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On the Origin of Ion Selectivity in the Cys-Loop Receptor Family

Steven M. Sine and Hai-Long Wang

Receptor Biology Laboratory, Departments of Physiology and Biomedical Engineering and Neurology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

Scott Hansen and Palmer Taylor

Department of Pharmacology, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA 92093, USA

Abstract

Agonist binding to Cys-loop receptors promotes a large transmembrane ion flux of several million cations or anions per second. To investigate structural bases for the rapid and charge-selective flux, we used all atom molecular dynamics (MD) simulations, X-ray crystallography, and single channel recording. MD simulations of the muscle nicotinic receptor, imbedded in a lipid bilayer with an applied transmembrane potential, reveal single cation translocation events during transient periods of channel hydration. During the simulation trajectory, cations paused for prolonged periods near several rings of anionic residues projecting from the lumen of the extracellular domain of the receptor, but subsequently the cation moved rapidly through the hydrophobic transmembrane region as the constituent alpha-helices exhibited back and forth rocking motions. Cococrystallization of acetylcholine binding protein with sulfate ions revealed coordination of five sulfates with residues from one of these charged rings; in cation-selective Cys-loop receptors this ring contains negatively charged residues, whereas in anion-selective receptors it contains positively charged residues. In the muscle nicotinic receptor, charge reversal of residues of this ring decreases unitary conductance by up to 80%. Thus in Cys-loop receptors, a series of charged rings along the ion translocation pathway concentrates hydrated ions relative to bulk solution, giving rise to charge selectivity, and then subtle motions of the hydrophobic transmembrane, coupled with transient periods of water filling, enable rapid ion flux.

Keywords

Cys-loop receptor family; Ion conductance and selectivity; Single channel recording; Acetylcholine binding protein; Molecular dynamics simulation

Ion channel proteins circumvent the enormous energetic barrier to ion transport imposed by the cell membrane, maintain cellular homeostasis, and mediate cell–cell communication. When an ion channel activates, permeant ions flow passively down their electrochemical gradients, changing the membrane potential and enabling communication between extra- and intracellular environments. The selectivity of ion translocation depends on the type of channel, and can be non-selective, charge-selective, or ion-selective, suggesting that a diversity of mechanisms underlie ion translocation. In recent years, our understanding of ion translocation has advanced greatly owing to the determination of atomic structures of ion

channels combined with established electrophysiological, mutational, and computational approaches.

Our present understanding of ion translocation comes from studies of voltage-gated ion channels where ions are first dehydrated and then coordinated with partial charges of carbonyl groups of the protein backbone as they travel single file through the channel (Doyle et al. 1998; Dutzler et al. 2002; Zhou et al. 2001). However, in Cys-loop receptors, functional studies suggest a fundamentally different mechanism of ion translocation. First, in all Cys-loop receptors, hydrophobic α -helices line the pore, suggesting water remains bound to the ions as they pass through. Second, in multiple locations along the translocation pathway, ions are coordinated by charged amino acid side chains. Third, in Cys-loop receptors, the diameter of the channel is larger than in voltage-gated ion channels.

The AChR from skeletal muscle is a prototypical member of the Cys-loop family of receptor-coupled ion channels. It is a hetero-pentamer of 250 kD, and contains an intrinsic channel that opens in response to nerve-released ACh to allow inward flow of cations that depolarizes the cell. The AChR channel selects for cations, mainly according to size, and is formed by α -helices from the second transmembrane domains (TMD2) of each of the five subunits. Selectivity for cations is achieved by anionic residues located on either side of TMD2 (Imoto et al. 1988), as well as in fenestrated structures in the cytoplasmic domain (Kelley et al. 2003), which stabilize cations and concentrate them relative to bulk solution. Hydrophobic residues extend from TMD2 and line the narrow region of the AChR channel (Miyazawa et al. 2003), providing little stabilization and thus minimally slowing passage of the hydrated ion. Cryo-electron microscopy applied to the AChR from Torpedo provided structural information at a resolution of 4 Å (Unwin 2005; Miyazawa et al. 2003), revealing the locations of α -carbon atoms of the protein main chain and large side chains.

