

# Prolonged Residence Time of a Noncovalent Molecular Adapter, $\beta$ -Cyclodextrin, within the Lumen of Mutant $\alpha$ -Hemolysin Pores

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**ABSTRACT** Noncovalent molecular adapters, such as cyclodextrins, act as binding sites for channel blockers when lodged in the lumen of the  $\alpha$ -hemolysin ( $\alpha$ HL) pore, thereby offering a basis for the detection of a variety of organic molecules with  $\alpha$ HL as a sensor element.  $\beta$ -Cyclodextrin ( $\beta$ CD) resides in the wild-type  $\alpha$ HL pore for several hundred microseconds. The residence time can be extended to several milliseconds by the manipulation of pH and transmembrane potential. Here, we describe mutant homoheptameric  $\alpha$ HL pores that are capable of accommodating  $\beta$ CD for tens of seconds. The mutants were obtained by site-directed mutagenesis at position 113, which is a residue that lies near a constriction in the lumen of the transmembrane  $\beta$  barrel, and fall into two classes. Members of the tight-binding class, M113D, M113N, M113V, M113H, M113F and M113Y, bind  $\beta$ CD  $\sim 10^4$ -fold more avidly than the remaining  $\alpha$ HL pores, including WT- $\alpha$ HL. The lower  $K_d$  values of these mutants are dominated by reduced values of  $k_{off}$ . The major effect of the mutations is most likely a remodeling of the binding site for  $\beta$ CD in the vicinity of position 113. In addition, there is a smaller voltage-sensitive component of the binding, which is also affected by the residue at 113 and may result from transport of the neutral  $\beta$ CD molecule by electroosmotic flow. The mutant pores for which the dwell time of  $\beta$ CD is prolonged can serve as improved components for stochastic sensors.

**KEY WORDS:**  $\alpha$ -toxin • molecular adapter • mutagenesis • pore • stochastic sensing

## INTRODUCTION

Considerable effort has been spent on the engineering of soluble proteins, such as enzymes and antibodies, both by natural and unnatural amino acid substitution, and by targeted chemical modification. By contrast, the engineering of transmembrane channels and pores is a relatively unexplored area (Bayley, 1999) that is worth examination because membrane proteins differ in environment and structure compared with soluble proteins. Further, just as in the case of soluble proteins, applications for engineered channels and pores are emerging in several areas of biotechnology (Bayley, 1999).

To further efforts in both the protein engineering and the biotechnology of membrane proteins, we have been working with the bacterial pore-forming toxin, staphylococcal  $\alpha$ -hemolysin ( $\alpha$ HL).<sup>\*</sup> The  $\alpha$ HL pore is a heptamer made up of identical subunits of 293 amino acids (see Fig. 1 A). Roughly globular molecules with molecular masses of up to  $\sim 2,000$  D (Füssle et al., 1981), or larger elongated polymers such as single-

stranded nucleic acids (Kasianowicz et al., 1996), can pass through a channel centered on the molecular sevenfold axis of the pore (see Fig. 1 A). Before the crystal structure of the  $\alpha$ HL pore was solved (Song et al., 1996), we used biochemical knowledge to engineer triggers and switches into the protein through which the assembly pathway could be controlled by a variety of agents including metal ions, enzymes, and light (Bayley, 1995, 1997). Potential applications of these molecules include the use of metal-regulated pores for reversible cell permeabilization (Russo et al., 1997; Otto-Bruc et al., 1998; Eroglu et al., 2000) and the development of cytotoxic agents activated by cell-surface proteases (Walker and Bayley, 1994; Panchal et al., 1996).

The availability of the structure of the  $\alpha$ HL pore (Song et al., 1996) allowed us to focus on the properties of fully assembled pores altered by direct mutagenesis (Braha et al., 1997) or by targeted covalent modification (Movileanu et al., 2000; Howorka et al., 2001a,b). From the viewpoint of biotechnology, the primary goal has been to produce pores that respond to various analytes as components for stochastic sensors operating at the single molecule level (Bayley et al., 2000; Bayley and Cremer, 2001). For example, direct mutagenesis has permitted the detection of divalent metal ions (Braha et al., 1997, 2000), whereas the covalent attachment of ligands has allowed the detection of proteins (Movileanu et al., 2000) and DNA (Howorka et al., 2001a).

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<sup>\*</sup>Abbreviations used in this paper:  $\alpha$ HL, staphylococcal  $\alpha$ -hemolysin;  $\beta$ CD,  $\beta$ -cyclodextrin.

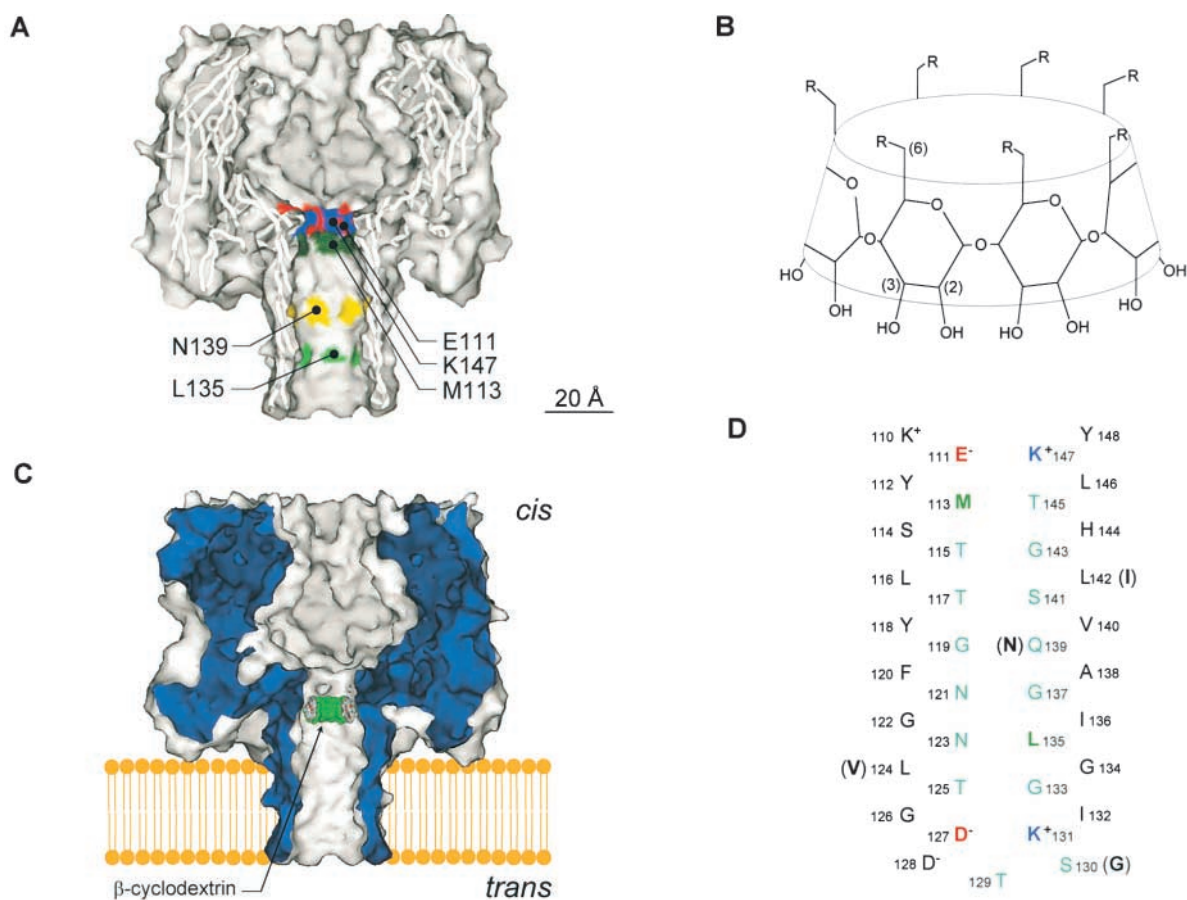


FIGURE 1. Representations of staphylococcal  $\alpha$ -hemolysin ( $\alpha$ HL) and  $\beta$ -cyclodextrin ( $\beta$ CD). (A) Sagittal section through the WT- $\alpha$ HL pore showing the location of Met-113 (dark green), Glu-111 (red), Lys-147 (blue), Asn-139 (yellow), and Leu-135 (green). (B) Structure of  $\beta$ CD. (C) Schematic of the WT- $\alpha$ HL pore showing  $\beta$ CD lodged in the lumen of the channel. The location is based on mutagenesis data (Gu et al., 1999, 2001), including that described here. (D) Sequence of the transmembrane  $\beta$  barrel of  $\alpha$ HL-RL2. WT residues are shown in parentheses.

The stochastic sensing of small organic molecules is a substantial challenge that was solved by the introduction of noncovalent molecular adapters (Fig. 1, B and C; Gu et al., 1999). Adapter molecules, such as cyclodextrins, can become lodged in the lumen of the  $\alpha$ HL pore, where they remain available for the host-guest interactions that are well-known to occur in solution (D'Souza and Lipkowitz, 1998). Hence, binding of a cyclodextrin within the lumen of the  $\alpha$ HL pore causes a reduction in current flowing through the pore. Additional transient reductions in the current are brought about by the subsequent binding of organic analyte molecules to the cyclodextrin, permitting the analyte to be identified and quantified (Gu et al., 1999). Other molecules, notably cyclic peptides, can act as adapters (Sanchez-Quesada et al., 2000). The adapters can bring about further changes in the properties of the  $\alpha$ HL pore; for example, they can alter ion selectivity (Gu et al., 2000; Sanchez-Quesada et al., 2000).

