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The Nicotinic Receptor Ligand Binding Domain

Steven M. Sine

Receptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55905

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ABSTRACT: The ligand binding domain (LBD) of the nicotinic acetylcholine receptor has served as a prototype for understanding molecular recognition in the family of neurotransmitter-gated ion channels. During the past fifty years, studies progressed from fundamental electrophysiological analyses of ACh-evoked ion flow, to biochemical purification of the receptor protein, pharmacological measurements of ligand binding, molecular cloning of receptor subunits, site-directed mutagenesis combined with functional analysis and recently, atomic structural determination. The emerging picture of the nicotinic receptor LBD is a specialized pocket of aromatic and hydrophobic residues formed at interfaces between protein subunits that changes conformation to convert agonist binding into gating of an intrinsic ion channel. © 2002 Wiley Periodicals, Inc. J Neurobiol 53: 431–446, 2002

Keywords: **allosteric protein; ligand-gated ion channel; acetylcholine binding protein; homology structural model; agonist-induced conformational change**

OVERVIEW

Acetylcholine (ACh) released from the motor nerve terminal diffuses the short distance across the synaptic cleft where it binds to muscle nicotinic receptors and triggers the all or nothing opening of the receptor ion channel. The synaptic protrusion of the receptor harbors structures specialized for binding ACh, known as ligand binding domains (LBD), as well as structures that transduce binding into gating of the ion channel. The LBD has attracted considerable attention not only because it initiates the biologic response of the receptor, but also because it is a target for drugs used clinically to paralyze voluntary muscle. Both functional and structural experimental approaches have been brought to bear on understanding the nicotinic receptor LBD. Early studies defined the number and stoichiometry of protein subunits in the receptor, as well as the number and location of ACh binding sites within the LBD. Later studies delineated the overall shape and dimensions of the receptor, localized ACh binding sites to interfaces between subunits, identified key residues at the subunit interfaces, revealed the fundamental molecular motion underlying receptor activation and resolved individual ACh binding and channel gating steps. Work in the past year heralds a new era in understanding the nicotinic receptor LBD at the atomic structural level. The structure of an acetylcholine binding protein was described (Brejc et al., 2001), reliable structural models of the receptor based on mutagenesis and homology modeling have emerged (Le Novere et al., 2002; Sine et al., 2002) and motions of β-sheets within receptor α-subunits have been found to accompany activation of the ion channel (Unwin et al., 2002). The emerging tenets established for the muscle nicotinic receptor will aid in understanding ligand recognition and triggering of biologic responses for all members of the nicotinic receptor superfamily.

Concepts and Controversies

In route to present-day understanding of the nicotinic receptor LBD, controversies have been resolved and

Correspondence to: S.M. Sine (sine@mayo.edu).
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key concepts have emerged. A sampling of them are listed as follows. The organization of the four different types of subunits within the muscle nicotinic receptor was only recently settled, as was the handedness of the arrangement of the subunits. Similarly, localization of the ACh binding sites to interfaces between subunits is now certain, but they were long thought to localize entirely within muscle receptor alpha subunits. The ACh binding site is not static in its affinity for ACh but is dynamic, and depends on functional state of the receptor ion channel: open, closed, or desensitized. Whether ACh binding or channel gating limits the rate of receptor activation was debated until single channel kinetic analysis resolved these two processes. Single-channel kinetics also pinpointed the origin of cooperativity in the ACh-induced response, but whether agonist binding itself is cooperative remains unknown. Physiologists long theorized that endplate current decay depends not only on the kinetics of opening and closing of the ion channel, but also on the rate of dissociation of agonist from the binding site. By slowing agonist dissociation and prolonging the endplate decay rate, a disease-causing mutation at the ACh binding site demonstrated the importance of agonist dissociation in governing the time course of the endplate response.

At the outset of this review, it is important to emphasize that the tremendous progress in the nicotinic receptor field owes to the innumerable contributions from a vast number of laboratories. Therefore, the interested reader should consult the many excellent and comprehensive reviews written over the years (Changeux, 1990; Lingle et al., 1992; Karlin and Akabas, 1995; Changeux and Edelstein, 1998; Unwin, 1998). The focus of this review is to provide a historic account of key advances that led to present-day understanding of the structure and function of the nicotinic receptor LBD.

Early Advances (1950s through 1970s)

The advent of microelectrode technology, and later the voltage clamp, led to establishment of fundamental properties of ACh-induced activation of the muscle nicotinic receptor (reviewed by Steinbach and Stevens, 1976). Small depolarizing responses elicited by iontophoretically applied ACh increased with the square of the ACh concentration, indicating activation of the receptor is a positively cooperative process (Katz and Thesleff, 1957). Also, during steady application of ACh, depolarizations elicited by a brief test pulse of ACh declined within several seconds, in a reversible process termed desensitization. Endplate and ACh-evoked currents were established to result from conductance increases in the muscle membrane (Takeuchi and Takeuchi, 1959), and this paved the way for quantitative dose–response measurements using the voltage clamp (Adams, 1975; Dreyer and Peper, 1975; Dionne et al., 1978). Also essential were more recent methods to deliver a known quantity of ACh in a pulse sufficiently brief to prevent desensitization of the response (Brett et al., 1986). Subsequent dose–response measurements confirmed that receptor activation is a positively cooperative process that develops on the submillisecond time scale (Dilger and Brett, 1986). Emerging from the early biophysical studies of receptor function, the following core mechanism was established and still serves today as a starting point for quantitatively describing receptor activation:

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AR + A \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^*$$

(Scheme 1)

where A is agonist, R is the receptor in the resting state, and R^* is the receptor in the open channel state. Scheme 1 provides a useful framework for understanding receptor activation because it provides a formal description of events at the ACh binding site and couples them to activation of the ion channel. This seemingly innocent scheme created several controversies. In particular, which step is rate limiting in receptor activation: ACh binding or channel gating? What is the origin of cooperativity? Is it induced-fit binding of ACh, as implied by sequential ACh binding steps, or is it gating of the channel as implied by the fact that only doubly occupied receptors open? Answers to these questions had to await discovery of the patch clamp and the ensuing development of single-channel kinetic analysis.