Here, we summarize work described in two recent papers from our laboratories (Wang et al. 2008; Hansen et al. 2008). As described in the first paper, we generate a homology model of the AChR from the human adult motor endplate, embed it in explicit lipids, and solvate it with water and ions. We apply a transmembrane electric field to mimic the cell membrane potential, and perform all atom molecular dynamics (MD) simulations. Our results reveal translocation of single cations, and show that in the course of translocation, individual ions are transiently stabilized by rings of negatively charged residues along the extracellular lumen prior to reaching the hydrophobic transmembrane region (Wang et al. 2008). As described in the second paper, using X-ray crystallography, sequence analysis, and single channel recording, we show that one of these rings of stabilizing residues is a novel determinant of ionic conductance (Hansen et al. 2008). Co-crystallization of acetylcholine binding protein (AChBP) with sulfate ions reveals coordination of sulfates by cationic lysine residues within the extracellular lumen. Analysis of multiple sequence alignments reveals that residues equivalent to the ring of lysines are negatively charged in cation-selective Cys-loop receptors, but are positively charged in anion-selective receptors. Charge-reversal of residues at homologous positions in the cation-selective muscle AChR reduces the single channel current amplitude up to 80%.

For MD simulation, we generated a macromolecular complex composed of one copy of the AChR protein, membrane lipids, water, Na^+ , and Cl^- ions. We then subjected the complex to a 10-ns equilibration step, applied a voltage bias equivalent to a physiological membrane potential of -100 mV, and generated a further MD simulation of 16 ns (Wang et al. 2008). The simulation reveals the trajectory of a single cation along an axis through the center of the channel (Fig. 1). The cation exhibits step-wise position changes along the central axis, with dwells at each position varying in duration. In the extracellular region bordering TMD2, dwells at each step are prolonged, whereas dwells within TMD2 are transient. The

position of the prolonged dwells immediately apical to TMD2 (upper gray arrow) corresponds to the “outer” ring of negatively charged residues previously shown to affect unitary conductance of the channel (Imoto et al. 1988). Further in the apical direction, two more positions of stability are detected (black arrows), corresponding to novel rings of polar or negatively charged residues within the lumen of the extracellular domain. Within TMD2, several positions are occupied transiently, corresponding to rings of hydrophobic side chains that line the channel and form the barrier to ion transport.

Color-encoded changes in the effective pore radius along TMD2 are superimposed upon the time course of cation transport (Fig. 1). Four horizontal stripes enriched in yellow and red colors indicate narrow regions formed by rings of hydrophobic side chains at the standard consensus positions 1', 5', 9', and 13' of TMD2 (Miller and Hughes 1992). The pore radius corresponding to each of these rings oscillates during the simulation trajectory, but the radius at position 9' shows much smaller oscillations. Until around 8 ns, the radius at the upper 13' position is narrow, but just before the cation approaches, it widens and stays wide until the cation passes by. Concurrent with the widening at position 13', the radius at position 1' constricts, even though the cation is far from this position. Next, the cation surpasses the central 9' position with little change in the local pore radius, but it hops back and forth across position 5' until the radius at position 1' widens enough to allow complete translocation of the cation. Simultaneous with widening at position 1', the more extracellular 13' position constricts. Thus, when the cation enters, the extracellular portion of TMD2 widens while the intracellular portion constricts, and when the cation exits the sequence of motions is reversed.