Because of the utility of noncovalent molecular adapters, it is important to maximize their dwell times

within the lumen of the pore. The dwell time of  $\beta$ -cyclodextrin ( $\beta$ CD) within the pore can be extended to several milliseconds at low pH and high negative transmembrane potentials or at high pH and high positive potentials (Gu and Bayley, 2000). However, it would be advantageous to lengthen the residency to seconds or longer. We have already shown that the mutant M113N binds  $\beta$ CD with high affinity (Gu et al., 1999) and, recently, this finding was extended when a nanocavity was built between two different cyclodextrins lodged in the transmembrane  $\beta$  barrel (Gu et al., 2001). Met-113 is located near the constriction at the internal end of the  $\beta$  barrel (Fig. 1 A); here, we report in detail the results of mutagenesis of Met-113 to each of the remaining nineteen natural amino acids.

## MATERIALS AND METHODS

### Mutagenesis of $\alpha$ HL at Position 113

All Met-113 mutants were made by cutting pT7- $\alpha$ HL-RL2 (Cheley et al., 1999) with SacII and HpaI and replacing the excised in-

ternal fragment with duplex DNA formed from 5'-GGAA-TTCGATTGATACAAAAGAGTATxyzAGTACGTT-3' (sense) and 5'-AACGTACTz'y'x'ATACTCTTTGTATCAATCGAATCCGC-3' (antisense), where xyz and z'y'x' represent, respectively, the codon and anticodon replacements for the following: Ala (xyz = GCA/ x'y'z' = TGC), Cys (TGC/GCA), Asp (GAT/ATC), Glu (GAG/CTC), Phe (TTT/AAA), Gly (GGG/CCC), His (CAT/ATG), Ile (ATT/AAT), Lys (AAA/TTT), Leu (CTA/TAG), Asn (AAT/ATT), Pro (CCA/TGG), Gln (CAG/CTG), Arg (AGA/TCT), Ser (AGC/GCT), Thr (ACT/AGT), Val (GTT/AAC), Trp (TGG/CCA), and Tyr (TAT/ATA). Because these changes were made in the RL2 background (Cheley et al., 1999), each mutant contains the following additional replacements with respect to WT- $\alpha$ HL: Lys-8  $\rightarrow$  Ala, Val-124  $\rightarrow$  Leu, Gly-130  $\rightarrow$  Ser, Asn-139  $\rightarrow$  Gln, and Ile-142  $\rightarrow$  Leu.

### Additional $\alpha$ HL Mutants

Replacements of charged residues near the constriction were also made by constructing the mutants K147N, E111N/K147N, and E111N/M113N/K147N. First, mutants  $\alpha$ HL-E111N and  $\alpha$ HL-K147N were constructed by cutting pT7- $\alpha$ HL-RL2 with the enzyme pairs SacII-HpaI and AflIII-XhoI, respectively, and replacing the excised internal fragments with, respectively, the duplexes formed from 5'-GGAATTCGATTGATACAAAAAATTATATGAGTACGTT-3' (sense) and 5'-AACGTACTCATATAATTTTTTTGTATCAATCGAATCCGC-3' (antisense) and from 5'-TTAATTATGTCAACCTGATTTCAAAAACAATTC-3' (sense) and 5'-TCGAGAA-TTGTTTTGAATCAGGTTGAACATAA-3' (antisense). To construct the double mutant E111N/K147N, pT7- $\alpha$ HL-K147N was digested with HpaI and HindIII, and the resulting internal fragment was inserted into pT7- $\alpha$ HL-E111N that had been cut with the same enzymes. The triple mutant E111N/M113N/K147N was prepared by cutting pT7- $\alpha$ HL-K147N with SacII and HpaI and replacing the small fragment with duplex DNA prepared from 5'-GGAATTCGATTGATACAAAAAATTATAATAGTACGTT-3' (sense) and 5'-AACGTACTATTATAATTTTTTTGTATCAATCGAATCCGC-3' (antisense). Because these changes were made in the RL2 background (Cheley et al., 1999), each mutant contained the additional replacements listed above.

Mutants M113N(WT), N139Q(WT), and M113N/N139Q(WT) were constructed as described previously (Gu et al., 2001).  $\alpha$ HL-L135N was constructed by cutting pT7- $\alpha$ HL-RL1 (Cheley et al., 1997) with SpeI and ApaI and replacing the fragment with duplex DNA prepared from 5' CTAGTAAAATTGGAGGCCAATTTGGGGCCAGG-3' (sense) and 5' GGCCCCAATATTGCC-TCCAATTTTA-3' (antisense). The StuI site that is present in the  $\alpha$ HL-RL1 gene, but absent in  $\alpha$ HL-L135N, was used to screen for cassette replacement before DNA sequencing. RL1 encodes the same amino sequence as RL2, but contains different restriction sites. Therefore, in the text, L135N is treated as having the RL2 background.  $\alpha$ HL-M113N/L135N was constructed in a similar fashion. pT7- $\alpha$ HL-M113N (RL2 background; see above) was cut with SpeI and ApaI, and the fragment was replaced with duplex DNA prepared from the same oligonucleotides used to construct  $\alpha$ HL-L135N (see above). As before, plasmids were screened by digestion with StuI.

### DNA Sequencing

The genes of all  $\alpha$ HL mutants used in this work were sequenced entirely. Sequencing was performed with SC001 (upstream forward primer) 5'-CACTATAGGGAGACCACAACGG-3' and SC003 (internal forward primer) 5'-CAGGGTTTTCCACCAGACTTCGC-3'. No changes were found, except for those intended.

### $\alpha$ HL Homoheptamer Formation and Purification

Heptameric WT- $\alpha$ HL was formed by treating monomeric  $\alpha$ HL, purified from *Staphylococcus aureus*, with deoxycholate (Bhakdi et al., 1981; Walker et al., 1992) and isolated from SDS-polyacrylamide gels as described previously (Braha et al., 1997). The remaining  $\alpha$ HL polypeptides were synthesized in vitro by coupled transcription and translation (IVTT) and assembled into homoheptamers by the inclusion of rabbit red cell membranes during synthesis, as described previously (Cheley et al., 1999). The heptamers were purified by SDS-PAGE and stored in 50- $\mu$ l aliquots at  $-80^{\circ}\text{C}$  (Cheley et al., 1999). All mutant polypeptides were synthesized and purified twice.

### Planar Bilayer Recordings

Planar bilayer recordings were made as described at  $22 \pm 2^{\circ}\text{C}$  (Gu and Bayley, 2000; Gu et al., 2000). Buffers for bilayer recording contained 1 M NaCl and 10 mM dibasic sodium phosphate (Sigma-Aldrich), in deionized water (Millipore Corp.), and were titrated to pH 7.5 with aqueous HCl (EMScience). The bilayer was formed from 1,2-diphytanoyl-*sn*-glycero-phosphocholine (Avanti Polar Lipids) over an orifice 50–100  $\mu\text{m}$  in diameter (Montal and Mueller, 1972). The orifice had been pretreated with hexadecane in pentane, and the lipid was transferred to the chambers in pentane. Protein was added to the cis chamber, which was at ground. A positive potential indicates a higher potential in the trans chamber of the apparatus, and a positive current is one in which cations flow from the trans to the cis side. Experiments were initiated by the addition of heptameric  $\alpha$ HL to the cis chamber, to a final concentration of 3–30  $\text{ng ml}^{-1}$ , with stirring until a single channel appeared.  $\beta$ CD (Sigma-Aldrich) was added to the trans chamber at 40  $\mu\text{M}$ , unless otherwise specified. The amplifier's internal low-pass Bessel filter was set at 5 kHz. Data were acquired at a sampling rate of 20 kHz. The current recordings were analyzed essentially as described previously (Gu and Bayley, 2000; Gu et al., 2000). To determine each set of kinetic constants, three or more experiments had been performed and, in each case, data were analyzed that were acquired for at least 2 min for weak binding mutants (class 2) and at least 1 h for tight binding mutants (class 1).  $\tau_{\text{on}}$  and  $\tau_{\text{off}}$  for  $\beta$ CD for each mutant, for data obtained at either  $-40$  mV or  $+40$  mV and 40  $\mu\text{M}$   $\beta$ CD, were obtained from dwell-time histograms fitted to single exponentials by the Levenberg-Marquardt procedure. In all cases, the coefficient of determination of the fits was  $R \geq 0.85$ . The data were replotted in semilogarithmic form for display in the paper. Separate segments of the data yielded similar  $\tau$  values, suggesting that stationary kinetics prevailed. Kinetic constants were calculated by using  $k_{\text{off}} = 1/\tau_{\text{off}}$ ,  $k_{\text{on}} = 1/\tau_{\text{on}}[\beta\text{CD}]$ , and  $K_d = k_{\text{off}}/k_{\text{on}}$ , where  $[\beta\text{CD}]$  is the concentration of  $\beta$ CD. Values for unitary conductance,  $k_{\text{on}}$  and  $k_{\text{off}}$ , and  $K_d$  are quoted as the mean  $\pm$  SD. Ion selectivity ( $P_{\text{K}^+}/P_{\text{Cl}^-}$ ) was determined from reversal potentials measured with the following solution: cis 1,000 mM KCl, 10 mM potassium phosphate, pH 7.5 (dibasic salt titrated with HCl), trans 200 mM KCl, and 10 mM potassium phosphate, pH 7.5 (Gu et al., 2000).

### Online Supplemental Material

Supplemental figures are available at <http://www.jgpp.org/cgi/content/full/118/5/481/DC1>. They show examples of dwell-time histograms used to obtain values of  $\tau_{\text{on}}$  for M113E, M113K, M113N, and M113W. The figures also display histograms obtained from independent segments of the recordings to show that the channel kinetics were stationary.

