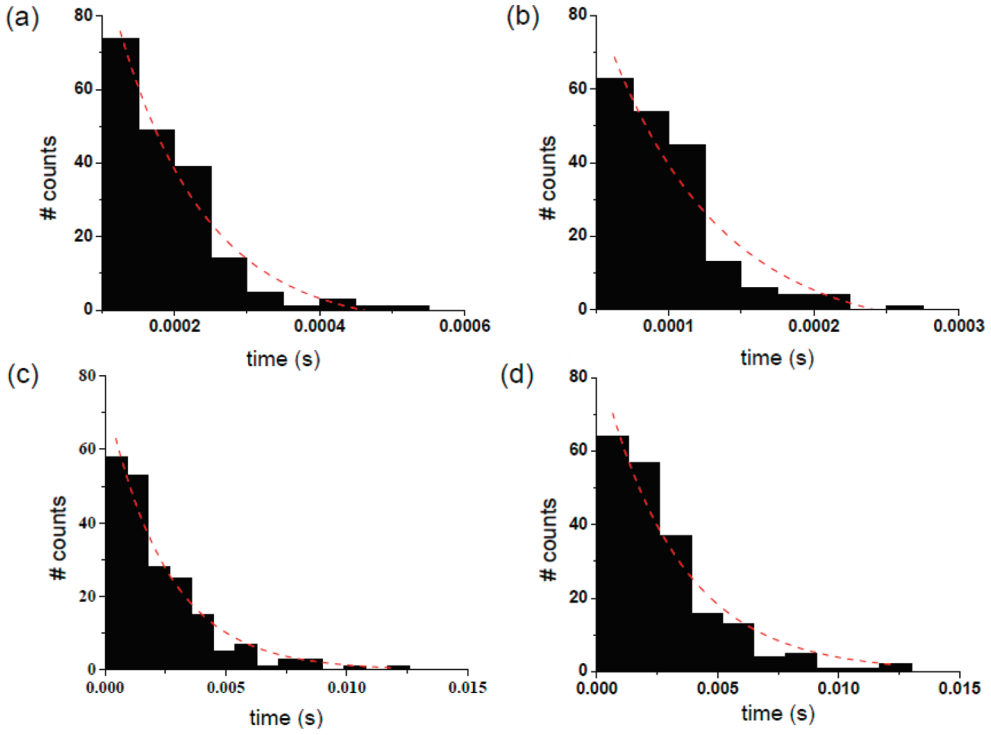

Figure 6. Typical histograms of "off" (a,b) and "on" (c,d) transient times (τ) characterizing the reversible interaction between trans-added ampicillin molecules, at a bulk concentration of 400 μM, with a single α-HL·γ-CD complex, at pH = 2.84 (a,c) and pH = 7.14 (b,d), at an applied potential of −90 mV. Around 200 τ values corresponding to the association and dissociation interaction between ampicillin and α-HL·γ-CD were hand-picked and used to construct such histograms. The smooth curves are single-exponential fits giving time-constants of τ_{decay} = 0.12 ms (a), 0.077 ms (b), 2.5 ms (c) and 3 ms (d), which were preferred over other models (e.g., two-exponentials fit) based on an Akaike information criterion test.

By invoking a minimalist interaction model, one may posit that when binding from the trans solution to its site within the α-HL channel and close to the lumen-entrapped γ-CD, ampicillin has to cross an energy barrier. The chemical dissociation step of ampicillin from the trapped γ-CD is also associated with surpassing an energy barrier, which is followed via a diffusion mechanism by either translocation of ampicillin through the α-HL channel, to the cis solution, or its returning from the binding site to the trans side of the bilayer. In a simplified model, the overall dissociation scheme of the α-HL·γ-CD−ampicillin complex (α-HL·γ-CD−amp) may be viewed as below:

$$\text{α-HL}\cdot\ \gamma\text{-CD}-\text{amp} \xrightarrow{k_{off}} \text{α-HL}\cdot\ \gamma\text{-CD}$$