Advances in understanding nicotinic receptor structure paralleled the early advances in understanding receptor function (reviewed by Changeux, 1990). Here, Mother Nature helped advance the field in two ways. First, the electric organ from the *Torpedo* ray provided an extremely rich source of cholinergic synapses (Feldberg et al., 1940), including milligram amounts of nicotinic receptors suitable for protein chemical analysis. Second, the snake venom α-toxins (Lee and Chang, 1966) served as high-affinity labels that allowed tracking and quantification of receptor number under a wide variety of experimental conditions. Initial investigations focussed on determining the number, stoichiometry, and types of protein subunits in affinity-purified receptors from *Torpedo* (Ol-

sen et al., 1972). The emerging consensus held that each receptor oligomer contains α-, β-, γ-, and δ-subunits giving rise to a 250-kDa protein complex (Reynolds and Karlin, 1978; Lindstrom et al., 1979). The number of α-bungartoxin binding sites approached 2 per oligomer, and the ratio of ACh to α-toxin sites was close to 1:1 (Neubig and Cohen, 1979). In all studies, the α-subunit was roughly twice as abundant as the other subunits, and was labeled by covalent affinity reagents (Reiter et al., 1972; Weiland et al., 1979); this led to the idea that the receptor pentamer contains two α-subunits, each of which contains one binding site. The idea that the binding sites are located within the α-subunits was later shown to be only half true through work demonstrating intrinsic differences in ligand affinity between the two binding sites of an individual receptor. That the isolated receptor pentamer accounted for all aspects of receptor function was demonstrated by years of work reconstituting the purified receptor in artificial lipid membranes and documenting key aspects of its function (reviewed by Montal, 1987).

The ACh binding sites are expected to localize in the synaptic protrusion of the receptor because the positively charged quaternary ammonium group of ACh prevents it from diffusing across the cell membrane. The ACh binding sites were shown to localize near a readily reducible disulfide bond, because following reduction, bromoacetylcholine could be covalently linked to the receptor and seen to cause perpetual activation (Silman and Karlin, 1969). Subsequent site-directed labeling studies showed that two moles of affinity label coincided with 1 mol of receptor (Weiland and Taylor, 1979).

Measurements of ligand binding to the receptor became possible using radiolabeled α-toxins in competition with unlabeled agonists and antagonists (Webber and Changeux, 1974). These studies quickly demonstrated the dynamic nature of the ligand binding site in the presence of agonist. In particular, affinity for the agonist increased during tens of seconds of agonist exposure to receptor-rich membranes in a process reminiscent of desensitization (Webber et al., 1975; Weiland et al., 1976; Quast et al., 1978). In fact, some 20 years earlier Katz and Thesleff (1957) proposed a cyclic mechanism involving a desensitized state with increased agonist affinity relative to the resting state, and such a mechanism accounted for the observed time and concentration dependencies of the agonist-induced affinity increase (Weiland et al., 1977). Coincidence between the agonist-induced affinity increase and functional desensitization was demonstrated using dual measurements of agonist binding and activation (Sine and Taylor, 1979; Neubig et al., 1982; Heidmann et al., 1983). The emerging picture of the ligand binding site indicated a dynamic structure designed to shuttle among functional states to elicit the biological response following brief exposure to ACh and an attenuated response following prolonged exposure. Teleologic explanations for a dynamic binding site arose again some 10 years later in the context of receptor activation at the single channel level (Jackson, 1989).

Measurements of binding of competitive antagonists such as curare demonstrated site selectivity, or different affinities of the two binding sites in an individual receptor. A variety of antagonists bound to the receptor with Hill coefficients much less than unity (Neubig and Cohen, 1979; Weiland and Taylor, 1979), and this was shown to result from intrinsic differences in the binding sites, as opposed to induced changes in the sites (Sine and Taylor, 1981). Since these early studies, a host of competitive antagonists with site selectivity have been discovered and used to delineate differences between the two binding sites at the level of individual amino acids. These ligands include the cyclic diterpine lophotoxins (Fenical et al., 1981), the short peptides, α-conotoxins (Sine et al., 1995a), and Waglerins (Molles et al., 2002), as well as the larger Naja mossambica mossambica α-neurotoxin (Osaka et al., 1999). Two possible explanations for intrinsic differences between the sites were (1) the two alpha subunits had different amino acid sequences, or (2) different subunit neighbors of the alpha subunits either directly or allosterically caused the sites to be different. These possibilities remained unresolved until cloning of receptor subunits and subsequent establishment of expression systems that allowed subunit omission and mutagenesis experiments.