T A B L E I

*Conductance Values and Kinetic Parameters for the Interaction of  $\beta$ CD with WT- $\alpha$ HL and Met-113 Mutants<sup>a</sup>*

$\alpha$ HL pore	Side chain <sup>b</sup>	-40 mV					+40 mV				
		$g_p$	$g_{pc}$	$k_{on}$	$k_{off}$	$K_d$	$g_p$	$g_{pc}$	$k_{on}$	$k_{off}$	$K_d$
		$pS$	$pS$	$M^{-1}s^{-1}$	$s^{-1}$	$M$	$pS$	$pS$	$M^{-1}s^{-1}$	$s^{-1}$	$M$
WT- $\alpha$ HL	N	651 $\pm$ 4	240 $\pm$ 3	4.0 $\pm$ 0.3 $\times 10^5$	1.3 $\pm$ 0.1 $\times 10^3$	3.4 $\pm$ 0.4 $\times 10^{-3}$	721 $\pm$ 6	253 $\pm$ 4	2.8 $\pm$ 0.2 $\times 10^5$	2.1 $\pm$ 0.2 $\times 10^3$	7.8 $\pm$ 0.3 $\times 10^{-3}$
RL-2	N	691 $\pm$ 4	255 $\pm$ 6	2.9 $\pm$ 0.1 $\times 10^5$	9.8 $\pm$ 0.3 $\times 10^2$	3.3 $\pm$ 0.2 $\times 10^{-3}$	739 $\pm$ 7	220 $\pm$ 7	2.4 $\pm$ 0.3 $\times 10^5$	1.5 $\pm$ 0.2 $\times 10^3$	6.9 $\pm$ 0.1 $\times 10^{-3}$
M113K	+	558 $\pm$ 10	241 $\pm$ 3	3.3 $\pm$ 0.3 $\times 10^5$	7.0 $\pm$ 0.5 $\times 10^2$	2.1 $\pm$ 0.1 $\times 10^{-3}$	629 $\pm$ 8	181 $\pm$ 5	1.6 $\pm$ 0.2 $\times 10^5$	2.0 $\pm$ 0.1 $\times 10^3$	1.4 $\pm$ 0.1 $\times 10^{-2}$
M113R	+	585 $\pm$ 6	289 $\pm$ 4	4.3 $\pm$ 0.1 $\times 10^5$	6.6 $\pm$ 0.2 $\times 10^2$	1.6 $\pm$ 0.1 $\times 10^{-3}$	666 $\pm$ 8	266 $\pm$ 3	2.2 $\pm$ 0.2 $\times 10^5$	3.1 $\pm$ 0.3 $\times 10^3$	1.3 $\pm$ 0.1 $\times 10^{-2}$
M113H	+	596 $\pm$ 7	193 $\pm$ 2	3.0 $\pm$ 0.6 $\times 10^5$	2.1 $\pm$ 0.4 $\times 10^{-1}$	7.0 $\pm$ 1.7 $\times 10^{-7}$	677 $\pm$ 4	189 $\pm$ 5	3.3 $\pm$ 0.4 $\times 10^5$	1.1 $\pm$ 0.3 $\times 10^{-1}$	3.0 $\pm$ 1.0 $\times 10^{-7}$
M113E	-	743 $\pm$ 6	217 $\pm$ 3	1.1 $\pm$ 0.2 $\times 10^5$	1.4 $\pm$ 0.1 $\times 10^3$	1.2 $\pm$ 0.2 $\times 10^{-2}$	628 $\pm$ 7	153 $\pm$ 5	4.0 $\pm$ 0.3 $\times 10^5$	1.4 $\pm$ 0.1 $\times 10^3$	3.3 $\pm$ 0.5 $\times 10^{-3}$
M113D	-	717 $\pm$ 3	228 $\pm$ 6	2.9 $\pm$ 0.4 $\times 10^5$	4.9 $\pm$ 1.0 $\times 10^{-2}$	1.7 $\pm$ 0.8 $\times 10^{-7}$	662 $\pm$ 3	211 $\pm$ 6	3.7 $\pm$ 0.7 $\times 10^5$	4.1 $\pm$ 1.0 $\times 10^{-2}$	1.2 $\pm$ 0.4 $\times 10^{-7}$
M113G	P	689 $\pm$ 5	252 $\pm$ 5	2.3 $\pm$ 0.1 $\times 10^5$	8.9 $\pm$ 0.8 $\times 10^2$	3.7 $\pm$ 0.3 $\times 10^{-3}$	736 $\pm$ 9	214 $\pm$ 7	2.3 $\pm$ 0.1 $\times 10^5$	2.0 $\pm$ 0.2 $\times 10^3$	8.4 $\pm$ 1.7 $\times 10^{-3}$
M113N	P	632 $\pm$ 7	311 $\pm$ 3	2.7 $\pm$ 0.6 $\times 10^5$	3.8 $\pm$ 1.1 $\times 10^{-2}$	1.3 $\pm$ 0.6 $\times 10^{-7}$	646 $\pm$ 7	297 $\pm$ 4	2.9 $\pm$ 0.6 $\times 10^5$	5.0 $\pm$ 0.8 $\times 10^{-2}$	1.8 $\pm$ 0.8 $\times 10^{-7}$
M113Q	P	714 $\pm$ 11	242 $\pm$ 6	3.2 $\pm$ 0.2 $\times 10^5$	1.2 $\pm$ 0.1 $\times 10^3$	4.2 $\pm$ 0.2 $\times 10^{-3}$	769 $\pm$ 10	218 $\pm$ 3	3.2 $\pm$ 0.2 $\times 10^5$	1.6 $\pm$ 0.2 $\times 10^3$	5.1 $\pm$ 0.8 $\times 10^{-3}$
M113C	P	576 $\pm$ 7	231 $\pm$ 5	2.6 $\pm$ 0.2 $\times 10^5$	1.2 $\pm$ 0.1 $\times 10^3$	4.3 $\pm$ 0.2 $\times 10^{-3}$	651 $\pm$ 3	199 $\pm$ 6	2.0 $\pm$ 0.2 $\times 10^5$	2.7 $\pm$ 0.1 $\times 10^3$	1.3 $\pm$ 0.2 $\times 10^{-2}$
M113S	P	744 $\pm$ 6	264 $\pm$ 3	2.5 $\pm$ 0.2 $\times 10^5$	8.0 $\pm$ 0.7 $\times 10^2$	3.4 $\pm$ 0.4 $\times 10^{-3}$	761 $\pm$ 9	242 $\pm$ 4	1.8 $\pm$ 0.2 $\times 10^5$	1.5 $\pm$ 0.1 $\times 10^3$	8.1 $\pm$ 0.6 $\times 10^{-3}$
M113T	P	705 $\pm$ 9	241 $\pm$ 4	1.8 $\pm$ 0.2 $\times 10^5$	8.5 $\pm$ 0.9 $\times 10^2$	5.0 $\pm$ 0.6 $\times 10^{-3}$	761 $\pm$ 5	195 $\pm$ 3	2.3 $\pm$ 0.2 $\times 10^5$	7.5 $\pm$ 0.5 $\times 10^2$	3.0 $\pm$ 0.5 $\times 10^{-3}$
M113Y	P	682 $\pm$ 6	209 $\pm$ 5	1.8 $\pm$ 0.5 $\times 10^5$	3.2 $\pm$ 0.9 $\times 10^{-2}$	1.9 $\pm$ 0.9 $\times 10^{-7}$	698 $\pm$ 12	235 $\pm$ 8	2.0 $\pm$ 0.4 $\times 10^5$	3.5 $\pm$ 0.6 $\times 10^{-2}$	1.7 $\pm$ 0.8 $\times 10^{-7}$
M113A	N	658 $\pm$ 8	243 $\pm$ 5	2.3 $\pm$ 0.2 $\times 10^5$	7.7 $\pm$ 0.4 $\times 10^2$	3.5 $\pm$ 0.3 $\times 10^{-3}$	721 $\pm$ 9	209 $\pm$ 5	2.3 $\pm$ 0.2 $\times 10^5$	1.5 $\pm$ 0.1 $\times 10^3$	6.0 $\pm$ 0.6 $\times 10^{-3}$
M113V	N	722 $\pm$ 7	183 $\pm$ 7	2.3 $\pm$ 0.3 $\times 10^5$	6.3 $\pm$ 0.7 $\times 10^{-1}$	2.8 $\pm$ 0.3 $\times 10^{-6}$	786 $\pm$ 7	202 $\pm$ 7	2.4 $\pm$ 0.2 $\times 10^5$	3.0 $\pm$ 0.2 $\times 10^{-1}$	1.2 $\pm$ 0.3 $\times 10^{-6}$
M113L	N	666 $\pm$ 7	243 $\pm$ 3	2.7 $\pm$ 0.2 $\times 10^5$	9.5 $\pm$ 0.7 $\times 10^2$	3.8 $\pm$ 0.2 $\times 10^{-3}$	763 $\pm$ 4	237 $\pm$ 5	2.2 $\pm$ 0.2 $\times 10^5$	1.6 $\pm$ 0.2 $\times 10^3$	7.3 $\pm$ 0.9 $\times 10^{-3}$
M113I	N	637 $\pm$ 5	232 $\pm$ 5	1.4 $\pm$ 0.1 $\times 10^5$	3.0 $\pm$ 0.1 $\times 10^2$	2.1 $\pm$ 0.1 $\times 10^{-3}$	773 $\pm$ 6	273 $\pm$ 4	1.4 $\pm$ 0.1 $\times 10^5$	2.6 $\pm$ 0.3 $\times 10^2$	1.7 $\pm$ 0.4 $\times 10^{-3}$
M113P	N	816 $\pm$ 10	285 $\pm$ 4	2.1 $\pm$ 0.2 $\times 10^5$	1.7 $\pm$ 0.1 $\times 10^2$	8.4 $\pm$ 0.5 $\times 10^{-4}$	849 $\pm$ 8	246 $\pm$ 7	3.5 $\pm$ 0.2 $\times 10^5$	1.1 $\pm$ 0.1 $\times 10^2$	2.9 $\pm$ 0.3 $\times 10^{-4}$
M113F	N	624 $\pm$ 4	165 $\pm$ 2	3.1 $\pm$ 0.2 $\times 10^5$	4.0 $\pm$ 0.7 $\times 10^{-2}$	1.2 $\pm$ 0.2 $\times 10^{-7}$	702 $\pm$ 5	204 $\pm$ 5	1.7 $\pm$ 0.3 $\times 10^5$	8.6 $\pm$ 1.2 $\times 10^{-2}$	5.4 $\pm$ 0.2 $\times 10^{-7}$
M113W	N	524 $\pm$ 5	165 $\pm$ 5	4.2 $\pm$ 0.4 $\times 10^5$	1.5 $\pm$ 0.1 $\times 10^1$	3.6 $\pm$ 0.3 $\times 10^{-5}$	630 $\pm$ 7	192 $\pm$ 3	2.1 $\pm$ 0.3 $\times 10^5$	2.0 $\pm$ 0.1 $\times 10^1$	9.7 $\pm$ 2.1 $\times 10^{-5}$

<sup>a</sup>1 M NaCl, 10 mM sodium phosphate, pH 7.5, in both the cis and trans chambers.  $g_p$ , conductance of the unoccupied  $\alpha$ HL pore;  $g_{pc}$ , conductance of the occupied pore,  $\alpha$ HL $\bullet$  $\beta$ CD.

