

Reversal of charge selectivity in transmembrane protein pores by using noncovalent molecular adapters

Li-Qun Gu*, Mauro Dalla Serra*[†], J. Bryan Vincent[‡], Gyula Vigh[‡], Stephen Cheley*, Orit Braha*, and Hagan Bayley*^{‡§}

*Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, College Station, TX 77843-1114; [†]Consiglio Nazionale delle Ricerche-Istituto Trentino di Cultura, Centro Fisica Stati Aggregati, I-38050 Povo (Trento), Italy; and [‡]Department of Chemistry, Texas A&M University, College Station, TX 77843-3255

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In this study, the charge selectivity of staphylococcal α -hemolysin (α HL), a bacterial pore-forming toxin, is manipulated by using cyclodextrins as noncovalent molecular adapters. Anion-selective versions of α HL, including the wild-type pore and various mutants, become more anion selective when β -cyclodextrin (β CD) is lodged within the channel lumen. By contrast, the negatively charged adapter, hepta-6-sulfato- β -cyclodextrin ($s_7\beta$ CD), produces cation selectivity. The cyclodextrin adapters have similar effects when placed in cation-selective mutant α HL pores. Most probably, hydrated Cl^- ions partition into the central cavity of β CD more readily than K^+ ions, whereas $s_7\beta$ CD introduces a charged ring near the midpoint of the channel lumen and confers cation selectivity through electrostatic interactions. The molecular adapters generate permeability ratios ($P_{\text{K}^+}/P_{\text{Cl}^-}$) over a 200-fold range and should be useful in the *de novo* design of membrane channels both for basic studies of ion permeation and for applications in biotechnology.

In keeping with their roles in the cell, natural transmembrane channel proteins are ion selective (1–3). For example, voltage-gated K channels do not handle anions, and they transport K^+ ions with a more than 100-fold preference over Na^+ ions (1, 3, 4). Other channels are less selective, but their preferences are also of physiological importance. For example, the nicotinic acetylcholine receptor, a ligand-gated channel, is charge selective; it does not transport anions but only weakly discriminates between various cations (5). Poorly selective channels that allow both cations and anions to pass, such as many of the porins (6–8), are not ion channels in their primary physiological roles, yet they are usually weakly charge selective (9–11).

Studies of ion selectivity have been directed toward an understanding of the selectivity of natural channels and, less commonly, the creation of engineered channels with a range of selectivities. The ion selectivity of natural channels has been investigated primarily by site-directed mutagenesis, which has in some cases allowed the identification of one or a few key amino acid residues. More often, the basis of selectivity has been found to be more complex and subtle. For example, in the case of voltage-gated K channels, where structural information is available to temper studies by mutagenesis, selectivity is seen to involve both electrostatic effects and the coordination of transported ions by backbone carbonyls (3, 4). The creation of engineered channels with a range of ion selectivities has been a spin-off from mechanistic studies. For example, the acetylcholine receptor has been converted from a cation-selective to an anion-selective channel by mutations that most likely result in structural changes involving the polypeptide backbone (5). In β -barrel proteins, such as porins, charge selectivity has been altered by manipulating charged residues in the channel lumen (e.g., refs. 9 and 11). In addition, ion selectivity has been achieved in the *de novo* design of channels and pores (12),

primarily by the chemical synthesis of membrane-active peptides and related amphipathic molecules (13–15).

In a different approach for manipulating the properties of transmembrane channels and pores, we demonstrated the remarkable ability of cyclic oligosaccharides comprising glucose units (cyclodextrins) to act as molecular adapters for the pore formed by staphylococcal α -hemolysin (α HL) (16). The α HL pore is a heptamer made up of identical subunits of 293 amino acids (17). Molecules of up to ≈ 2000 Da can be transported through a wide channel in the pore that is centered on the molecular sevenfold axis (18–20). Polynucleotide strands of much higher mass can move through the pore in extended form (21). Measurements of ionic currents indicate a weak anion selectivity for the unmodified heptamer (22). Cyclodextrins are able to reduce the conductance of the pore by lodging at a point about half way through the channel, where the diameter is at its narrowest (≈ 14 Å) (16). Further, channel blockers can bind to a cyclodextrin while it is in the channel. For example, β -cyclodextrin (β CD) reduces the conductance of wt (wild-type) α HL from 658 pS to 240 pS in 1 M NaCl (pH 7.5), and a large variety of organic molecules cause transient channel blockades by binding within the wt- α HL- β CD complex (16). The results with channel blockers suggest that a substantial fraction of the ionic current flows through the center of the cyclodextrin molecule when it is lodged in the channel lumen. Therefore, we reckoned that cyclodextrins might change the charge selectivity of the α HL pore. Here we show that this is the case.

Materials and Methods

Reagents. β CD was from Aldrich and γ -cyclodextrin from ACROS (Geel, Belgium). Hepta-6-sulfato- β -cyclodextrin ($s_7\beta$ CD) was prepared as described (23) and is available from J & W Scientific (Folsom, CA). Buffers for planar bilayer recording contained various concentrations of KCl or NaCl and 10 mM K_2HPO_4 or Na_2HPO_4 (Sigma) in deionized water (Millipore) and were titrated to pH 7.5 with 1 M HCl (EM Science). Experiments with the mutant α HL-CH1 were done in KCl containing 10 mM potassium phosphate buffer (pH 7.4) and 5 μM EDTA.