Early Advances (1980s through 1990s)

The 1980s yielded the greatest advances yet in understanding nicotinic receptor structure and function. These advances are indebted to the powerful insights provided by the patch clamp, discovered by Neher, Sakmann, and colleagues (Hamill et al., 1981), and cloning of receptor subunits by the Numa (Noda et al., 1982) and the Heinemenn and Patrick (Ballivet et al., 1982) laboratories. At first, patch clamp and molecular biology remained within separate research groups, but later individual groups took advantage of the synergy provided by combining the two approaches. A third advance complemented these, namely the use of electron microscopy to generate high-resolution images of the receptor in active and inactive states (Unwin, 1986).

Soon after the patch clamp was introduced and single channel currents could be resolved with unprecedented time resolution, investigators worked in earnest to identify the rate-limiting step in receptor activation. This inevitably required fitting kinetic schemes to single-channel dwell times and subsequent estimation of rate constants governing transitions between closed and open states (Colquhoun and Hawkes, 1981). The early findings showed that the fully occupied receptor switches between closed and open states very rapidly, occurring in tens of microseconds (Colquhoun and Sakmann, 1981), but there was uncertainty in assigning exponential components in the dwell time distributions to particular states in a kinetic scheme (Sine and Steinbach, 1986, 1987). Eventually, by fitting descriptions such as Scheme 1 to dwell times obtained over a wide range of ACh concentrations, a complete set of rate constants governing receptor activation was achieved (Sine et al., 1990; Zhang et al., 1995). The measured rate constants indicated that ACh binds at a rate some 10-fold slower than the rate of diffusion, the rate of ACh dissociation from the *Torpedo* receptor differed markedly at the two binding sites (Sine et al., 1990), and opening of the receptor channel occurred within tens of microseconds (Maconochie et al., 1995). The conclusion about which step was rate limiting was settled: neither step is rate limiting because binding of synaptic concentrations of ACh and channel opening both occur on the tens of microseconds time scale.

The cloning revolution provided the richest information yet on the structure of the nicotinic receptor. That cloning was possible owed to advances in biochemistry that enabled N-terminal microsequencing of the *Torpedo* receptor subunits (Raftery et al., 1980). The resulting partial amino acid sequences yielded two important advances. First, the stoichiometry of receptor subunits was finally confirmed to be $\alpha_2\beta\gamma\delta$. Second, partial amino acid sequences allowed use of degenerate oligonucleotides to probe a *Torpedo* cDNA library in search of open reading frames corresponding to receptor subunits. The results of the cloned receptor subunits emerged in dramatic fashion, first from the Numa laboratory based in Japan (Noda et al., 1982, 1983), and then from the Heinemann and Patrick laboratory at the Salk Institute in the United States (Ballivet et al., 1982; Claudio et al., 1983).

Comparison of the primary sequences of the four muscle nicotinic receptor subunits clearly showed homology, indicating that they evolved from a common ancestor. Already researchers began to think of a receptor superfamily, but this would not be clear until cloning of subunits from other members of the nicotinic receptor superfamily (reviewed by Heinemann et al., 1991; Patrick et al., 1993). Cloning also led to models of the folding of each subunit in the cell membrane based on hydropathy analysis of the sequences (Claudio et al., 1983). The emerging, and now accepted, model of the folded subunit comprises a large extracellular domain including about half of the primary sequence, four transmembrane domains, and a large cytoplasmic domain between transmembrane domains 3 and 4.

Cloning also had other important down stream consequences. It enabled site-directed mutagenesis aimed at identifying key structural counterparts of function, and spurred establishment of expression systems essential for monitoring function. It also allowed identification of sites of covalent attachment of site-directed labels targeted to the ACh binding site (Kao and Karlin, 1986; Abramson et al., 1989; Middleton and Cohen, 1991). Cloning of receptors from a variety of species allowed identification of conserved residues as prime targets for mutagenesis, as well as providing the basis for modeling protein secondary structure (Le Novere et al., 1999). Finally, cloning allowed identification of disease-causing mutations from patients with congenital myasthenic syndromes (Ohno et al., 1995; reviewed by Engel et al., 1998).

Direct structural information emerged with discovery of conditions for forming tubular two-dimensional crystals of *Torpedo* receptor coupled with electron microscopy. The initial images resolved the receptor to approximately 18 Å, revealing its cylindrical shape and dimensions (Unwin et al, 1988; Mitra et al., 1989). The majority of the structure was seen to protrude into the synapse to form the LBD, which contained a central vestibule through which cations flow in route to the transmembrane channel (Fig. 1). Individual subunits appeared as barrel staves surrounding the vestubule, and differences in electron densities within each subunit could be discerned. Assignment of subunit location was tentatively achieved using α-bungarotoxin and subunit-specific antibodies resolvable as new densities in the electron density maps (Zingsheim et al., 1982; Kubalek et al., 1987). From these studies, the two α-subunits were shown to be separated by another subunit, apparently the β-subunit, and the δ-subunit, which covalently joined pairs of receptors, was shown not to be the subunit between the two α-subunits (reviewed by Karlin, 1987). This assignment of subunit organization created considerable controversy because subsequent subunit omission experiments, site-directed labeling, and mutagenesis experiments indicated that the γ- and not the β-subunit lodges between the two α-subunits. Because the two ligand binding sites are formed at α–γ- and α–δ-subunit interfaces, the structural data could