<sup>b</sup>Side chain attributes: +, positively charged; -, negatively charged; P, polar; and N, nonpolar.

## R E S U L T S

### *The WT- $\alpha$ HL Pore and $\alpha$ HL-RL2, with the Background Used for Mutagenesis, Have Similar Properties*

The Met-113 replacements examined in this work were made in  $\alpha$ HL-RL2. RL2 is the product of a semisynthetic gene that was originally devised to permit ready cassette mutagenesis of the sequence encoding the transmembrane  $\beta$  barrel. RL2, and the mutants described here, contain the following additional mutations over WT- $\alpha$ HL: Lys-8  $\rightarrow$  Ala, Val-124  $\rightarrow$  Leu, Gly-130  $\rightarrow$  Ser, Asn-139  $\rightarrow$  Gln, and Ile-142  $\rightarrow$  Leu (Fig. 1 D). Therefore, the properties of the WT and RL2  $\alpha$ HL pores were first compared. They were found to be similar in terms of unitary conductance values and rectification ratios ( $g_{+40mV}/g_{-40mV}$ ; Table I), but their properties do differ in detail. For example, the extent of channel block by  $\beta$ CD at +40 mV is lower for RL2 (30% residual current) than for WT- $\alpha$ HL (35% residual current), and whereas WT- $\alpha$ HL $\bullet$  $\beta$ CD is incompletely blocked by a variety of organic molecules (e.g., ada-

mantane-1-carboxylic acid; Gu et al., 1999), RL2 $\bullet$  $\beta$ CD is almost completely blocked by the same molecules (unpublished data).

### *$\beta$ CD Exhibits a Prolonged Dwell Time within the Lumen of Certain Met-113 Replacement Mutants*

The interaction of  $\beta$ CD was tested with pores formed from 20  $\alpha$ HL polypeptides, representing Met and all 19 natural amino acid substitutions at position 113. As documented above, the mutations were made in the "RL2" background. In single-channel recordings, in all 20 cases, the addition of 40  $\mu$ M  $\beta$ CD to the trans compartment produced reversible partial blockades of the ionic current (Fig. 2). The dwell times for  $\beta$ CD in the lumen of the pore ( $\tau_{off}$ , level 2) were strikingly prolonged for certain mutants (Figs. 2 and 3 A), whereas the dwell times for the unoccupied state of each mutant (inter-event interval,  $\tau_{on}$ , level 1) did not vary as much and are similar to  $\tau_{on}$  values for  $\beta$ CD and WT- $\alpha$ HL (Fig. 3 A).

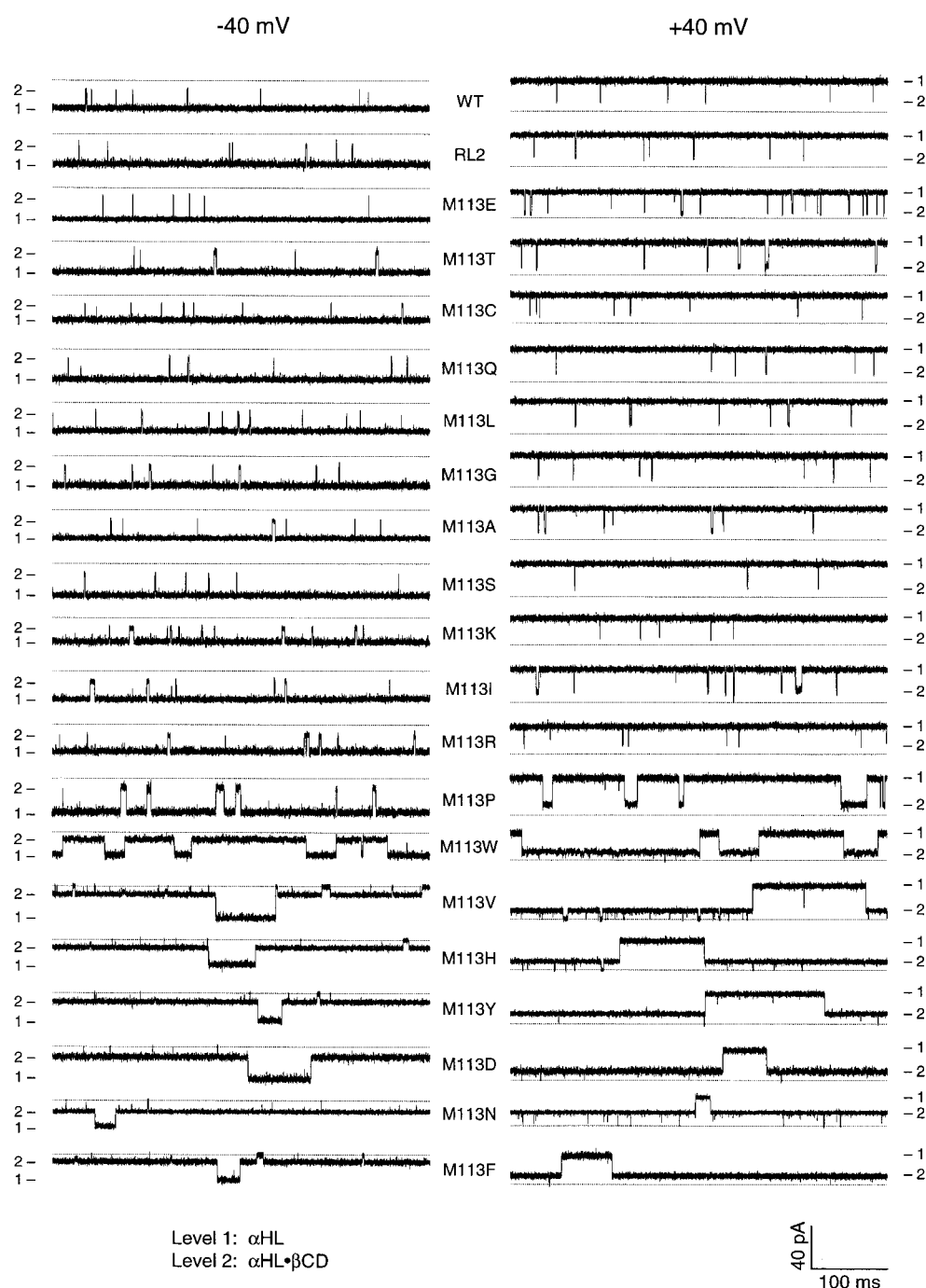


FIGURE 2. Representative current traces from single  $\alpha$ HL pores showing the blockade of Met-113 mutants by  $\beta$ CD. All traces were recorded under symmetrical conditions in buffer containing 1 M NaCl, 10 mM sodium phosphate, pH 7.5; 40  $\mu$ M  $\beta$ CD was added to the trans chamber. (left) Traces recorded at  $-40$  mV; (right) traces recorded at  $+40$  mV. The mutants shown are all derived from RL2 (see the first paragraph of RESULTS). The broken line indicates zero current. The mutants are ordered (top to bottom) according to increasing affinity for  $\beta$ CD.

Interestingly, most of the pores tested could be grouped into two classes according to their  $\tau_{\text{off}}$  values.  $\beta$ CD binds to mutant pores of class 1, namely M113D, M113N, M113V, M113H, M113F, and M113Y, with extended dwell times ( $\tau_{\text{off}}$ ) compared with WT- $\alpha$ HL (Fig. 3 A). For example at  $-40$  mV, the dwell time of M113N $\cdot\beta$ CD ( $\tau_{\text{off}} = 27$  s) is over  $10^4$ -fold longer than that of WT- $\alpha$ HL $\cdot\beta$ CD ( $\tau_{\text{off}} = 0.76$  ms) or RL2 $\cdot\beta$ CD ( $\tau_{\text{off}} = 1.0$  ms). By contrast,  $\beta$ CD binds to the mutants in class 2 (all but two of the mutants not in class 1) with a dwell time similar to that of WT- $\alpha$ HL or RL2. For ex-

ample at  $-40$  mV, the dwell time for M113K $\cdot\beta$ CD is  $\tau_{\text{off}} = 1.4$  ms. M113W and M113P are borderline cases and were not included in class 1 or 2.