$$\neq\ \text{amp} \xrightarrow{k_{diff}} \text{α-HL}\cdot\ \gamma\text{-CD}$$

where "α-HL·γ-CD ≠ amp" denotes the substate whereby ampicillin is chemically dissociated from the α-HL·γ-CD−ampicillin complex, with the "k_{off}" rate constant, and yet still within the protein pore, and "α-HL·γ-CD" represents the truly ampicillin-free protein pore, once ampicillin diffuses away in the outer buffer solution, with the diffusion-related rate constant "k_{diff}". It should be noted that by electrophysiology means only, it is impossible to discern between :α-HL·γ-CD ≠ amp" and "α-HL·γ-CD−amp" substates, both of which give rise to similar partial blockades of the single-channel currents through the α-HL·γ-CD complex, as seen in Figure 2.

The voltage dependence of the current blockades revealed a decrease in the τ_{off} event durations with the applied transmembrane potential (*vide infra*). Because the rate limiting step of arriving at the ampicillin-free α-HL·γ-CD complex is the chemical dissociation constant of the γ-CD−ampicillin interaction (e.g., a small molecule with a diffusion coefficient $D = 0.5 \times 10^{-9}$ m^{2}s^{-1}, located 2.5 nm from the trans entrance, would have a pore mean residence time of only 6.25 ns[3]), the above finding suggests that electric and electro-osmotic driving forces have the potential to alter the chemical dissociation energy barrier of ampicillin, via a still elusive mechanism.

In addition, by altering the energetic landscape along the reaction coordinate of the α-HL·γ-CD−ampicillin interaction, the presence of an electric field across the α-HL channel, as well as the electro-osmotic flow through the α-HL·γ-CD complex, are prone to induce changes in the probabilities of "open" (ampicillin-free α-HL·γ-CD) and "closed" (ampicillin-rich α-HL·γ-CD) substates.

By approaching the reversible α-HL·γ-CD−ampicillin interaction scheme within Eyring's transition state theory, the association (on) and dissociation (off) rate constants are exponentially sensitive to the activation Gibbs standard free energy, which is described in terms of interactions manifested between the ampicillin and the γ-CD, as well as molecular structure of the solvent confined within the α-HL lumen, and terms associated with the interaction energy between ampicillin and the applied transmembrane potential, or water electro-osmotic flow. Because ampicillin is found with a probability of ~0.3 in an anionic state at pH = 7.14, caused by the partial deprotonation of phenyl's amino group and practically fully deprotonated penam's carboxyl group, it is not unexpected that ampicillin's association rate constant increases with more negative potentials. That is, this may be explained in simplest terms by the voltage-induced decrease of the association activation energy barrier of the partially anionic

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ampicillin, passing from the negatively biased, trans-side of the membrane, toward the α-HL-entrapped γ-CD. Nevertheless, to explain the same kinetic tendency seen at pH = 2.84, whereby ampicillin is partly cationic and the α-HL • γ-CD complex is almost twice as anion selective as at pH = 7.14, one should consider the prevailing effect of the electro-osmotic flow of water toward the binding site inside the α-HL·γ-CD complex, that would result in an augmentation of ampicillin concentration at the *trans* entrance and be enhanced under a high negative voltage.[55]

Interestingly, our single-molecule kinetic analysis reveals a consistent decrease, visible especially at low potentials where electric and electro-osmotic effects manifested on the ampicillin molecules are reduced, of the association rate constant between the ampicillin and a trapped γ-CD molecule, as the pH changes from acidic (pH = 2.84) to neutral values (pH = 7.14) (Figure 4b). To explain this, we admit that at acidity values well above penam's carboxyl group pK_a, the most likely γ-CD−ampicillin interaction mechanism involves penicillin's phenyl group; therefore, we posit that as the pH changes to neutral values and approaches the phenyl's moiety amino group pK_a, its protonated state is achieved with lower probability, so that hydrogen bonding formation with the hydroxyl-groups of γ-CD becomes hindered.