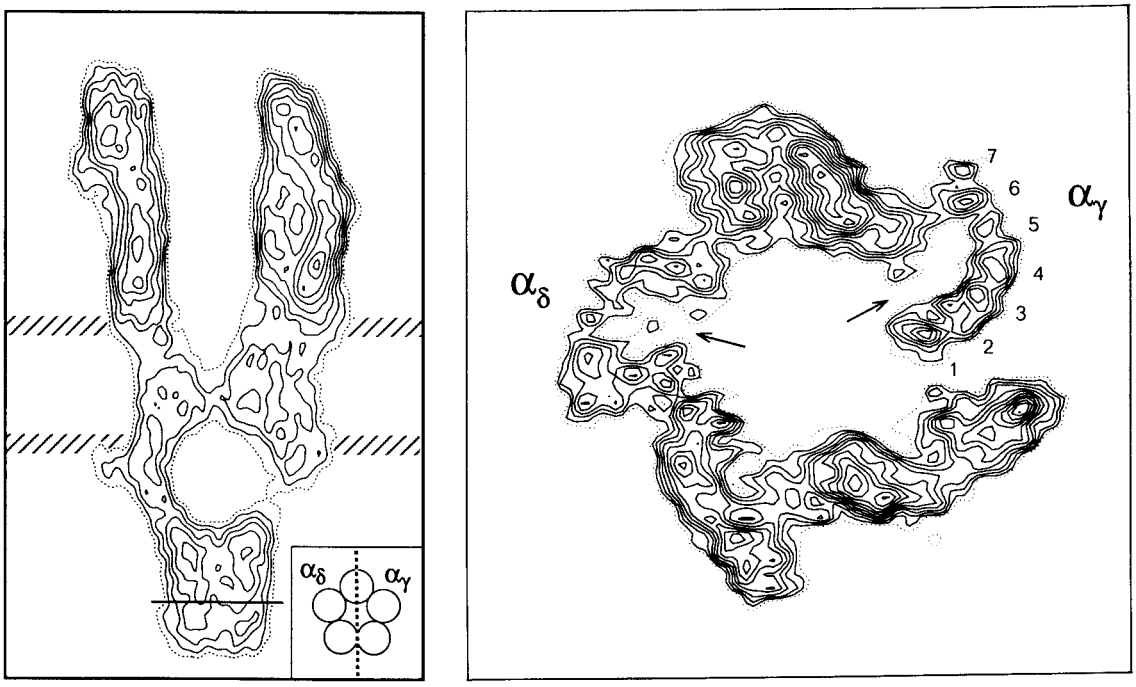


Figure 1 Electron microscopic images of the AChR from tubular crystals of *Torpedo* postsynaptic membranes at 4.6 Å resolution (generously provided by Nigel Unwin; see Miyazawa et al., 1999). Left panel shows a cross-section through the receptor along the plane shown in the inset, with the ligand binding domain protruding upward in a synaptic direction, the membrane indicated by horozontal hatch marks and the cytoplasmic domain extending downward and joining with a nonreceptor protein (horozontal line). Right panel shows a cross-section parallel to the membrane at the level of the ligand binding sites. The labels indicate the two α-subunits; one of these shows a regular pattern of densities (1–7) suggesting a β-sheet structure.

not be explained unless the two binding sites rotated opposite to each other in the pentamer, a possibility incompatible with symmetry considerations, as well as with symmetry of the subunits visible in the electron density maps.

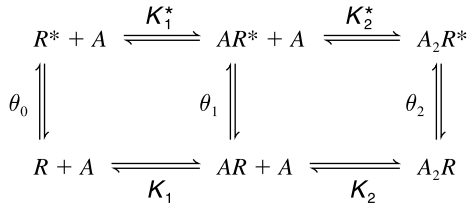
Higher resolution images at 9 Å resolution provided a glimpse of the internal structures of the subunits, including electron densities in the vicinity of the ACh binding sites (Unwin et al., 1993). Small rods consistent with α-helices were present in all subunits at the level of the ACh binding site, and those in the α-subunit were proposed to form the site. Perhaps the most telling insight into structural correlates of function came from images obtained before and after brief exposure to ACh (Unwin, 1995). The results showed that the fundamental motion underlying receptor activation is rotation of the subunits about an axis running through the ion channel. This rotation could be discerned at the levels of the transmembrane domains and the ACh binding sites. The images also pointed to the location of the channel gate roughly half way through the membrane and formed by the conjunction of conserved leucine residues contributed by each subunit. Still higher resolution images at 4.6 Å clarified the structure of the ACh binding sites (Miyazawa et al., 1999). The rod-like structures apparent at lower resolution instead appeared as rows of β-sheet structures at higher resolution, and the images suggested entry of ACh through the central vestibule rather than from the periphery of the pentamer (Fig. 1).