Proteins. The mutant α HL genes M113N, M113N/L135N, and E111N/K147N were prepared by cassette mutagenesis in the

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Abbreviations: α HL, staphylococcal α -hemolysin; β CD, β -cyclodextrin; $s_7\beta$ CD, hepta-6-sulfato- β -cyclodextrin; wt, wild-type.

[§]To whom reprint requests should be addressed at: Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, 440 Reynolds Medical Building, College Station, TX 77843-1114. E-mail: bayley@tamu.edu.

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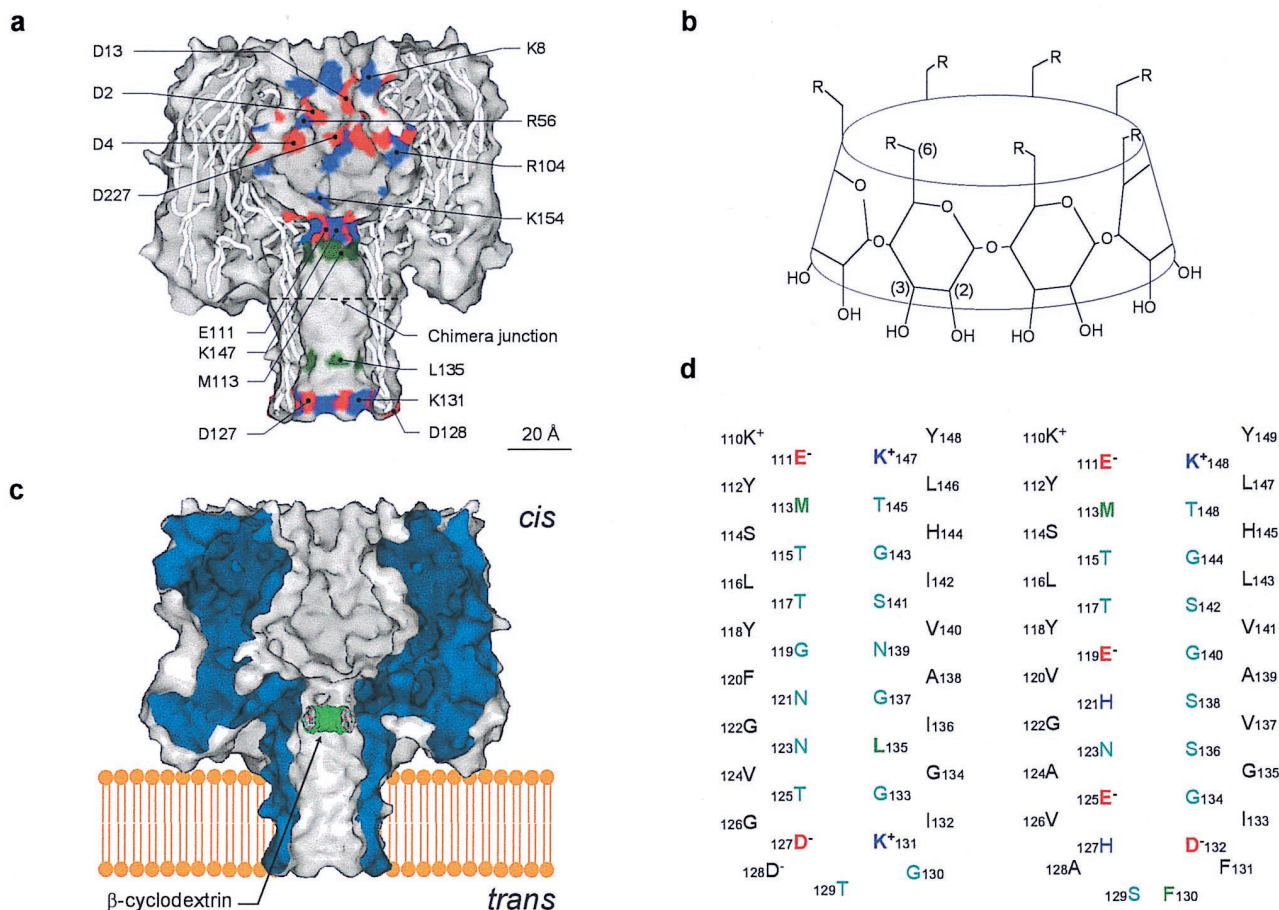


Fig. 1. Representations of the proteins and cyclodextrins used in this work. (a), Sagittal section through the wt- α HL pore showing the location of all of the charged side chains in the channel lumen (red, negative; blue, positive) and the key hydrophobic residues M113 and L135 (green). The site of the junction in the chimera α HL-CH1 is indicated; (b), Structures of the β CDs used in this work. β CD, R = -OH; $\gamma\beta$ CD, R = -OSO₃⁻; (c), Schematic of the wt- α HL pore showing β CD lodged in the lumen of the channel. The location is based on mutagenesis data (16); (d), Sequences of the transmembrane β barrels in wt- α HL (Left) and α HL-CH1 (Right).