Once the cation reaches TMD2, hydrophobic interactions dominate the transport process. The rings of non-polar side chains lining TMD2 form a narrow constriction analogous to the hydrophobic channel of MscS (Spronk et al. 2006; Sotomayor et al. 2006, 2007) and hydrophobic nanotubes (Hummer et al. 2001; Beckstein et al. 2004). As we observe in the case of AChR (Wang et al. 2008), these systems exhibit increases in hydration with only slight increases in channel radius, and the cycles of water filling and emptying diminish with increasing transmembrane potential, likely through increasing the degree of order of the water molecules. The collective observations suggest that the switch from an inactive to an active channel could occur with increases in channel radius of an Å or less (Cymes et al. 2005). The non-polar side chains along TMD2 provide little stabilization for a hydrated cation, minimally slowing transport, and suggest that a steric interplay between the rings of non-polar side chains and the cation may limit transport in this region. The non-polar side chains also create an environment of low electric permittivity, extending the distance range of inter-cation Coulombic repulsion, perhaps explaining why only a single cation at a time occupies TMD2 (Fig. 1).

Summarizing the results up to this point, simulation of single cation trajectories reveals fundamental features of ion selectivity and protein motions that accompany ion translocation through a hydrophobic channel. Rings of charges are arranged in series along the ion translocation pathway and give rise to charge selectivity. The changes in channel radius reveal a back and forth tilting of TMD2 about an axis centered at the 9' position, and the hydrophobicity of TMD2 minimizes stabilization of the ion, permitting rapid translocation. A back and forth tilting of TMD2 upon channel opening was recently suggested by high resolution structures of the bacterial pentameric channel GLIC crystallized in the open state (Hilf and Dutzler 2009; Bocquet et al. 2009).

The soluble acetylcholine binding protein from mollusks is an established structural and functional surrogate of the N-terminal ligand binding domain of Cys-loop receptors amenable to high-resolution crystallographic studies (Reeves et al. 2002; Hansen et al. 2004,

2005). AChBP from *Aplysia californica* (*Ac_AChBP*) was co-crystallized in the presence of the anions, sulfate and cacodylate. The resulting crystals diffracted to 3.1 Å resolution, and the data were refined to an R/Rfree of 21/25. The structure reveals that within the central vestibule, Arg 97 and Lys 42 occupy a single conformation in each subunit and coordinate one of a total of five sulfate ions per pentamer (Fig. 2). When viewed from an apical direction and perpendicular to the central vestibule, the ring of sulfates is located below the narrowest constriction in the extracellular domain and ~15 Å apical to what would be the outer membrane interface in a full length receptor.

Sequence alignment of Cys-loop receptors from humans shows that residues at positions equivalent to Arg 97 in *Ac_AChBP* are conserved as Asp in cation-selective receptors, whereas they are conserved as Lys or adjacent Lys residues in anion-selective receptors (Fig. 3); Lys 42 is not conserved in the Cys-loop receptor family. In the *Torpedo* AChR, Asp 97 extends from a loop that forms the narrowest region of the central vestibule of the N-terminal ligand binding domain. We reasoned that the position and negative charge of Asp 97 may allow it to filter ions, analogous to the selectivity filters that flank the α -helical transmembrane domain (Imoto et al. 1988) or within the cytoplasmic domain (Kelley et al. 2003).

To determine whether the ring of charged residues in the extracellular vestibule contributes to ion translocation, we examined Asp 97 of the AChR α -subunit and anionic residues at equivalent positions of the β -, δ -, and ϵ -subunits. We reversed the charges of residues in all five subunits, co-expressed the subunits to form heteropentameric receptors and recorded single channel currents elicited by ACh (Fig. 4). Compared to the wild-type receptor, the mutant receptor exhibits decreased unitary current amplitude at each test potential (Fig. 4a, b); a plot of unitary current amplitude against transmembrane potential reveals a straight line, the slope of which yields the unitary conductance and shows a decrease of 70% compared to the wild type AChR (Fig. 4f). The conductance decrease depends non-linearly on the number of charge-reversal mutations in the pentamer, showing no change after mutation of the two α subunits, and only a slight decrease with mutations in three subunits (Fig. 4d, e). However, the net charge of the ring is less important than the side chain substitutions and the locations of the mutant subunits. Introducing four Lys and maintaining one Asp (net charge +3) decreases unitary conductance by 55%, whereas introducing three Lys and two Ala residues (net charge +3) decreases unitary conductance by 80%. The observation that the ring of charge aligned at Asp 97 affects unitary conductance agrees with predictions from our preceding all atom molecular dynamics simulations in which cations paused for extended periods at this location in the course of passing through the channel (Fig. 1; Wang et al. 2008). Thus, positioned close to the point at which permanent ions enter the narrow region of the channel, this vestibular ring of charge acts to select and concentrate cations for translocation.