The availability of cDNAs encoding receptor subunits brought about enormous creative tension to establish expression systems, and yielded the *Xenopus* oocyte (Mishina et al., 1985) and clonal fibroblast (Claudio et al., 1987; Blount and Merlie, 1988; Sine, 1993) systems. These soon led to advances in understanding the LBD, first by enabling subunit omission experiments, and subsequently by allowing expression of site-directed mutations. Expression of pairs of subunits in clonal fibroblasts showed that the α–γ- and α–δ-subunit pairs bound the competitive antagonist curare with different affinities, and that the sum of binding for the two subunit pairs accounted for the broad profile characteristic of binding to the fully assembled wild-type receptor (Blount and Merlie, 1989). However, these subunit pairs remained con-

fined within the cell, and the contribution of the β-subunit remained unknown. Expression of triplets of subunits, however, solved both of these problems because both types of triplets expressed on the cell surface as symmetrical pentamers, $\alpha_2\beta\gamma_2$ and $\alpha_2\beta\delta_2$, each containing two binding sites composed of either α–γ or α–δ subunit pairs (Sine and Claudio, 1991). Moreover, curare binding to these symmetrical pentamers showed high affinity for $\alpha_2\beta\gamma_2$ and low affinity for $\alpha_2\beta\delta_2$, to quantitatively account for binding to the wild type $\alpha_2\beta\gamma\delta$ receptor expressed on the cell surface. The symmetrical pentamers also exhibited loss of cooperative, low-affinity binding of agonist characteristic of the pentamer containing the full complement of subunits. The β-subunit emerged as a structural subunit that does not contribute to the ACh binding site, but the β along with the other non-α-subunits is necessary to confer allosteric properties of the pentamer. Thus, subunit omission studies clearly showed that the two ACh binding sites are formed by α–δ and α–γ subunit pairs, but this conclusion could not be explained by the electron microscopic data, suggesting the β-subunit lodges between the two α-subunits.

Benefiting from knowledge of primary sequences of the subunits, mutagenesis and chemical labeling studies provided a detailed picture of residues from both α- and non-α-subunits present at the ACh binding site (reviewed by Prince and Sine, 1997; Corringer et al., 2000). In the α-subunits, residues in three distinct regions along the linear chain were found to converge at the binding site, giving rise to the nomenclature: loops A, B, and C. Key residues in these loops include αY93 in loop A (Galzi et al., 1990), αW149 and αG153 in loop B (Galzi et al., 1991; Sine et al., 1995b), αY190, αS187, αV188, αT189, and αY198 in loop C (Abramson et al., 1989; Krienkamp et al., 1994; Sine et al., 1994; Malany et al., 2000). In the γ-, δ-, or ε-subunits, residues in four regions in equivalent positions of each subunit were found to converge at the binding site, corresponding to loops D, E, F, and G. Key residues in these loops include γK34 and δS34 in loop D (Sine et al., 1995a; Bren and Sine, 2000), γW55 and εD59 in loop E (Chiara et al., 1998; Bren and Sine, 1997), γL109, γY111, εS115, γT117, γL119, and εP121 in loop F (Ohno et al., 1996; Sine, 1993, 1997; Sine et al., 1995a; Wang et al., 2000; Molles et al., 2002), and δD180 and γD174 in loop G (Martin et al., 1996). The overall results from mutagenesis and covalent labeling gave rise to two new concepts: the multiloop and basic scaffold hypotheses. The multiloop hypothesis held that the protein main chain of each subunit folds multiple times to place residues well separated along the linear sequence into a local region specialized for binding ACh (Prince and Sine, 1997). Formation of the multiloop structure can be visualized by extending a piece of thread, grasping it in several places, and bringing the nodes together to form the walls of a binding site cavity. The basic scaffold hypothesis emerged from experiments showing that key residues could be swapped between γ-, δ-, or ε-subunits and create ligand affinity of the fully assembled receptor corresponding to the parent subunit from which the key residues originated (Sine, 1993; Sine et al., 1995a). Thus, the basic scaffold hypothesis held that the protein main chains of γ-, δ-, ε-subunits are equivalent.

Application of allosteric theory, advances in single-channel kinetic analysis, and discovery of a disease-causing mutation in the receptor provided a deeper understanding of how ACh occupancy of the binding site mediates receptor activation. The results from these studies elucidated the origin of cooperativity in activation, and described the dynamic nature of the ACh binding site as necessary for activation. The analysis centered around the following mechanism first proposed by Monod et al. (1965):



where R is the closed state, R^* the open state, A is ACh, the Ks are dissociation constants for ACh binding, and the θs are channel gating equilibrium constants with zero, one, or two ACh molecules bound. Jackson (1989) applied this mechanism to receptor activation based on upper bound estimates of θ_0 and θ_1, and a lower bound estimate for K_2. The remaining equilibrium constants came from single-channel kinetic measurements of the microscopic rate constants underlying them (Jackson, 1988). Subsequent kinetic analysis during the 1990s provided precise measurements of K_1, K_2, θ_1 and θ_2 from several species of receptor (Sine et al., 1990; Zhang et al., 1995; Ohno et al., 1996), which roughly agreed with the values used in Jackson's analysis.

Receptor channels activate in the absence of ACh but at a very low frequency (Jackson, 1984). Therefore, the task for agonist is to increase the likelihood of this improbable channel opening event. Jackson (1989) realized that the only way to increase the probability of channel opening is to increase K_1^* relative to K_1, which by detailed balancing, will in-

crease θ_1 relative to θ_0. However, the estimated binding energy available from interaction of ACh with the protein suggested a K_1* in the nanomolar range, but given a measured K_1 in the micromolar range, this could only increase the channel opening equilibrium from $\sim 10^{-6}$ to 10^{-3}. Thus, including a second ACh binding step, with K_2 of hundreds of a micromolar and K_2* of tens of a nanomolar, becomes necessary to increase the channel opening equilibrium the remaining extent from 10^{-3} to 25. The emerging concept viewed low-affinity binding to the resting, closed state of the receptor as necessary to provide a large ratio of closed- to open-state affinities that promotes efficient channel gating. Also in the course of activation, the binding site changes conformation from a loose complement of the shape of the ACh molecule to a good complement. The importance of a high-affinity open state was further established by analysis of a mutation causing a congenital myasthenic syndrome. The mutation ϵP121L localized to loop F at the binding site and selectively reduced affinity of ACh for the open state, but had little effect on affinity for the closed state (Ohno et al., 1996). ACh-evoked single channel currents through the ϵP121L mutant opened with a maximum probability of 0.05, some 500-fold worse than in wild-type receptor, showing the importance of a high-affinity open state in promoting efficient activation.