plasmid α HL-RL2 (24). These constructs contain the following additional changes over wt- α HL: Lys-8 \rightarrow Ala, Val-124 \rightarrow Leu, Gly-130 \rightarrow Ser, Asn-139 \rightarrow Gln, and Ile-142 \rightarrow Leu. α HL polypeptides with these mutations behave similarly to wt- α HL in hemolysis assays and in planar bilayer recordings, at the salt concentrations used here (24). The chimeric protein α HL-CH1 features a transmembrane domain derived from the protective antigen of anthrax toxin fused to the cap domain of α HL (laboratories of R. J. Collier and H.B., unpublished data). Residues 119–139 inclusive of α HL (21 residues) were replaced with 22 residues, 302–323, from protective antigen. The register of the β strands in the transmembrane domain is that given by Petosa and colleagues (25).

Heptameric wt- α HL was formed by treating monomeric α HL, purified from *Staphylococcus aureus*, with deoxycholate (26, 27) and isolated from SDS/polyacrylamide gels as described (28). Mutant α HL polypeptides were prepared by coupled *in vitro* transcription and translation, with an S30 extract from *Escherichia coli* (no. L114A, Promega) (24). Heptamers were prepared from the mutants by assembly on rabbit red cell membranes, followed by preparative SDS/PAGE (24).

Bilayer Recordings. A 25- μ m-thick Teflon film (Goodfellow, Malvern, MA) with a 100- to 150- μ m diameter orifice was used as the partition between the two chambers (2 ml each) of a Teflon bilayer apparatus. The orifice was pretreated with 1:10

hexadecane (Aldrich)/pentane (Burdick and Jackson). A solvent-free planar lipid bilayer of 1,2-diphytanoyl-*sn*-glycerophosphatidylcholine (Avanti Polar Lipids) was formed over the orifice (29). A potential was applied across the bilayer with Ag/AgCl electrodes with 1.5% agarose (Ultra Pure DNA Grade, Bio-Rad) bridges containing 3 M KCl. Protein was added to the *cis* chamber, which was at ground. A positive potential indicates a higher potential in the trans chamber, and a positive current is one in which cations flow from trans to *cis*. Single-channel currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) in the whole cell ($\beta = 1$) mode with a CV-203BU headstage and filtered at 5 kHz with a built-in 4-pole low-pass Bessel Filter. Data including short events (≈ 1 msec) were acquired directly by computer by using a Digidata 1200 A/D converter (Axon). The other data were recorded on DAT tape and subsequently transferred to the computer after filtering at 1 kHz through a low-pass 8-pole Bessel filter (model 900, Frequency Devices, Haverhill, MA). The data were acquired by using Clampex 7.0 software (Axon) after sampling at a rate of 20 kHz and analyzed with pClamp 6.03 (Axon) and Origin (Microcal Software, Northampton, MA) software.

Experiments were initiated by the addition of heptameric α HL to the *cis* compartment with stirring until a single channel inserted into the bilayer. For the wt heptamer, oligomerized with deoxycholate, the final concentration was 3–30 ng/ml. For the

Table 1. Permeability ratios (P_{K^+}/P_{Cl^-}) and conductance values (g) for various α -HLs with and without adapters

Adapter	Charge, e	Minimum diameter, Å	wt- α HL			α HL-M113N			α HL-E111N/K147N			α HL-CH1		
			V_r , mV	P_{K^+}/P_{Cl^-}	g , pS*	V_r , mV	P_{K^+}/P_{Cl^-}	g , pS*	V_r , mV	P_{K^+}/P_{Cl^-}	g , pS*	V_r , mV	P_{K^+}/P_{Cl^-}	g , pS*
None		14	+9.1 [†] -3.7 [‡]	0.55 ± 0.02 0.79 ± 0.02	658 ± 11	+5.96 [†] -2.21 [‡]	0.68 ± 0.03 0.87 ± 0.04	622 ± 9	+2.71 [§]	1.2 ± 0.1	634 ± 12	+27.1 [¶]	5.1 ± 0.2	541 ± 11
β CD	0	6.2	+19.6 [†] -20.5 [‡]	0.25 ± 0.01 0.23 ± 0.01	240 ± 5	+29.7 [†] -32.3 [‡]	0.079 ± 0.005 0.046 ± 0.006	261 ± 4	-24.9 [§]	0.15 ± 0.02	207 ± 7	-3.9 [¶]	0.82 ± 0.01	109 ± 9
$s_7\beta$ CD	-7	<6.2	-24.1 [†] +28.1 [‡]	6.7 ± 0.4 10.0 ± 0.2	53 ± 6	-19.3 [†] +23.2 [‡]	4.1 ± 0.02 6.1 ± 0.1	40 ± 1	ND	ND	ND	ND	ND	ND
γ CD	0	7.9	-14.0 [§]	0.38 ± 0.04	328 ± 4	ND	ND	287 ± 5	ND	ND	275 ± 4	ND	ND	ND

For each entry, three or more separate experiments were performed, and data acquired for at least 1 min were analyzed. The reversal potentials (V_r) are mean values under the conditions stated. Permeability ratios are quoted as the mean ± SD. ND, not determined.