In contrast to  $\tau_{\text{off}}$ , the  $\tau_{\text{on}}$  values of the mutants do not vary greatly and are similar to WT- $\alpha$ HL. For example at  $-40$  mV, the inter-event interval for M113N at 40  $\mu$ M  $\beta$ CD ( $\tau_{\text{on}} = 91$  ms) is comparable to that of WT- $\alpha$ HL ( $\tau_{\text{on}} = 68$  ms) or RL2 ( $\tau_{\text{on}} = 93$  ms). Therefore, the mutants in class 1, with prolonged  $\tau_{\text{off}}$  values, show high affinity for  $\beta$ CD, whereas mutants in class 2, with short  $\tau_{\text{off}}$  values, show a similar affinity for  $\beta$ CD to WT- $\alpha$ HL (Fig. 2

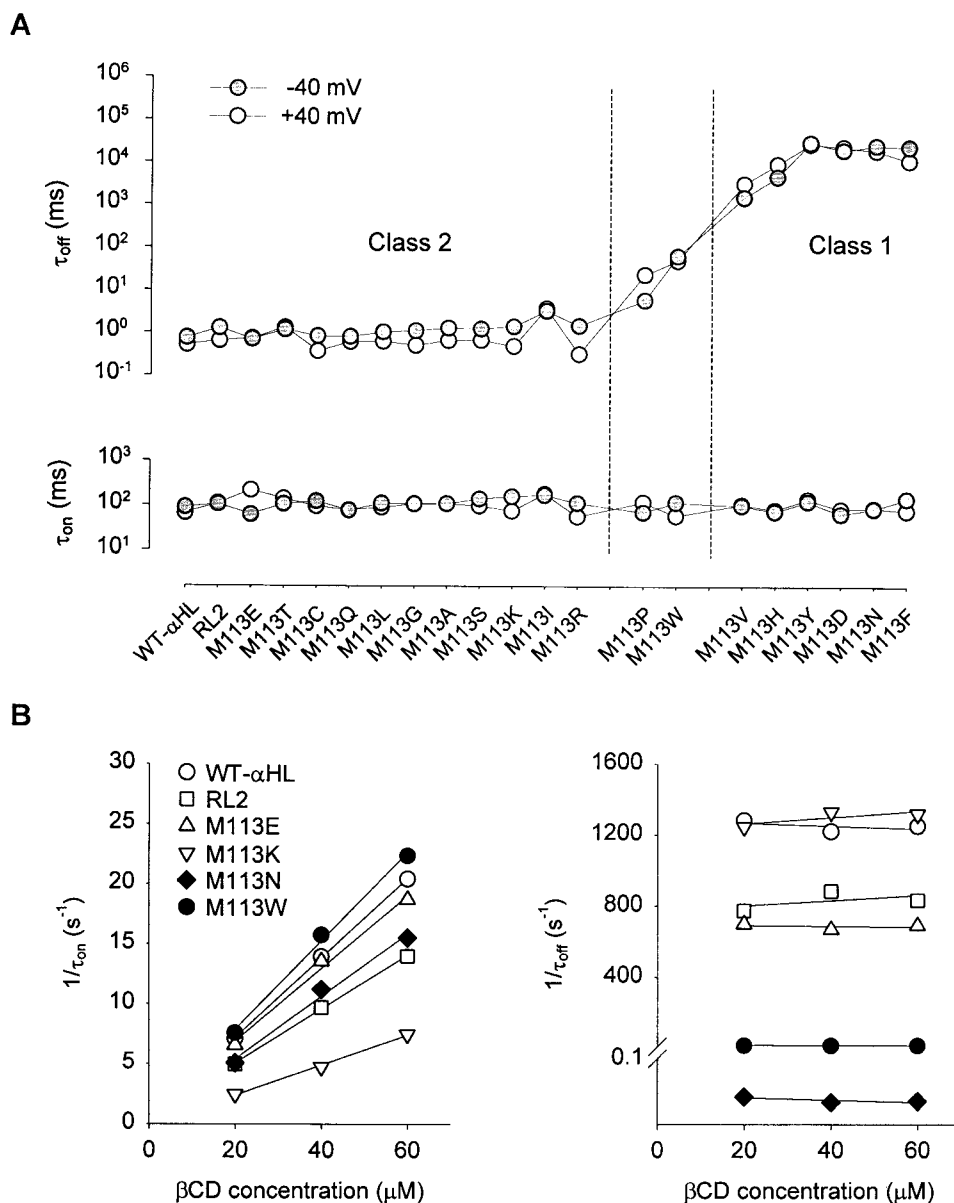


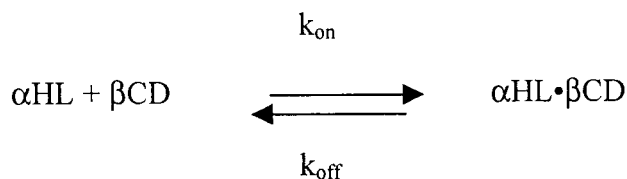
FIGURE 3. Dwell times for the interaction between  $\beta$ CD and the Met-113 mutants. (A) Values of  $\tau_{\text{off}}$ , the dwell time of  $\beta$ CD in the pore, and  $\tau_{\text{on}}$ , the inter-event interval at 40  $\mu\text{M}$  trans  $\beta$ CD. (closed gray circle) -40 mV; ( $\circ$ ) +40 mV. The mutants are ordered (left to right) according to increasing affinity for  $\beta$ CD. (B) Dependence of  $\tau_{\text{off}}$  and  $\tau_{\text{on}}$  on the concentration of  $\beta$ CD (trans) for WT- $\alpha$ HL and selected mutants.

and Table I). For example at -40 mV, M113N in class 1 ( $K_{\text{d}} = 1.3 \pm 0.6 \times 10^{-7}$  M) binds  $\beta$ CD over  $10^4$ -fold more tightly than WT- $\alpha$ HL ( $K_{\text{d}} = 3.4 \pm 0.4 \times 10^{-3}$  M) and RL2 ( $K_{\text{d}} = 3.3 \pm 0.2 \times 10^{-3}$  M), whereas M113K in class 2 ( $K_{\text{d}} = 2.1 \pm 0.1 \times 10^{-3}$  M) is similar to WT- $\alpha$ HL in affinity.

#### $\beta$ CD Binds to $\alpha$ HL Pores in a Simple Bimolecular Interaction

Level 2 is the only major current blockade level seen in the amplitude histograms of all 20 mutants, which suggests that there is only one major binding site for  $\beta$ CD within the lumen of each  $\alpha$ HL pore (Gu and Bayley, 2000). The binding kinetics are also in keeping with this interpretation. As in the case of WT- $\alpha$ HL (Gu and Bayley, 2000),  $\tau_{\text{on}}$  and  $\tau_{\text{off}}$  for  $\beta$ CD for each mutant could be fitted by single-exponential distributions for data obtained at either -40 or +40 mV (1 M NaCl and 10 mM

sodium phosphate, pH 7.5) and 40  $\mu\text{M}$   $\beta$ CD in the trans chamber (*Supplemental Material*). Therefore, the kinetics of the interaction between  $\beta$ CD and the mutant  $\alpha$ HL pores most likely obey the simple Scheme I, with  $k_{\text{off}} = 1/\tau_{\text{off}}$ ,  $k_{\text{on}} = 1/\tau_{\text{on}}[\beta\text{CD}]$ , and  $K_{\text{d}} = k_{\text{off}}/k_{\text{on}}$ , where  $[\beta\text{CD}]$  is the concentration of  $\beta$ CD. The kinetic constants were calculated accordingly (Table I).



(SCHEME I)

In selected cases, WT- $\alpha$ HL, RL2, M113E, M113K, M113N and M113W, the concentration dependence of  $1/\tau_{\text{on}}$  was examined and found to be proportional to  $[\beta\text{CD}]$  (Fig. 3 B), in further support of a simple bimolecular interaction between the pores and  $\beta\text{CD}$ .

For mutants in class 1, short additional blockades could be seen while  $\beta\text{CD}$  was bound at either  $-40$  or  $+40$  mV. Because their frequency of occurrence was independent of  $\beta\text{CD}$  concentration (tested for M113N and M113D; unpublished data), these events are probably not due to the binding of a second  $\beta\text{CD}$ . Instead, they may correspond to a second conformation of the occupied state,  $\alpha\text{HL}\cdot\beta\text{CD}$ . Because, these events occupied  $<3\%$  of the total  $\beta\text{CD}$  binding time, they were merged with level 2 for the kinetic analysis.

#### *$\beta\text{CD}$ Affinity and Side Chain Properties at Position 113*

The properties of the side chains at position 113 in the tight-binding class 1 mutants show no obvious relationship with one other. For example, when  $1/K_d$ ,  $k_{\text{on}}$  and

$k_{\text{off}}$  are plotted versus side-chain van der Waals volume no pattern is seen, although this is a convenient way to display the data (Fig. 4). Patterns are revealed when the data are examined in detail. Small side chains of  $<85 \text{ \AA}^3$  fall into class 2. Whereas M113N and M113D fall into class 1, mutants with homologous substitution, M113Q and M113E, are in class 2. Similarly, of mutants with nonaromatic hydrophobic amino acid substituents, the one with the lowest volume side chain, M113V, is in class 1, whereas the others M113L, M113I, and RL2 itself (Met-113) are in class 2. Mutants with aromatic substitutions fall into class 1, with the exception of the mutant with the bulkiest substituent, M113W, which has a  $K_d$  value intermediate between those characteristic of the two classes.