The kinetics and voltage dependence of ampicillin dissociation are also interesting. We found that the dissociation constant of the ampicillin interaction with the transient α-HL·γ-CD complex is also pH- and voltage-dependent, whereby neutral pH values and more negative potentials facilitate more unstable α-HL·γ-CD−ampicillin complexes. To rationalize this, we posit that at pH = 7.14 and in the presence of trans, negatively applied potentials, the interaction of the partially anionic ampicillin with the existing transmembrane electric fields leads to a decrease in the dissociation standard free energy barrier of the α-HL·γ-CD−ampicillin complex, whose mechanism we still investigate. In addition to this, an electro-osmotic water flow-favored ampicillin dissociation, induced by net ion movement through a slightly anion-selective α-HL·γ-CD complex at negative potentials, cannot be disregarded.[55] Because ampicillin molecules cross the pore too rapidly to be resolved within our measurement bandwidth (*vide supra*), we cannot conclude that a decrease of residence times of ampicillin within the protein pore at more negative potentials reflects faster translocation, driven by favorable interactions between the transmembrane electric field and anionic ampicillin, although favored ampicillin electrodiffusion to the cis side of the membrane cannon be disregarded in this case.

To explain the increase in the α-HL·γ-CD−ampicillin dissociation constant seen at pH = 2.84, at more negative potentials, one must consider that the cationic ampicillin unbinding is favored when it occurs in the direction of the net movement of ions from the trans to the cis side of the membrane, and implicitly of water flow induced by ion movement.

For future work, we plan to address the complementary issue of proposing a simple, yet realistic model of the free energy landscape able to describe the α-HL·γ-CD−ampicillin interactions, under a wide range of conditions (e.g., pH, voltage, ionic strength), starting from the analysis of discrete kinetics and free energy of interactions involved. This desiderate has been successfully approached previously, for a number of distinct protein channels and pores.[56,57] The apparent intricacies of the kinetics and voltage dependence of ampicillin interaction with the α-HL·γ-CD complex (*vide supra*), suggest that more experimentation and computational biophysics are needed to arrive at a suitable model, as envisioned above, able to offer a better understanding of the dynamical behaviors of inclusion of various analytes within α-HL-trapped CDs.

CONCLUSIONS

Knowledge of the binding constants and the chemical parameters of the interaction are of paramount importance for describing the molecular interaction of a guest with CDs. So far, a wide range of techniques and effort have been devoted to understand such phenomena at a collective level, including spectroscopic techniques, NMR, fluorescence, and calorimetry. Herein we provide a single-molecule analysis of thermodynamic parameters that characterize ampicillin complexation with a γ-CD molecule transiently trapped within a α-HL pore, which may have practical implications for such antibiotic delivery.

Since ampicillin exists as a cation, a zwitterion, or an anion, due to the specific pK_a values of its ionizable groups, the formation and stability of inclusion complexes with a γ-CD molecule is influenced by pH in the solution, and our data support this. Besides the overall charge of the ampicillin, pH influences the anion-selectivity of the α-HL·γ-CD complex, so that hydrogen bonding and electrostatic interactions become essential factors to the inclusion complexation behaviors of ampicillin to γ-CD, besides hydrophobic interactions. These results further demonstrate the benefits of the α-HL-based method for monitoring at the single-molecule level molecular recognition between CDs and guests, in a label-free manner, which could be potentially attractive for use in drug discovery and delivery and pharmaceutical applications. We demonstrate that factors which modulate the molecular-binding ability of CDs to therapeutic antibiotics can be investigated in fine detail. This may prove to be useful in several areas of basic science and biotechnology, as well as the construction of nanodevices devoted to understanding biomolecular recognition interactions, with practical implications for developing tunable drug delivery systems.

ASSOCIATED CONTENT

Supporting Information. Statistical analysis of data recorded at −70, −80, and −100 mV, showing the effect of ampicillin concentration on reversible current blocking events of the α-HL·γ-CD complex, at pH = 2.84 and 7.14. This material is available free of charge via the Internet at http://pubs.acs.org.

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