* -40 mV, 1 M NaCl, 10 mM sodium phosphate (pH 7.5).

† -40 mV 1 M KCl, 10 mM potassium phosphate (pH 7.4).

‡ pH 7.5, KCl in mM (cis/trans) was (200/1000).

§ pH 7.5, KCl in mM (cis/trans) was (1000/200).

¶ pH 7.4, KCl in mM (cis/trans) was (1000/100).

mutant heptamers, oligomerized on red cell membranes, the final concentration was ≈ 0.2 ng/ml. β CD or $s_7\beta$ CD was added to the trans chamber to 40 μ M. Experiments were at 22 ± 2°C.

Data Analysis. Single-channel conductances were determined by fitting the peaks in amplitude histograms to Gaussian functions. The permeability ratios (P_{K^+}/P_{Cl^-}) were calculated from reversal potentials by using the Goldman-Hodgkin-Katz (GHK) equation (1):

$$\frac{P_{K^+}}{P_{Cl^-}} = \frac{[a_{Cl^-}]_t - [a_{Cl^-}]_c e^{V_r F/RT}}{[a_{K^+}]_c e^{V_r F/RT} - [a_{K^+}]_t}$$

where V_r is the reversal potential (i.e., the electrical potential giving zero current), a_X is the activity of ion X (30), subscripts c and t represent the cis and trans compartments, and the other symbols have their usual meanings. V_r was obtained by a polynomial fit of the current-voltage (I - V) data near zero current. For asymmetrical conditions, one chamber (cis or trans) contained 1000 mM KCl, whereas the other chamber contained 200 mM KCl, except for the α HL-CH1 pore where cis contained 1000 mM KCl and trans, 100 mM KCl. After the measurements, the membrane was broken to determine the contribution of electrode junction potentials (normally smaller than 0.5 mV). Permeability ratios for α HL depend on several variables including pH and bilayer composition (31). The results obtained here are valid only for the conditions stated.

Results

Cyclodextrins Act as Molecular Adapters To Enhance the Anion Selectivity of α HL Pores. Various α -hemolysins and cyclodextrins were used in this study (Fig. 1). We showed previously that β CD lodges transiently in the lumen of the wt- α HL pore, where it acts as a noncovalent molecular adapter reducing the unitary conductance (Table 1) (16). The homoheptameric wt- α HL pore is weakly anion selective (22). To determine whether the selectivity is altered while β CD is in the channel lumen, single-channel currents were recorded under asymmetric conditions: 1000 mM KCl cis, 200 mM KCl trans (pH 7.5) (Fig. 2a). I - V curves were plotted for the contributions arising from the unmodified wt- α HL pore and the wt- α HL· β CD complex (Fig. 2c). Experiments were also performed with the opposite KCl asymmetry: 200 mM KCl cis, 1000 mM KCl trans (pH 7.5) (Fig. 2d). The charge selectivities under the various conditions were then calculated from V_r and the GHK equation. The wt- α HL· β CD complex ($P_{K^+}/P_{Cl^-} = 0.23$ – 0.25) is significantly more anion selective than the unmodified wt- α HL pore ($P_{K^+}/P_{Cl^-} = 0.55$ – 0.79) (Table 1).

In addition, the effect of β CD on the mutant M113N was examined. M113N binds the cyclodextrin far more tightly than wt- α HL (16). The almost nonselective M113N pore ($P_{K^+}/P_{Cl^-} = 0.68$ – 0.87) became highly anion selective with β CD bound ($P_{K^+}/P_{Cl^-} = 0.046$ – 0.079) (Table 1). The mutant M113N/L135N (16) gave similar results (data not shown). The effect of γ -cyclodextrin (γ CD) which contains eight glucose units, on the selectivity of the wt- α HL pore was also tested. γ CD enhanced the anion selectivity of the pore ($P_{K^+}/P_{Cl^-} = 0.38$), but to a lesser extent than β CD ($P_{K^+}/P_{Cl^-} = 0.23$ – 0.25) (Table 1).

The Anionic Adapter Hepta-6-sulfato- β -cyclodextrin ($s_7\beta$ CD) Creates a Cation-Selective α HL Pore. We wished to test the effects of a charged adapter on the ion selectivity of α HL. Many commercially available derivatives of β CD are complex mixtures of regioisomers with different extents of substitution. Therefore, we tested hepta-6-sulfato- β -cyclodextrin ($s_7\beta$ CD, Fig. 1) of 97 mole % isomeric purity. $s_7\beta$ CD bound to the wt- α HL pore from the trans side of the membrane producing a substantial single-channel block (Fig. 2b) (Table 1). The dwell time of $s_7\beta$ CD at pH 7.5 ($\tau = 846 \pm 37$ msec at -40 mV, $n = 3$) was far greater than β CD ($\tau = 0.84 \pm 0.09$ msec at -40 mV, $n = 8$) and, as expected, it was voltage-dependent (data not shown). I - V curves were constructed for currents recorded under both cis/trans and trans/cis KCl gradients (Fig. 2 c and d) and charge selectivities calculated from V_r . The wt- α HL· $s_7\beta$ CD complex is strongly cation selective ($P_{K^+}/P_{Cl^-} = 6.7$ – 10) (Table 1). α HL-M113N also became cation selective with $s_7\beta$ CD bound ($P_{K^+}/P_{Cl^-} = 4.1$ – 6.1) (Table 1).