The extracellular beta sheet filter appears late in prokaryote evolution and is maintained in all eukaryotes (Tasneem et al. 2005). *Ac_AChBP* contains an Arg at position 97 (Fig. 3; Hansen et al. 2005) and anion conducting AChRs are present in *Aplysia* (Kehoe and McIntosh 1998), suggesting *Ac_AChBP* evolved from an anion channel. Recently the first high-resolution structure of a nicotinic receptor homolog, called ELIC, was solved from a bacterial species (Hilf and Dutzler 2008). Asp 86 from ELIC occupies a position near Asp 97 in the AChR, and the diameter defined by α -carbon atoms is 19.1 Å. ELIC may also have a more apical selectivity filter at residue Glu 64 where an alpha helix narrows the extracellular vestibule to a diameter of 20 Å and the aspartate carbonyl side chains tilt inward for hydrogen bonding.

The molecular basis of ion selectivity correlates well with the charge-selective nature of Cys-loop receptors. Filters are located at relatively wide regions of the extracellular domain, where flexible side chains project into the central vestibule. The channel selects for charge but is limited in its ability to select for size or valence. Ions are thought to be hydrated at the level of the filter (Lewis and Stevens 1983), and bound SO_4^{2-} coordinates several waters. Notably, a pentameric ring forms angles of 108° amenable to water and ion coordination. Potentially, these geometrical constraints and the need to translocate hydrated ions may have contributed to the rise of pentameric ion channels with an elongated vestibule for ion entry.

Characterization of ion selectivity filters in the N-terminal domain and a structural description of ion translocation are fundamental to understanding how Cys-loop receptors function. Our computational, structural, and electrophysiological studies combine to show that β -sheet regions of pentameric Cys-loop receptors form a vestibular structure capable of filtering ions during the sub-millisecond times that the channel is open. The data demonstrate that the structure of the extracellular domain contributes to an integrated function extending beyond that of ligand binding and its linkage to allosteric gating. The location of the filter is also significant because when nicotinic receptor channels open, sodium ions flow from outside the cell to the cytoplasm and are thus first exposed to the most extracellular selectivity filters. As a region of conserved structure and critical sequence positions, the vestibular rings of charge could become targets for developing highly selective non-competitive modulators of ion conductance.

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Figure 1.

Position of a single cation along the channel axis from all atom MD simulation (*black trace*) is superimposed upon a color-encoded plot of the effective pore radius (see *calibration scale*), measured using the program HOLE (Smart et al. 1996). The Z axis position of the lipid membrane spans from -20 to 20 Å. *Gray arrows* indicate previously described extracellular and intracellular rings of charged residues (Imoto et al. 1988), while the *black arrows* indicate two more rings of charge coinciding with prolonged dwell times of the cation. Homology model of the human adult muscle AChR used in the simulations (*right*). Rings of charged residues in the extracellular lumen flanking TMD2 are shown. *Rings and labels in gray* indicate previously described rings of charged residues that affect conductance and selectivity (Imoto et al. 1988), whereas *rings in black* indicate newly identified residues associated with prolonged cation dwell times

Figure 2.