Single-channel conductance values also show no clear correlation with side-chain van der Waals volume (Fig. 5, A and B, and Table I). Although the presence of seven copies of the bulkiest residue, Trp, at position 113 does yield the pore with the lowest conductance, the effect is not dramatic. The rectification ratios

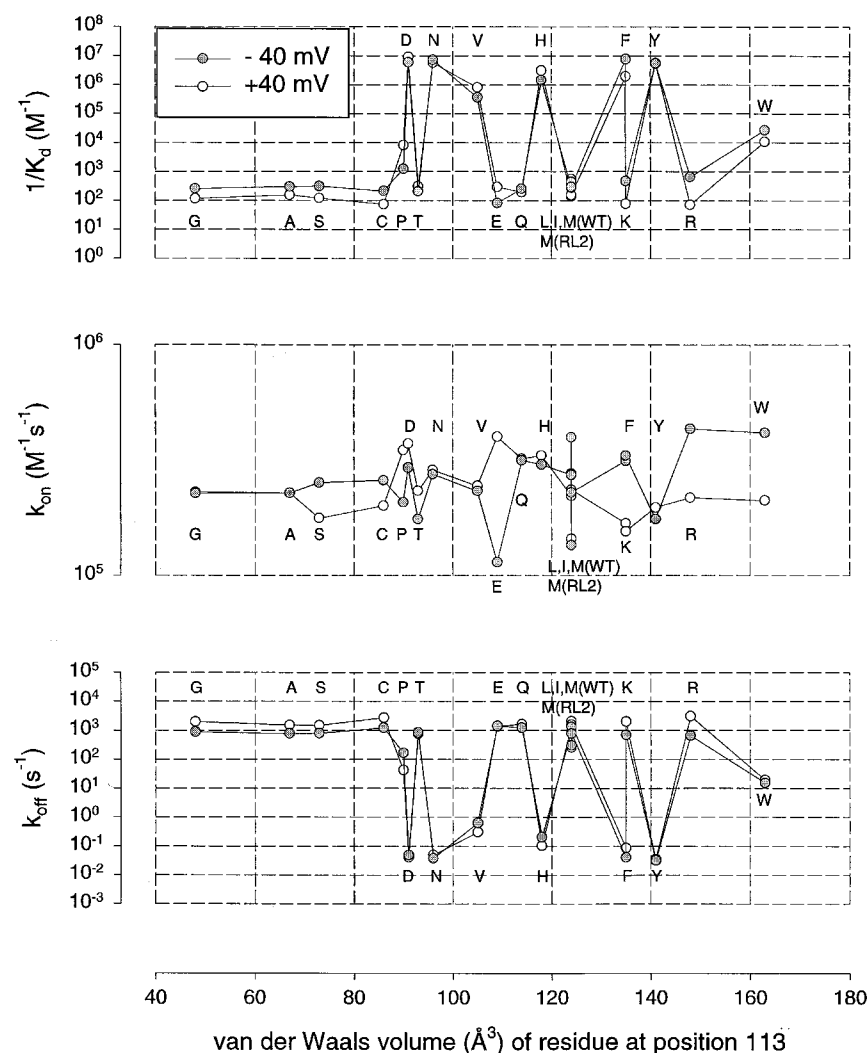


FIGURE 4. Plots of kinetic constants for the Met-113 mutants versus the van der Waals volume (Creighton, 1993) of the residue at position 113. (A)  $1/K_d$ ; (B)  $k_{\text{on}}$ ; and (C)  $k_{\text{off}}$ . (closed gray circle)  $-40$  mV; (○)  $+40$  mV. Some of the points are obscured, but the values can be found in Table I.

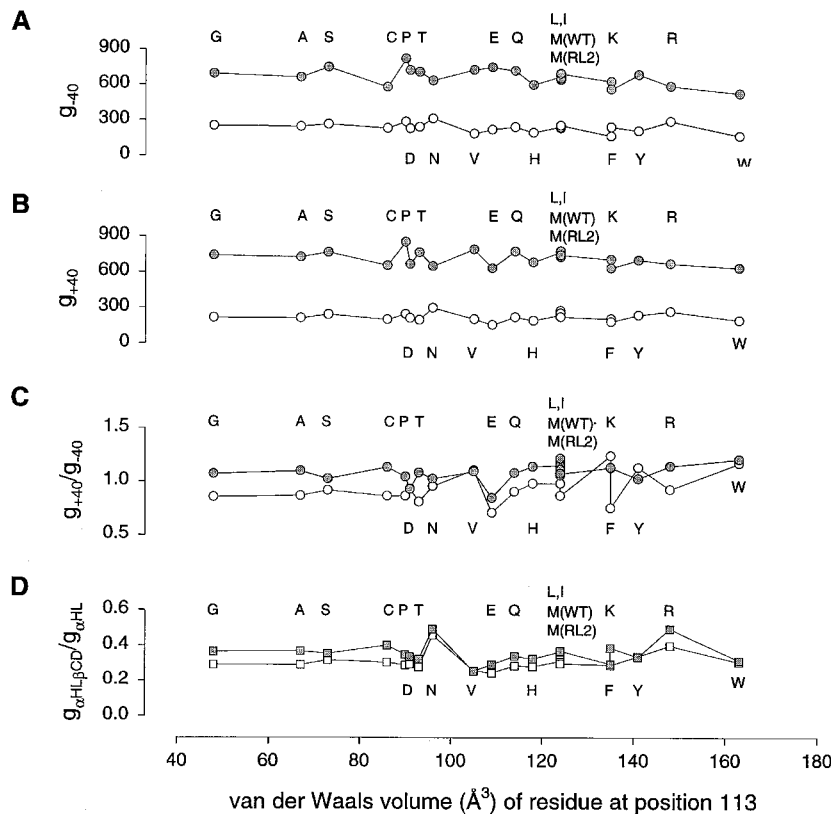


FIGURE 5. Plots of single-channel conductance values for the Met-113 mutants with and without  $\beta$ CD bound versus the van der Waals volume of the residue at position 113. (A) Conductance values at  $-40$  mV: (closed gray circle),  $\alpha$ HL conductance; (O)  $\alpha$ HL $\cdot\beta$ CD conductance. (B) Conductance values at  $+40$  mV. (C) Ratio of conductance at  $+40$  mV to conductance at  $-40$  mV ( $g_{+40}/g_{-40}$ ). (D) Conductance of the pore with  $\beta$ CD bound divided by the conductance of the pore itself. (closed gray square),  $-40$  mV; (□)  $+40$  mV.

( $g_{+40 \text{ mV}}/g_{-40 \text{ mV}}$ ) also appear to be independent of the van der Waals volume (Fig. 5 C). The extent of block by  $\beta$ CD (Fig. 5 A, B, and D, and Table I) and the rectification ratios with  $\beta$ CD bound (Fig. 5 C) are also uncorrelated with van der Waals volume. There is also no correlation between the examined electrical properties and whether a mutant is a member of class 1 or class 2.

#### *The Interaction of $\beta$ CD with Pores Mutated at Position 113 Is Independent of Various Other Mutations in the $\beta$ Barrel*

To check the effect of additional mutations in the  $\beta$  barrel on the affinity of  $\beta$ CD, five additional mutants were tested: M113N(WT), N139Q(WT), M113N/N139Q(WT), L135N(RL2), and M113N/L135N(RL2) (Fig. 6). As noted earlier, mutants with the RL2 background contain the following additional mutations over WT- $\alpha$ HL: Lys-8  $\rightarrow$  Ala, Val-124  $\rightarrow$  Leu, Gly-130  $\rightarrow$  Ser, Asn-139  $\rightarrow$  Gln, and Ile-142  $\rightarrow$  Leu.

WT- $\alpha$ HL, N139Q(WT), and L135N(RL2) share the residue Met-113, and all three mutants bind  $\beta$ CD weakly: WT- $\alpha$ HL,  $K_d = 3.4 \times 10^{-3}$  M; N139Q(WT),  $K_d = 2.9 \times 10^{-3}$  M; and L135N(RL2),  $K_d = 3.9 \times 10^{-3}$  M. By contrast, M113N(WT), M113N/N139Q(WT) (also named  $P_{NQ}$ ; Gu et al., 2001) and M113N/L135N(RL2) share Asn at position 113, and all three bind  $\beta$ CD strongly: M113N(WT),  $K_d = 2.1 \times 10^{-7}$  M; M113N/N139Q(WT),  $K_d = 3.1 \times 10^{-7}$  M; and M113N/

L135N(RL2),  $K_d = 9.3 \times 10^{-7}$  M. Therefore, the affinity of the  $\alpha$ HL pore for  $\beta$ CD is dependent on the residue at position 113, but independent of other sites, notably positions 135 and 139, at least for the substitutions examined here. Residues 135 and 139 project into the lumen of the pore (Fig. 1) and we have previously shown that the mutation N139Q forms a binding site for a different cyclodextrin, hepta-6-sulfato- $\beta$ CD, but not for  $\beta$ CD (Gu et al., 2001).

A similar pattern was observed when the extent of current block by  $\beta$ CD was examined for the same mutants.  $\beta$ CD reduced the conductance of WT- $\alpha$ HL from 651 to 240 pA (37% residual current), N139Q(WT) from 635 to 253 pA (40%), and L135N(RL2) from 640 to 243 pA (38%). In contrast, the residual conductance values for the pores containing the M113N mutation were as follows: M113N(WT) from 623 to 283 pA with  $\beta$ CD (46%); M113N/N139Q(WT) from 668 to 285 pA (43%); and M113N/L135N(RL2) from 645 to 283 pA (45%). Therefore, the extent of current block by  $\beta$ CD is more dependent on the amino acid at position 113 than those at positions 135 and 139.