We also compared the preference of wt- α HL· $s_7\beta$ CD for Na^+ and K^+ . I - V data were recorded with 1000 mM NaCl in one chamber and 1000 mM KCl in the other (Fig. 2e). Ion selectivities were calculated from V_r and the average values of P_{K^+}/P_{Cl^-} by using the GHK equation expanded to include Na^+ , K^+ and Cl^- (1). The permeability ratio, $P_{K^+}/P_{Na^+} = 0.87$, was the same for both cis/trans and trans/cis gradients. The value for the unmodified wt- α HL pore under the same conditions was $P_{K^+}/P_{Na^+} = 1.0$.

Cation-Selective Mutant Pores Become Anion Selective with β CD as an Adapter. The increase in anion selectivity observed when β CD was used as an adapter for the wt- α HL pore was modest and we wished to determine whether β CD would produce anion selectivity in a cation-selective pore. To this end, we examined α HL-CH1, a chimeric protein that features a transmembrane domain derived from the protective antigen of anthrax toxin fused to the cap domain of α HL. The net charge in the

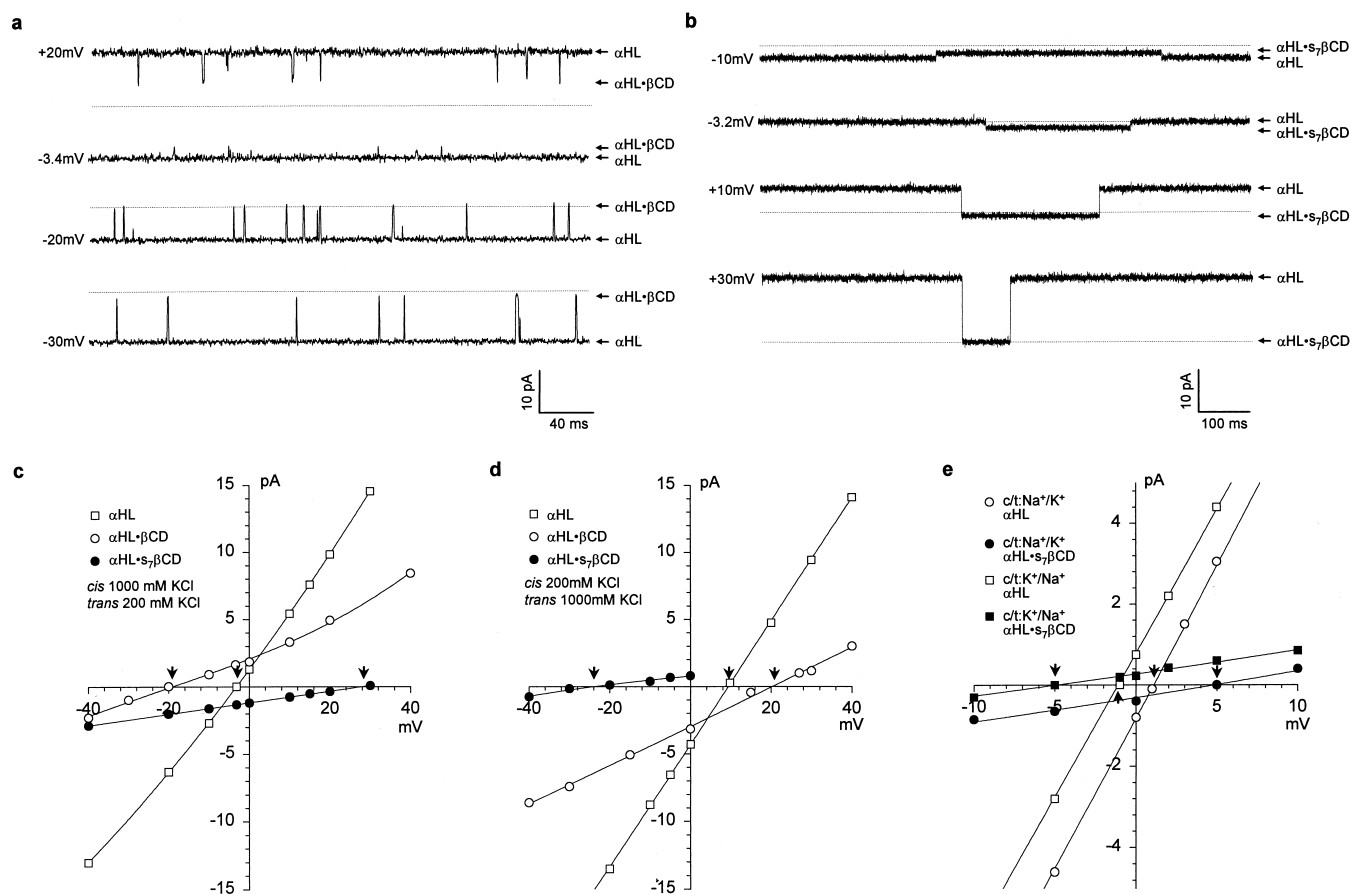


Fig. 2. Representative bilayer recordings and I-V curves showing modulation of single-channel currents through the wt- α HL pore by cyclodextrin adapters. (a), Current recording in the presence of $40 \mu\text{M}$ βCD added to the trans side of the bilayer. The chambers contained 10 mM K phosphate (pH 7.5), with cis, 1000 mM KCl; trans, 200 mM KCl. The transmembrane potential is indicated. The thin line indicates zero current. (b), Current recording in the presence of $40 \mu\text{M}$ $s_7\beta\text{CD}$ added to the trans side. Other conditions as in a. (c), I-V curves for αHL (\square), $\alpha\text{HL}\cdot\beta\text{CD}$ (\circ), and $\alpha\text{HL}\cdot s_7\beta\text{CD}$ (\bullet) based on recordings made with cis: 1000 mM KCl; trans, 200 mM KCl. Reversal potentials (V_r) are marked by arrows. (d), I-V curves for αHL (\square), $\alpha\text{HL}\cdot\beta\text{CD}$ (\circ), and $\alpha\text{HL}\cdot s_7\beta\text{CD}$ (\bullet) based on recordings made with cis: 200 mM KCl; trans, 1000 mM KCl. (e), I-V curves recorded to test cation selectivity in 10 mM phosphate buffer (pH 7.5): \square and \blacksquare , αHL and $\alpha\text{HL}\cdot s_7\beta\text{CD}$ with cis: 1000 mM KCl; trans, 1000 mM NaCl; \circ and \bullet , αHL and $\alpha\text{HL}\cdot s_7\beta\text{CD}$ with cis: 1000 mM NaCl; trans, 1000 mM KCl.