X-ray structure of the ion selectivity filter identified in *Aplysia* AChBP. **a, b** Sulfate bound to *Ac*_AChBP. **a** Side view with one subunit removed. Arg 97 is shown in *blue*; a ring of five sulfates (sulfur *orange*, oxygen *red*) is located in a plane 15 Å above where the membrane would be located in the AChR. **b** View down the fivefold axis. **c** Sulfate coordinated between Arg 97 and Lys 42

	EC Filter	TM Filters	CP Filter
	97	241 gate 262 266	432 436 440
h_α7	NSA ^{ERF}	SG- ^{IKISL} GITVLLSLTVFLLVAIMPATSD	FRQDESEAVCS ^{WK}
h_α2	NNA ^{GEF}	CG- ^{IKITL} CISVLLSLTVFLLIT ^{II} IPSTSL	LRSEDADSSVKE ^{WK}
h_α3	NNAVGD ^F	CG- ^{IKVTL} CISVLLSLTVFLLVIT ^{II} IPSTSL	MKAQNEAKEIQD ^{WK}
h_α4	NNA ^{GDF}	CG- ^{IKITL} CISVLLSLTVFLLIT ^{II} IPSTSL	LKAEDTDFSVKE ^{WK}
h_α5	DNA ^{GRF}	EG- ^{IKICL} CTSVLVS ^{LT} VFLLVIE ^{II} IPSSSK	IMKENDVREVE ^{WK}
h_α6	NNAVGD ^F	CG- ^{IKVTL} CISVLLSLTVFLLVIT ^{II} IPSTSL	MKSHNETKEVE ^{WK}
h_α9	NKA ^{DES}	SG- ^{IKVSL} GVITILLAMTVFQLMVAIMPASE	LKDHKATSSKGS ^{WK}
h_α10	NKA ^{AQP}	SG- ^{IKVSL} GVITVLLALT ^{VF} QLLA SM PPAE	FRSHRAAQRCH ^{WK}
h_β2	NNA ^{GMY}	CG- ^{IKMTL} CISVLLALT ^{VF} LLISKIVPPTSL	MRSEDDQSVSE ^{WK}
h_β3	ENA ^{GRF}	EG- ^{IKLSL} STSVLVS ^{LT} VFLLVIE ^{II} IPSSSK	VKKEHFISQVQ ^{WK}
h_β4	NNA ^{GTY}	CG- ^{IKMTL} CISVLLALT ^{VF} LLISKIVPPTSL	MKNDDQSVVE ^{WK}
h_α1	NNA ^{GDF}	SG- ^{IKMTL} CISVLLSLTVFLLVIT ^{II} IPSTSS	MKSDQESNNAA ^{WK}
h_β1	NNN ^{GNF}	AG- ^{IKMGL} SIFALL ^{TL} TVFLLLA ^{KVP} TSL	LQEQEDHDALKE ^{WK}
h_δ	NNN ^{GSE}	SG- ^{IKTSV} AI ^{SV} LLAQSVFLLISKRLPATSM	MRDQNNYNEEKDSW ^N
h_γ	NNV ^{GVF}	AGGQKCTVA ^{IN} VLLAQTVFLFLVAKKVP ^{TS} Q	RHQQSHFDNGNE ^{WF}
h_ε	NNI ^{GQF}	AGGQKCTV ^{IN} VLLAQTVFLFLIAQKIP ^{TS} L	TRDQATGEVSV ^{WV}
Tca_α	NNA ^{GDF}	SG- ^{IKMTL} CISVLLSLTVFLLVIT ^{II} IPSTSS	MKSDEESSNAAE ^{WK}
Tca_β	NNN ^{GSE}	AG- ^{IKMGL} SISALL ^{AV} TVFLLLA ^{KVP} TSL	LESASEFDDLKK ^{WK}
Tca_δ	NNN ^{GQY}	SG- ^{IKMST} AI ^{SV} LLAQAVFLLLSQRLP ^{TAL}	IKEKNAYDEEVGNW ^N
Tca_γ	NNV ^{GQF}	AGGQKCTLS ^{IV} LLAQTVFLFLIAQKVP ^{TS} L	TKEQNDSSGENENW ^V
5HT3_A	EFV ^{VG}	SG- ^{IKRVS} FKITLL ^{LG} YSVFLIIVS ^{TL} PATAI	LEK ^{DEI} SEVANDWL
5HT3_B	EFV ^{IE}	CR- ^{IKRIV} FKTSVL ^{VG} YTVFRVNMNSQVPRSVG	LQT ^{DDQ} TQQE ^{EW} L
GABA_α1	NGK ^K SVA	SVPA ^{TV} FGVTTVLTMTT ^{LS} ISARNSLP ^{VAY}	EP----- ^K TF