#### *The Voltage Dependence of $\beta$ CD Binding Is Correlated with the Charge Selectivity of the Mutant Pores*

The affinity of each mutant  $\alpha$ HL pore for  $\beta$ CD is voltage dependent.  $\beta$ CD binds to some mutants more



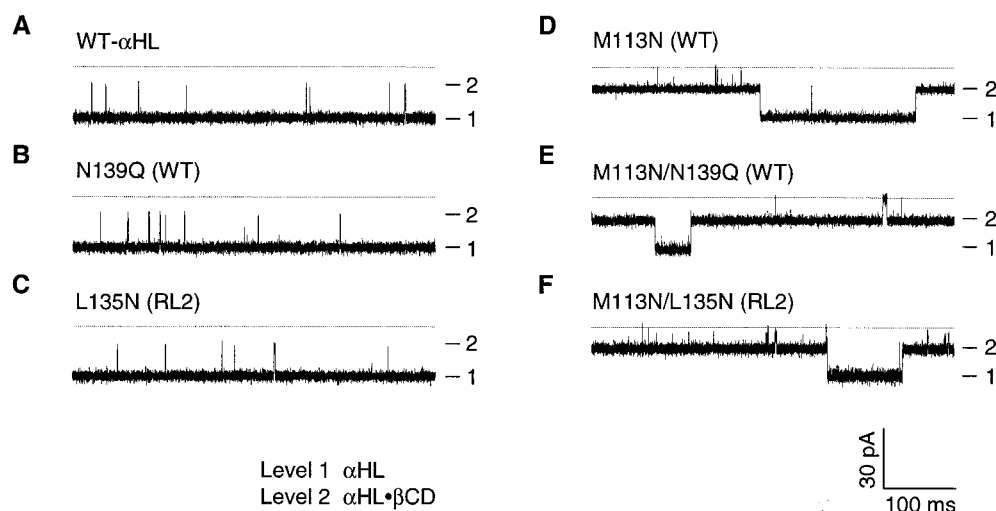


FIGURE 6. Representative current traces from single  $\alpha$ HL pores showing blockades by  $\beta$ CD. All traces were recorded under symmetrical conditions in buffer containing 1 M NaCl, 10 mM sodium phosphate, pH 7.5.  $\beta$ CD (40  $\mu$ M) was present on the trans side of the membrane. (A) WT- $\alpha$ HL, (B) N139Q(WT), (C) L135N(RL2), (D) M113N(WT), (E) M113N/N139Q(WT), and (F) M113N/L135N(RL2). The broken line indicates zero current.

weakly at positive transmembrane potentials than at negative potentials, but binds to others with the opposite dependence on potential (Fig. 2 and Table I). For example,  $\beta$ CD binds to M113E more strongly at +40 mV ( $K_d = 3.3 \pm 0.5 \times 10^{-3}$  M) than at -40 mV ( $K_d = 1.2 \pm 0.2 \times 10^{-2}$  M), whereas  $\beta$ CD binds to M113R more weakly at +40 mV ( $K_d = 1.3 \pm 0.1 \times 10^{-2}$  M) than at -40 mV ( $K_d = 1.6 \pm 0.1 \times 10^{-3}$  M).

These data were quantified by using  $\beta_{1/K} = \log(K_{d-40}/K_{d+40})$ .  $\beta_{1/K} > 0$  reflects a stronger affinity for  $\beta$ CD at +40 mV than at -40 mV, and  $\beta_{1/K} < 0$ , reflects the opposite. Similarly,  $\alpha = \log(P_{K^+}/P_{Cl^-})$  was used as a measure of the charge selectivity of each mutant. Where  $\alpha > 0$ , a pore is cation selective, and where  $\alpha < 0$ , a pore is anion selective (Table II). When  $\alpha$  and  $\beta_{1/K}$  were displayed on a scatter plot, they were seen to be

correlated (Fig. 7 A). This means that  $\beta$ CD binds to a cation-selective pore more strongly at positive potentials than at negative potentials, whereas  $\beta$ CD binds to an anion-selective pore more strongly at negative potentials than at positive. Although the effect of mutagenesis on the affinity for  $\beta$ CD was largely reflected in  $k_{off}$ , the smaller effect of voltage was manifested in both  $k_{on}$  and  $k_{off}$  (Fig. 7, B and C).

## DISCUSSION

### *$\beta$ CD Binds at a Single Site Near Residue 113 in the $\alpha$ HL Pore*

Previous work demonstrated that  $\beta$ CD binds within the lumen of the  $\alpha$ HL pore (Gu et al., 1999), where it reduces the single-channel conductance (Gu et al., 1999;

TABLE II  
Charge Selectivity of Pores and Voltage Dependence of Affinity for  $\beta$ CD

$\alpha$ HL pore	Charge selectivity <sup>a</sup>			Voltage dependence of affinity for $\beta$ CD	
	$V_r$ mV	$P_{K^+}/P_{Cl^-}$	$\alpha$	$(1/K_{d+40})/(1/K_{d-40})$	$\beta$
WT- $\alpha$ HL (pH 5.0)	-15.8	0.34	-0.45	0.25	-0.60
M113R	-13.7	0.39	-0.41	0.11	-0.96
M113K	-11.6	0.46	-0.34	0.16	-0.80
E111N	-7.2	0.63	-0.20	0.13	-0.89
WT- $\alpha$ HL (pH 7.5)	-3.7	0.79	-0.10	0.53	-0.28
M113T	+0.4	1.03	0.01	1.5	0.18
M113V	+1.7	1.12	0.05	2.2	0.34
E111N/K147N	+2.7	1.20	0.08	1.3	0.10
M113P	+3.3	1.24	0.09	2.6	0.42
K147N	+11.5	2.19	0.34	2.9	0.46
WT- $\alpha$ HL (pH 11.0)	+11.9	2.20	0.34	6.6	0.82
M113D	+12.4	2.34	0.37	1.5	0.18
M113E	+13.5	2.54	0.40	3.6	0.56

<sup>a</sup>The reversal potential ( $V_r$ ) was determined using 1,000 mM KCl (cis) and 200 mM KCl (trans). The charge selectivity ( $P_{K^+}/P_{Cl^-}$ ) was calculated by using the GHK equation as described previously (Gu et al., 2000).  $\alpha = \log(P_{K^+}/P_{Cl^-})$ ,  $\beta = \log(K_{d-40}/K_{d+40})$ .

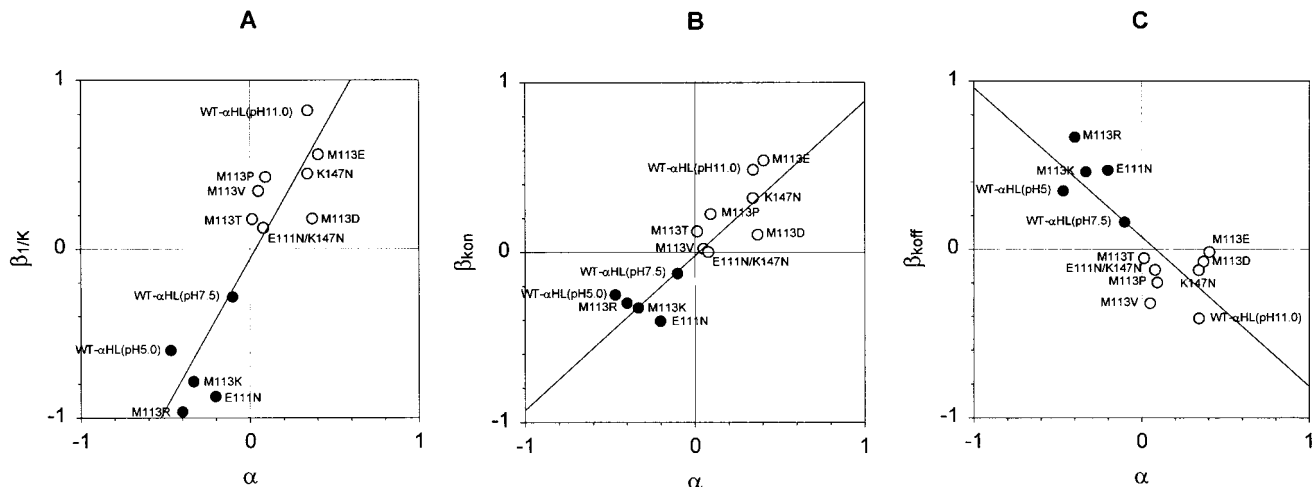


FIGURE 7. Relationships of the voltage dependence of kinetic constants for the interaction of  $\alpha$ HL and  $\beta$ CD, and the charge selectivity of the pore. (A) Plot of  $\beta_{1/K}$  versus  $\alpha$ , where  $\beta_{1/K} = \log[(1/K_{d+40})/(1/K_{d-40})] = \log(K_{d-40}/K_{d+40})$  for the mutants in Table II.  $\beta_{1/K} > 0$  reflects a stronger affinity at +40 mV than -40 mV, and  $\beta_{1/K} < 0$ , the opposite.  $\alpha = \log(P_{K^+}/P_{Cl^-})$ , a measure of the charge selectivity of each mutant. Where  $\alpha > 0$ , a pore is cation selective, and where  $\alpha < 0$ , a pore is anion selective. A line was fitted to the data by linear regression. The correlation coefficient is  $R^2 = 0.80$ . (B) Plot of  $\beta_{kon}$  versus  $\alpha$ , where  $\beta_{kon} = \log(k_{on+40}/k_{on-40})$ .  $R^2 = 0.79$ . (C) Plot of  $\beta_{koff}$  versus  $\alpha$ , where  $\beta_{koff} = \log(k_{off+40}/k_{off-40})$ .  $R^2 = 0.77$ .

Gu and Bayley, 2000), alters ion selectivity (Gu et al., 2000) and acts as a blocker site for various small organic molecules (Gu et al., 1999, 2001). Mutagenesis experiments implied that  $\beta$ CD binds in the vicinity of residue 113. The seven Met-113 side chains in the WT pore project into the lumen of the transmembrane  $\beta$  barrel near its cis end (Fig. 1 A). When the Met-113 residues are replaced with Asn, in M113N,  $\beta$ CD binds  $4.4 \times 10^4$  times more tightly (Gu et al., 1999, 2001).

The present work, in which all possible natural amino acid substitutions at position 113 were examined and six were found to bind  $\beta$ CD tightly (Table I and Fig. 4), supports the idea that  $\beta$ CD binds at or near residue 113. The variation in the affinity of  $\beta$ CD ( $K_d$ ) for the mutants at 113 spans a range of about five orders of magnitude, far greater than the roughly two orders observed for  $\beta$ CD and WT- $\alpha$ HL when pH and transmembrane potential were varied (Gu and Bayley, 2000). Further, mutations at positions in the  $\beta$  barrel other than 113, notably 135 and 139, have little effect on the interaction with  $\beta$ CD. WT- $\alpha$ HL (Leu-135, Asn-139), N139Q(WT) (Leu-135, Gln-139), and L135N(RL2) (Asn-135, Gln-139) all share Met-113 and all bind  $\beta$ CD weakly. M113N(WT), M113N/N139Q(WT), and M113N/L135N(RL2) all share Asn-113 and all bind  $\beta$ CD strongly. Similarly, the three pores with Met-113, show conductance blockades by  $\beta$ CD in the range of 37–40%, whereas the three pores with Asn-113 have blockades in the range of 43–46%.