The origin of cooperativity emerged from the single channel kinetic analyses. The channel-gating equilibrium constant increases with increasing numbers of ACh bound to the receptor such that diliganded receptors open with about 10,000-fold greater probability than monoliganded receptors (Ohno et al., 1996; Wang et al., 1999), which in turn, open with greater probability than unliganded receptors (Jackson, 1984). Thus, cooperativity originates in the channel-gating steps. That diliganded receptors activate with highest probability was demonstrated about a decade earlier from experiments showing that occupancy of one of the two binding sites by a competitive antagonist fully blocked the macroscopic permeability increase elicited by the agonist (Sine and Taylor, 1980, 1981). The possibility that there is also cooperativity at the level of the ACh binding steps seems unlikely because kinetic analyses show identical, moderately different or markedly different affinities for the two binding sites, in the adult mouse (Wang et al., 1997; Salamone et al., 1999), adult human (Wang et al., 1999) and *Torpedo* (Sine et al., 1990) receptors, respectively. Thus, each of the two ACh binding sites is tightly coupled to the channel gate, but they do not appear coupled to each other.

The agonist occupancy step nevertheless contributes to the time course of the endplate response. Physiologists long theorized that endplate current decay depends not only on the kinetics of opening and closing of the ion channel, but also on the rate of dissociation of agonist from the binding site (Magleby and Stevens, 1972; Colhoun and Hawkes, 1981; Colquhoun and Sakmann, 1985). By slowing agonist dissociation and prolonging the endplate decay rate, a disease-causing mutation at the ACh binding site demonstrated the importance of agonist dissociation in governing the time course of the endplate response (Sine et al., 1995b). In wild-type AChR, the rate of ACh dissociation is comparable to the rate of channel opening, which allows an average of two openings per ACh occupancy and prolongs the response of the doubly liganded receptor about twofold compared with the limiting case of very fast ACh dissociation. However, for receptors harboring the disease-causing mutation αG153S, ACh dissociation is slowed by more than an order of magnitude, which allows an average of 20 openings per ACh occupancy to greatly prolong the response.

The molecular basis for ACh recognition by the receptor has been intensively investigated by mutagenesis combined with measurements of ligand binding or receptor function (reviewed by Prince and Sine, 1997; Corringer et al., 2000). However, the multiloop structure of the binding site, and the many residues found to localize at the site, confounded assignment of the actual site of ACh docking. Investigators thought the site had to localize within a pocket framed by aromatic residues, as these are abundant at the binding site and their chemistry would stabilize the quaternary ammonium group of ACh. Measurements of agonist affinity following mutagenesis, as expected, revealed large decreases in affinity following mutation of the aromatic residues, αW149, αY190, αY93, and αY198 (Galzi et al., 1991b; O'Leary and White, 1992; Sine et al., 1994). However, even the question of which subunit is involved was uncertain because large decreases in agonist affinity were also observed following mutation of δW57, δS36, δI178, and δD175 (Czajkowski, et al. 1993; Prince and Sine, 1996; Xie and Cohen, 2001). Perhaps the strongest evidence for the identity of the primary ACh docking site came from studies of unnatural mutations in which varying numbers of fluorines were substituted on tryptophan at position 149 in the α-subunit, and EC_{50}s for receptor activation were compared to quantum mechanical calculations of interaction energies between the tryptophan analogs and a cation (Zhong et al., 1998). The EC_{50}s decreased linearly with increasing cation interaction energy,

suggesting that αW149 is a good candidate for interacting with the quaternary ammonium group of ACh.

Recent Advances (2000s)

The new millennium heralded the atomic structural age in our understanding of the nicotinic receptor. This began at the Annual Society for Neuroscience meeting in 2000 where Smit, Sixma, and colleagues presented a poster describing the cloning and atomic structural determination of an acetylcholine binding protein (AChBP). News of the AChBP structure spread, and the work was published with great anticipation in the Spring of 2001 (Brejc et al., 2001). Although other proteins that bind ACh had been described at the atomic structural level (Sussman et al., 1991), this one was special. It was a homopentamer, and was homologous to the major extracelluler domain from nicotinic receptor α-subunits, the closest relative being the homomeric α7. More important, AChBP contained many of the structural cornerstones that give nicotinic receptors their unique signature: a size compatible with that of the *Torpedo* receptor ECD imaged at a resolution of 4.6 Å, the presence of binding site loops A–G, the disulfide bonded loop between C128 and C142, key aromatic residues conserved in α- and non-α-subunits (αY93, αW149, αY190, αY198, and γW55), the vicinal cysteines (αC192, αC193) and the main immunogenic region (MIR) at the top of the α subunit.