GABA_α2	NGK ^K SVA	SVPA ^{TV} FGVTTVLTMTT ^{LS} ISARNSLP ^{VAY}	EA----- ^K TF
GABA_α3	NGK ^K SVA	SVPA ^{TV} FGVTTVLTMTT ^{LS} ISARNSLP ^{VAY}	KATYVQDSPTET ^{TY}
GABA_α5	NGK ^K SIA	SVPA ^{TV} FGVTTVLTMTT ^{LS} ISARNSLP ^{VAY}	ES----- ^K TY
GABA_β2	NDK ^K SFV	ASAA ^{RV} ALGITTVLTMTT ^{IN} THLETLP ^{IPY}	RAS--- ^Q LKITIPDL
GABA_β3	NDK ^K SFV	ASAA ^{RV} ALGITTVLTMTT ^{IN} THLETLP ^{IPY}	RSS--- ^Q LKIKIPDL
GABA_γ2	NSK ^K ADA	AVPA ^{RT} SLGITTVLTMTT ^{LS} IAKSLP ^{VSY}	AWR----- ^H GIH
GABA_γ3	NSK ^K AEA	ATPA ^{RT} ALGITTVLTMTT ^{LS} IAKSLP ^{VSY}	SWR----- ^K GIH
Gly_α1	NEK ^K AHF	AAPA ^{RV} LGITTVLTMTT ^Q SSGSASLP ^{VSY}	EMR----- ^L FI
Gly_α2	NEK ^K ANF	AAPA ^{RV} LGITTVLTMTT ^Q SSGSASLP ^{VSY}	AIK----- ^K FV
Gly_α3	NEK ^K ANF	AAPA ^{RV} LGITTVLTMTT ^Q SSGSASLP ^{VSY}	EMR----- ^L VFI
Gly_β	NEK ^K ANF	ASAA ^{RV} PLGIFSVLSLASECTTLAAELP ^{VSY}	PAK----- ^P VIP
ELIC	NVVGSP ^Q	SFS ^{RL} QTSFTLMLTVVAY ^{AY} TSNLPRLPY	
AChBP	SST ^{IP} VQ		

Figure 3.

Sequence alignment of ion selectivity filters: *EC* extracellular, *TM* transmembrane, *CP* cytoplasmic. Basic residues presumably involved in anion selectivity are shaded *blue* and acidic residues involved with cation selectivity are shaded *red*. Residues implicated in channel gating are shaded *gray*. Sequences are human except *Torpedo californica* (Tca), *Erwinia chrysanthemi* (ELIC), and *Aplysia californica* (AChBP)

Figure 4.

Electrostatic contribution of D97 to ion conductance. **a, b** and **d, e** single channel currents are shown at a bandwidth of 10 kHz for the indicated wild-type or mutant receptors; channel openings are upward deflections. All-points histograms of current amplitude are shown for each test membrane potential and fitted by the sum of two Gaussian functions. **c** current-voltage relationship for receptors with increasing numbers of Lys mutations per pentamer: *filled circle* wild-type, *unfilled circle* eL9'S, *filled diamond* αβδD97K+ε9'S, *unfilled diamond* αβδεD97K+ε9'S, and *filled square* αδD97K. **f** graph of conductance derived from the slope of the current-voltage relationship in **c**. In **e**, for the receptor with three Lys and two Ala substitutions, the current-voltage relationship yields a unitary conductance of 18 pS