transmembrane barrel of homoheptameric $\alpha\text{HL}\text{-CH1}$ is -21 , compared with -7 in the wt- αHL barrel, and it is cation selective. The altered barrel in $\alpha\text{HL}\text{-CH1}$ retains the site near Met-113, where cyclodextrins are believed to bind (16). βCD indeed converted the cation selectivity of $\alpha\text{HL}\text{-CH1}$ ($P_{\text{K}^+}/P_{\text{Cl}^-} = 5.1$ at pH 7.4) to weak anion selectivity ($P_{\text{K}^+}/P_{\text{Cl}^-} = 0.82$) (Fig. 3 a and b; Table 1). The weakly cation selective mutant E111N/K147N ($P_{\text{K}^+}/P_{\text{Cl}^-} = 1.2$) was also examined in the presence of βCD , which converted it to an anion selective pore ($P_{\text{K}^+}/P_{\text{Cl}^-} = 0.15$) (Table 1).

Discussion

The goal of this study was to determine whether molecular adapters are capable of altering the ion selectivity of a transmembrane pore. We have found that cyclodextrin adapters produce substantial changes in the charge selectivity of αHL . For example, in the most striking examples, the wt- αHL pore equipped with an anionic adapter, $s_7\beta\text{CD}$, was strongly cation selective ($P_{\text{K}^+}/P_{\text{Cl}^-} = 10$), whereas a mutant αHL , M113N, equipped with unmodified βCD was strongly anion selective ($P_{\text{K}^+}/P_{\text{Cl}^-} = 0.05$) (Fig. 4). In both these cases, a complete block is observed when various guest molecules bind to the adapter within the channel lumen (data not shown). Therefore, it is likely that most of the current flows through the center of the adapter, rather than around its edges. The direction of the salt gradient

had a significant effect on $P_{\text{K}^+}/P_{\text{Cl}^-}$ values (Table 1), which might be attributed to several causes, including differential screening of charged groups, effects on the conformation of the protein and the onset of multi-ion transport conditions. Nevertheless, the general trends are unaffected by these deviations. The cyclodextrin adapters produced no significant discrimination between cations, as manifest in $P_{\text{K}^+}/P_{\text{Na}^+}$ values.

Ion permeation remains one of the most disputatious areas of theoretical biophysics (2, 32–34). Computational demands place an understanding of permeation beyond present-day exact molecular dynamics simulations (34, 35). The two major practicable approaches, chemical kinetics and diffusion theory, are rich in adjustable parameters and it is not surprising they “explain” most experimental observations. For example, a permeability ratio of 10 amounts to a barrier difference of only $1.4 \text{ kcal mol}^{-1}$, which is readily accommodated. Here is a situation where our ability to measure far exceeds our ability to compute. Therefore, rather than confront the difficulties, we show that our findings are at least consistent with qualitative notions about selectivity.