Each AChBP monomer contains a core of 10 β-strands that form a sandwich analogous to the classical immunoglobulin fold, but with two additional strands and pronounced twisting of the strands to form a well-defined hydrophobic core (see structural model of the ε subunit in Fig. 2). The N-terminus at the top of the subunit forms an α-helix, which is followed by a linker to strand β1 that spans the length of the subunit and spirals around the hydrophobic core as it extends from the middle to the bottom of the subunit. Following a short linker, strand β2 establishes an antiparallel β-sheet with strand β1, terminating at the top of the subunit with the start of the MIR. Following the MIR, the short strands β3, β4, β5, and β5′ and intervening linkers encircle the middle of the subunit facing the central vestibule, and the subsequent strand β6 establishes an antiparallel β-sheet with strand β2 as it descends to the bottom of the subunit. Structural elements from strands β1 through β6 constitute the inner set of β-strands of the subunit.

Located at the bottom of the subunit, the linker between strands β6 and β7 is the signature *cis*-loop found in all members of the AChR superfamily. Following the *cis*-loop, strand β7 extends to the top of the subunit, and the short strand β8 forms a parallel β-sheet with strand β1. The ensuing β8 to β9 linker is very long, and extends the remaining distance from the top to the bottom of the subunit. From here, strand β9 projects to the top and periphery of the subunit where the disulfide-bonded vicinal cysteines create a hairpin. The ensuing strand β10 retraces strand β9, forming an antiparallel β-sheet and terminating at the bottom of the subunit. Structural elements from strands β7 through β10 constitute the outer set of β-strands of the subunit.

The AChBP structure confirmed long-held hypotheses and resolved controversies about the structure of the nicotinic receptor LBD. The first controversy resolved by AChBP was location of the ACh binding sites at interfaces between subunits; each binding site in AChBP contains all seven loops found at the ACh binding site of the receptor, as well as key residues in loops A–G (Prince and Sine, 1997; Corringer, et al., 2000). Second, AChBP contains five ACh binding sites arrayed symmetrically around the pentameric ring, and this symmetry indicated that the α–γ and α–δ binding sites could not be rotated opposite to each other in the *Torpedo* receptor; consequently, the β-subunit is not the subunit separating the two α-subunits. The intervening subunit must therefore be γ in the *Torpedo* receptor and ε in the adult mammalian muscle receptor. Third, the handedness of subunit organization was decided: the side of the α-subunit that forms the binding site faces in a counterclockwise direction when viewed from the synaptic cleft. Fourth, the binding site is accessible from the periphery of the pentamer rather than from the central vestibule, contrary to suggestions by electron density maps of *Torpedo* receptor at 4.6 Å resolution (Miyazawa et al., 1999). This apparent contradiction is tentative, however, because the AChBP structure most closely resembles the active state of the receptor, but the more opened up structure of the inactive state may enable agonist access from both the central vestibule and the periphery (see below; Unwin et al., 2002). A question that was not controversial, but that was nevertheless unanswered, was the location of the cys-loop, which was shown to protrude from the bottom of the subunit where it can interact with transmembrane domains and possibly couple agonist binding to gating of the ion channel.

Suggestion of the site of ACh binding also emerged from the atomic structure of AChBP. HEPES buffer was present in the "mother-liquor" used to crystalize AChBP, and the X-ray diffraction data revealed one molecule of HEPES within the binding site pocket where its positively charged nitrogen hovered over the indole ring of Trp-143 (Brejc