We cannot rule out the possibility that  $\beta$ CD binds at a site removed from position 113 and that mutations at 113 induce conformational changes that affect the binding site. Indeed, the existence of two distinct

classes of binding mutants (see next section) is suggestive of low and high affinity states. However, because a second cyclodextrin-binding site can be engineered nearer the trans entrance of the  $\beta$  barrel (Gu et al., 2001) and because  $\beta$ CD cannot bind from the cis side, there are few options for the location of the site under consideration here other than near, but not necessarily in contact with, residue 113. Obviously, additional support for the location of the  $\beta$ CD site through structural studies would be most welcome.

The exact location aside, several arguments suggest that  $\beta$ CD binds at a single site, rather than multiple sites, within the lumen of each  $\alpha$ HL mutant. First, for each mutant, there is only one major conductance state that can be assigned to  $\alpha$ HL $\cdot\beta$ CD. Second, there is no additional noise associated with the  $\alpha$ HL $\cdot\beta$ CD state, suggesting that the rather rigid  $\beta$ CD molecule is firmly held at the binding site (Fig. 2). Third, for each mutant, whether binding is strong or weak, the dwell time histograms for  $\tau_{off}$  and  $\tau_{on}$  can be fitted to single exponentials (*Supplemental Material*), which is consistent with simple bimolecular kinetics for the interaction of  $\beta$ CD with  $\alpha$ HL.

#### The $K_d$ Values for $\beta$ CD and Met-113 Replacement Mutants Fall into Two Major Classes

The  $K_d$  values for  $\beta$ CD and the Met-113 mutants of  $\alpha$ HL fall into two classes: class 1, tight binding mutants, mean  $K_d = 6.8 \times 10^{-7}$  M; and class 2, weak binding mutants, mean  $K_d = 4.1 \times 10^{-3}$  M. The major determinant of whether a mutant falls into class 1 or class 2 is the dissociation rate constant ( $k_{off}$ ), values of which fall into the same two classes (Fig. 4). The association rate constant ( $k_{on}$ ) is hardly changed by mutagenesis (Fig. 4),

suggesting that it may reflect transfer of  $\beta$ CD through the entrance to the pore, which would be expected to be little affected by mutations at position 113. The nature of the mutations that cause tight binding do not fall into an easily recognizable group. However, patterns do appear when the data are examined in detail. For example, whereas M113N and M113D fall into class 1, mutants with homologous substitutions, M113Q and M113E, are in class 2. The one mutant in class 1 with a nonaromatic hydrophobic side chain, M113V, is also the smallest of its kind. Mutants with aromatic substitutions, except the bulky tryptophan, are tight binding. As mentioned above, the existence of two distinct classes of binding mutants is suggestive of low and high affinity states of  $\alpha$ HL for  $\beta$ CD. However, this would require a conformational change that is not readily detected in measurements of primary electrical properties, such as single-channel conductance (Fig. 5).

*The Interaction of  $\beta$ CD with  $\alpha$ HL Is Voltage Dependent and Correlated with the Charge Selectivity of the Mutant Pores*

The interaction of  $\beta$ CD with the WT- $\alpha$ HL pore is voltage- and pH-dependent (Gu and Bayley, 2000). At low pH values,  $\beta$ CD (trans) binds more tightly at negative potentials; at high pH values,  $\beta$ CD binds more tightly at positive potentials. Because  $\beta$ CD is a neutral molecule, Woodhull's mechanism for the voltage-dependent binding of a charged blocker was ruled out. Further, because the dissociation rate constant of  $\beta$ CD from its binding site varies continuously with voltage, a mechanism involving the voltage-dependent interconversion of two different states was also discounted. Instead, a continuous change in the free energy of  $\alpha$ HL (and/or  $\alpha$ HL• $\beta$ CD) as a function of the membrane potential was postulated (Gu and Bayley, 2000). Voltage-dependent block by neutral molecules has been observed in other systems, but it has not been investigated in detail (Bezrukov et al., 2000).

Like the manipulation of pH, mutagenesis provides another way to change the charge distribution in a protein. In this work, we found that negative substituents at position 113 favor the binding of  $\beta$ CD at positive potentials, whereas positive substituents favor binding at negative potentials (Table II). It is possible that the charge status of residue 113 can determine the affinity of the pore for  $\beta$ CD in the same way that unidentified charged groups do in WT- $\alpha$ HL (Gu and Bayley, 2000). Therefore, an effect of membrane potential on the structure of the pore, as proposed for the pH-dependent properties of WT- $\alpha$ HL (Gu and Bayley, 2000), is also a reasonable explanation for the effects of mutagenesis.

However, the mutagenesis experiments prompt a second possible explanation. The affinity of  $\beta$ CD is correlated with the charge selectivity of the pore, which in  $\alpha$ HL is modulated in a predictable manner by the

charge at position 113 (Table II). For example, M113K with seven more positive charges than WT- $\alpha$ HL, is more anion selective than WT- $\alpha$ HL, whereas M113E with seven additional negative charges is cation selective. The anion-selective mutants bind  $\beta$ CD, applied from the trans chamber, more tightly at positive applied potentials, whereas the cation-selective mutants bind  $\beta$ CD more tightly at negative potentials (Fig. 7). Just as in the case of pH (Gu and Bayley, 2000), both  $k_{\text{on}}$  and  $k_{\text{off}}$  are affected (Fig. 7). Consistent with these data, pH also alters the charge selectivity of the  $\alpha$ HL pore (Table II; Krasilnikov et al., 1997). For example,  $\alpha$ HL is weakly anion selective at pH 7.5 ( $P_{\text{K}^+}/P_{\text{Cl}^-} = 0.77$ ), more anion selective at pH 5.0 ( $P_{\text{K}^+}/P_{\text{Cl}^-} = 0.34$ ), and cation selective at pH 11.0 ( $P_{\text{K}^+}/P_{\text{Cl}^-} = 2.2$ ). Again, the voltage dependence of the affinity for  $\beta$ CD is correlated with charge selectivity (Table II).

These results show that  $\beta$ CD binding is favored when association occurs in the direction of the net movement of ions and dissociation occurs against the net ion flow. For example, in the case of M113E, a cation-selective channel, net ion flow is from trans to cis in a positive-applied potential, and  $\beta$ CD binding from the trans side of the membrane is enhanced under these conditions. Thus, the second possible explanation is that the  $\beta$ CD molecules move into the pore carried by water flow induced by ion movement—an electroosmotic effect (Katchalsky and Curran, 1965). Because the main barrier to reaching the binding site is entry into the  $\beta$  barrel, it is likely that the water flow would cause accumulation or depletion of the  $\beta$ CD at the trans entrance compared with the bulk concentration and, hence, promote or impede binding.

Water flow caused by electroosmosis is considerable. Applying various simplifications, water flow in a charge-selective pore is given by (Katchalsky and Curran, 1965):

$$J = \frac{RT}{D} \frac{r^2}{8\eta F} I,$$

where D is the diffusion coefficient of the mobile ion; r is the radius of pore;  $\eta$  is the viscosity of water; I is the current flow through pore; and R, T, and F have their usual meanings. At a current of 20 pA,  $J \sim 10^9 \text{ s}^{-1}$ . Because 20 pA in a charge-selective pore corresponds to the transport of  $1.25 \times 10^8$  ions  $\text{s}^{-1}$ , approximately eight water molecules are transported for each ion that moves through the pore. This is in keeping with experimental findings. For example, in the case of a single cation-selective nafion-filled pore in mica of  $\sim 50 \mu\text{m}$  in radius,  $\sim 10$  water molecules flow per  $\text{Na}^+$  ion transported (Bath et al., 1998, 2000). In the same experiments, neutral organic molecules were shown to move with the water. For example, in the case of 0.2 M hydroquinone, about one molecule was transported for every 1,000 water molecules. For a 40- $\mu\text{M}$  solute, one mole-

cule would move with every  $5 \times 10^6$  waters. Therefore, if the water flow were  $\sim 10^9 \text{ s}^{-1}$  (see above), this would result in an appreciable concentration of  $\beta$ CD at the trans entrance.

To further examine the likelihood of an electroosmotic effect, the mutant E111N/K147N was studied. E111N/K147N is a weakly cation-selective pore ( $P_{K^+}/P_{Cl^-} = 1.20$ ). As expected,  $\beta$ CD applied from the trans chamber binds to E111N/K147N more strongly at positive than at negative potentials ( $\beta_{1/K} = 0.10$ ). In most cases,  $\beta$ CD binds to  $\alpha$ HL pores only from the trans side of the bilayer. However, in the case of E111N/K147N,  $\beta$ CD can also bind from the cis side and in this case, the voltage dependency was reversed ( $\beta_{1/K} = -0.15$ ). Although this result is available only for a weakly ion-selective pore, it is in keeping with an electroosmotic mechanism, as opposed to an effect of voltage on the protein. The effects of voltage are also independent of whether a mutant is in class 1 or class 2, and this also favors an electroosmotic mechanism. Otherwise, the effects of voltage would have to be similar on both the conformation of class 1 and that of class 2.

In conclusion, we have shown that long residence times for the noncovalent molecular adapter  $\beta$ CD within the lumen of the  $\alpha$ HL pore can be achieved at neutral pH by mutagenesis at position 113. For example, the mean residence time ( $\tau_{\text{off}}$ ) for  $\beta$ CD bound to M113N at  $-40 \text{ mV}$  is 27 s. In addition to the greatly enhanced affinity of the class 1 mutants, which most likely arises from alterations of the binding site for  $\beta$ CD in the vicinity of residue 113, the binding can be further enhanced by a voltage-dependent mechanism that may originate in an electroosmotic effect. The latter explanation requires verification through more detailed study. The improved residence times for  $\beta$ CD will contribute to our ability to build nanostructures from the  $\alpha$ HL pore (Gu et al., 2001) and to use it as a component of stochastic sensors (Bayley and Cremer, 2001).

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