Ion selectivity clearly depends to a large extent on the dimensions of a pore and the spatial distribution of charges at the entrance to and within the channel lumen (1, 3). A “wide pore” with a radius greater than the Debye length ($\approx 10 \text{ \AA}$ in 100 mM salt, $\approx 3 \text{ \AA}$ at 1000 mM) generally shows weak selectivity because ions in transit interact primarily with water and other ions, rather

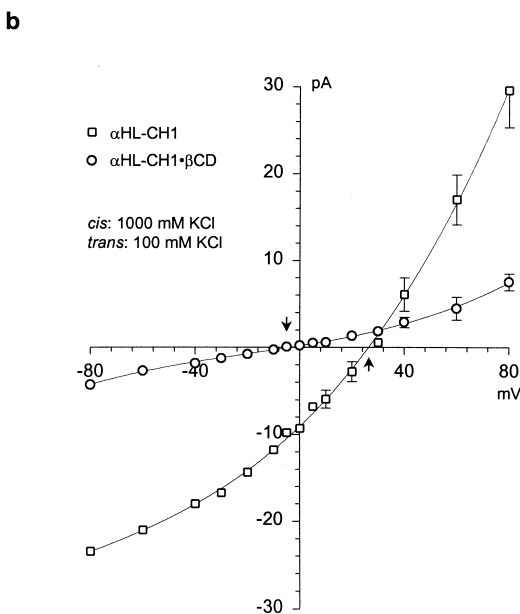
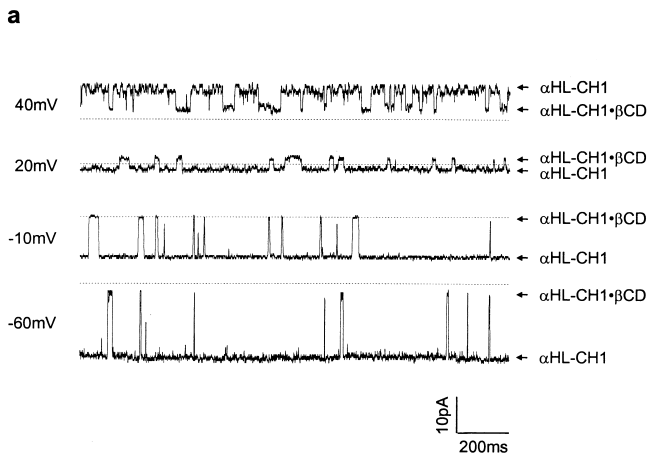


Fig. 3. Bilayer recordings and I-V curves showing modulation of single-channel currents through the α HL-CH1 pore by β CD. (a), Current recording in the presence of $40 \mu\text{M}$ β CD added to the trans side of the bilayer. The chambers contained 10 mM K phosphate (pH 7.4), and $5 \mu\text{M}$ EDTA, with cis, 1000 mM KCl; trans, 100 mM KCl. (b), I-V curves for α HL-CH1 (\square) and α HL-CH1- β CD (\circ). The data points represent mean values (\pm SD) from three different experiments for α HL-CH1 and two experiments for α HL-CH1- β CD. Arrows, V_r .

than with the wall of the lumen (36). In this case, ion selectivities roughly reflect the diffusion coefficients of individual ions in solution. Narrow pores, such as voltage-gated K channels ([diameter (d) = 3 Å], Na channels (d = 4 Å), and gramicidin A (d = 4 Å) are at the opposite extreme and show not only high charge selectivity, but also substantial discrimination among ions of the same charge. Here, high selectivity arises through dehydration of ions in the channel lumen and coordination by preorganized functional groups in a selectivity filter (1, 37), which in the case of K channels are the oxygen atoms of backbone carbonyls (3, 4). Between the extremes of wide and narrow channels, mid-sized channels, such as the nicotinic acetylcholine receptor and the anion-selective γ -aminobutyric acid type A receptor show high charge selectivity, but low selectivity among ions of the same charge. The selectivity of wide

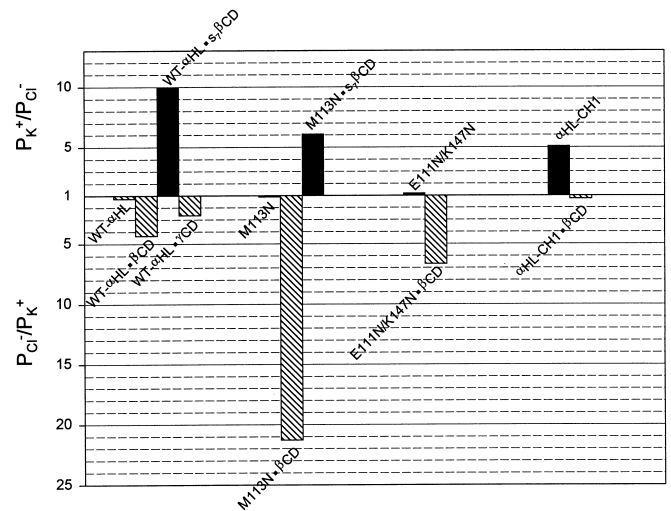


Fig. 4. Summary of the charge selectivity data. For cation selective pores P_{K^+}/P_{Cl^-} is shown (dark bars). For anion selective pores, the ratios in Table 1 have been inverted to give P_{Cl^-}/P_{K^+} (hatched bars).

and mid-sized channels can be altered by using mutagenesis to place or alter charged amino acid side chains along the conductive pathway and at its entrance (for recent examples see refs. 11, 15, 38, and 39). Even for highly selective channels electrostatics can provide a prefilter (3, 4).

wt- α HL should be considered a “wide pore” (22). The narrowest internal diameter is ~ 14 Å near Met-113, which is close to the cyclodextrin binding site (16, 17). In keeping with the assignment as a wide pore, wt- α HL is of high conductance (658 pS, 1 M NaCl at pH 7.5, -40 mV) and, despite the presence of charged residues throughout the channel lumen (Fig. 1a), charge selectivity is weak (22). Under the conditions used here $P_{K^+}/P_{Cl^-} = 0.55$ – 0.79 . The selectivity of α HL can be altered by introducing multiple charged side chains into the channel lumen, as seen here with α HL-CH1 ($P_{K^+}/P_{Cl^-} = 5.1$; 541 pS, 1 M KCl at pH 7.4, -40 mV), in which the net charge of the lower half of the transmembrane barrel is changed from -7 to -21 . wt- α HL and α HL-CH1 pores both bound the neutral adapter β CD, which introduces a mid-sized constriction [internal diameter 6.2 Å (40), still sufficient to admit a hydrated ion] as demonstrated by the reductions in conductance: wt- α HL- β CD, $g = 240$ pS, 1 M NaCl at pH 7.5, -40 mV; α HL-CH1- β CD, $g = 109$ pS, 1 M KCl at pH 7.4, -40 mV. Both pores are anion selective when the adapter is bound. Therefore, the β CD adapter dominates ion selection as judged by the similar outcomes in both an anion-selective and cation-selective background (Table 1). γ CD, which contains eight glucose units, rather than the seven of β CD, is also unchanged, with a larger internal diameter of 7.9 Å (40). As expected, γ CD has a lesser effect than β CD on the conductance of the wt- α HL pore (wt- α HL- γ CD: $g = 328$ pS, 1 M NaCl at pH 7.5, -40 mV). The influence on anion selectivity was also reduced ($P_{K^+}/P_{Cl^-} = 0.38$).

The seven sulfate groups of $s_7\beta$ CD form a negatively charged ring (-7) (Fig. 1b). This adapter greatly reduced the conductance of the wt- α HL pore: wt- α HL- $s_7\beta$ CD, $g = 53$ pS, 1 M NaCl (pH 7.5), -40 mV. Further, the pore became cation selective, $P_{K^+}/P_{Cl^-} = 6.7$ – 10 . In this case, electrostatics predominate over the preference of the interior of the cyclodextrin for anions. This interpretation is supported by the finding that the P_{K^+}/P_{Cl^-} value for wt- α HL- $s_7\beta$ CD is highly dependent on the absolute ionic strength (data not shown). It is also in keeping with many studies in the literature in which the introduction by mutagenesis of charged rings at channel entrances or in the lumen, altered

charge selectivity in a qualitatively predictable manner. In general, the placement of negatively charged side chains in the lumen favors cation selectivity, whereas positively charged side chains favor anion selectivity, as seen, for example, in the cases of the mitochondrial voltage-dependent anion channel (VDAC) (9), the porin of *Paracoccus denitrificans* (11), and alamethicin (15).

Interestingly, modified cyclodextrins have been used directly as ion channels. For example, Tabushi and colleagues made a β CD carrying hydrophobic chains on four of the seven 6-positions (41). It was claimed that two of these molecule form a transmembrane pore. In a more extensive study, Lehn and collaborators made a "bouquet" molecule from β CD by attaching seven PEG chains to each face. This molecule showed some of the properties expected of a transmembrane pore (42, 43). In another study, a condensed monolayer of β CD with all seven 6-positions modified with long alkyl chains was deposited on a graphite electrode. The modified surface was permeable to the electroactive marker *p*-quinone (44). Transport through the central cavity was invoked because it could be blocked with guest molecules. Unfortunately, in none of these cases were clear-cut measurements of charge selectivity made.

In conclusion, we have presented a different way to modulate the ion selectivity of a transmembrane pore. The adapters can be regarded as crude modular selectivity filters. By reducing the dimensions of the channel lumen from "wide" to "mid-sized," the adapters dominate the charge selectivity of the pore. Unmodified β CD has an affinity for anions, whereas the negatively

charged $s_7\beta$ CD rejects anions, allowing cations to pass in preference. Like site-directed mutagenesis, the adapter approach is versatile because various adapters can be used to program the same protein. Furthermore, mutagenesis and the adapter approach can be combined: for example, to increase the dwell time of the adapter on the protein (16). Protein pores with adapters should be useful model systems with which to study the details of ion permeation. In the case of the α HL- β CD system, the protein is of known structure and both the protein and the adapter have sevenfold symmetry. The adapter is not likely to produce any major rearrangements of the protein, although additional structural studies will be required to confirm this and to provide the exact location of the adapter. It may be possible to use a similar approach to alter the activity of other proteins, e.g., to modify the active site of an enzyme. In future work with pores, a great challenge will be to introduce yet greater selectivity; for example, by using an adapter with a ring of carbonyl groups similar to those found in the ionophore valinomycin (45) or eukaryotic potassium channels (4). This simple method to control ion selectivity may also have useful applications in aspects of biotechnology including drug delivery and biosensor design.

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