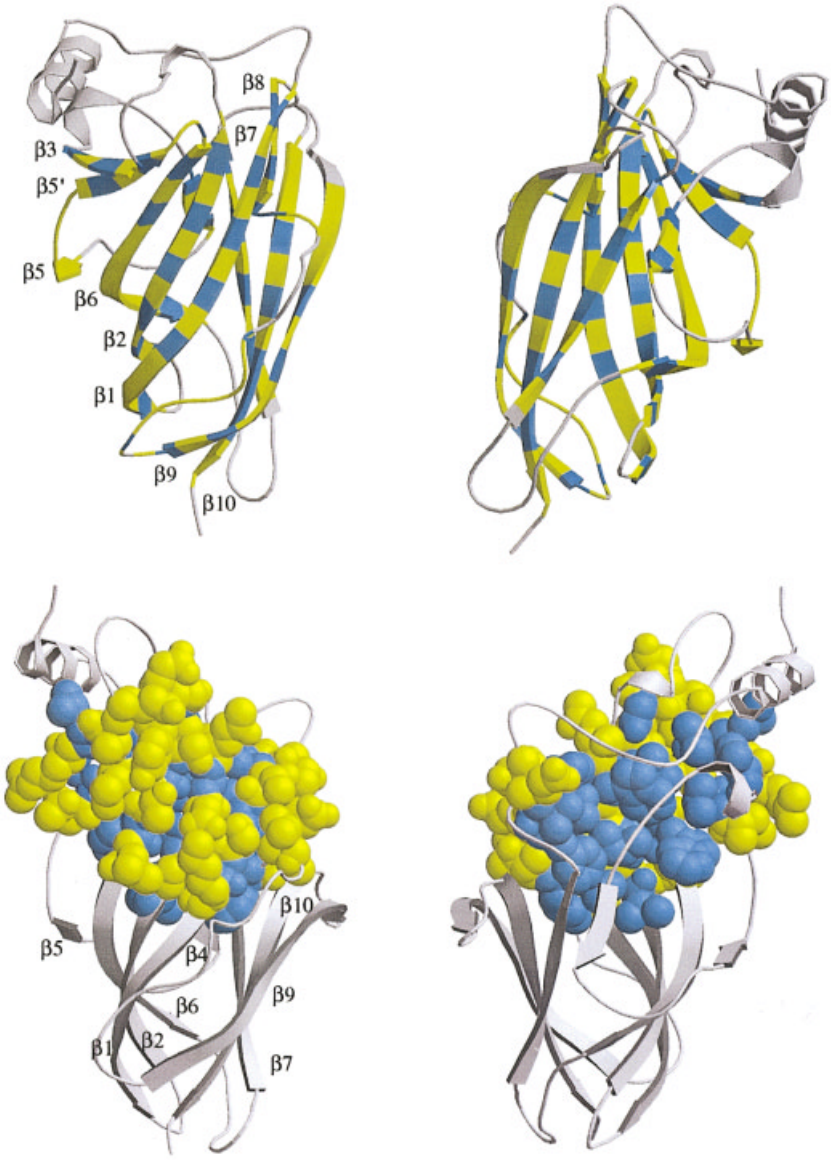


Figure 2 Structural model of the adult human muscle receptor ϵ-subunit based on lysine scanning and homology modeling according to AChBP (Sine et al., 2002). Upper panel shows secondary structure rendering with results from lysine scanning overlaid. Regions colored yellow correspond to positions where lysine mutation produced a folded subunit, whereas regions colored blue correspond to positions where lysine mutation produced a misfolded subunit. Left view is from the subunit interface that forms the ligand binding site with the α-subunit. Right view is rotated 180°, and shows the opposite interface and the region facing the inner vestibule. Lower panel shows space-filling rendering of residues in the upper portion of the subunit, color coded as above.

et al., 2001), which is equivalent to α-Trp-149 in the muscle nicotinic receptor. α-Tryp-149 was suggested to be the cation binding site from mutagenesis and quantum mechanical calculations of interaction energies between unnatural tryptophan analogs and a cation (Zhong et al., 1998). Thus, Trp-149 in the center of the binding pocket represents the best candidate for stabilizing the quaternary nitrogen of ACh.

The AChBP structure also pointed to new opportunities destined to deepen understanding of the nicotinic receptor LBD. The first opportunity was the potential to generate reliable structural models of LBDs of nicotinic receptors (Le Novere et al., 2002; Molles et al., 2002; Sine et al., 2002). The standard technique for doing this, homology modeling, begins with a known atomic structure and then superimposes

the unknown structure on this template, the result being essentially a copy of the template protein but with the different side chains of the unknown protein; energetically unfavorable interactions such as steric overlap are subsequently removed by simulated annealing and molecular dynamics to produce a physically plausible structure (Sali and Blundell, 1993). The key to homology modeling is correct alignment of the template sequence with the unknown protein sequence, which is traditionally done by aligning sequences of related proteins according to homology. However, in β-sheet proteins, an error in alignment of as little as one residue can produce substantial errors because that would place residue side chains on the wrong face of the β-sheet. Thus, prior to modeling, the question had to be answered of whether residues in receptor LBDs have the same side chain register as in AChBP.

The answer to this question emerged from mutagenesis combined with homology modeling (Sine et al., 2002). Lysine scanning of the muscle receptor ε-subunit revealed alternating patterns of folding and misfolding of the subunit as lysine was advanced along the protein chain, indicating the presence of β-strands and establishing orientation of the side chains toward core hydrophobic and surface hydrophilic environments (Fig. 2). Misfolding of the mutant subunits was interpreted as due to the large energetic penalty for placing a positively charged side chain in the low dielectric environment of the hydrophobic core, together with the hydrogen bonded β-strands that attempt to hold it in place. The results established side chain register between each of the 10 β-strands in the receptor ε-subunit and the corresponding β-strands in AChBP, providing the basis for homology modeling, and furthermore showed that side-chain register between the two proteins is maintained to a remarkable degree. The results of both lysine scanning and the emerging homology model showed that the structure of the ε-subunit is quite similar to that of the AChBP protomer. The similarity between the receptor and AChBP structures extends beyond equivalent side chain orientations of the β-stranded residues, and even applies to local regions within the β-sheets themselves, where identical parts of the strands were found to twist normal to the hydrophobic core (Sine et al., 2002). Because the human ε-subunit is very distant from the snail AChBP from an evolutionary standpoint, other members of the nicotinic receptor superfamily are also likely to mirror structurally AChBP.

Availability of a reliable structural model of a receptor LBD provides a wealth of information for further studies. Within the ACh binding pocket, relative positions of both conserved aromatic residues present in AChBP and conserved hydrophobic residues unique to the receptor are defined (Fig. 3), providing starting points for mutagenesis combined with functional measurements. Moreover, structures unique to receptor subunit interfaces emerge, and serve as candidates for stabilizing open or closed states of the receptor (Sine et al., 2002). For example, a salt bridge at the top of each of the five subunit interfaces is present in the structural model of the receptor, which differs from the bifurcated salt bridge in AChBP; the corresponding residues in the receptor, αR20 and εE4, are conserved across species, suggesting that these establish stabilizing interactions essential for receptor structure or function. Additionally, putative interactions specific to particular interfaces emerge from the structural model, such as a salt bridge between the conserved residues εE93 and αR55 at the nonligand binding ε-α interface, an amide-aromatic hydrogen bond between the conserved αY127 and εN39 at the α–ε interface, and a hydrogen bond between the conserved εN182 and the main chain carbonyl of αC128 at the α–ε interface. Thus, residues close to each other in the three-dimensional structural model provide powerful starting points for defining structural interactions essential for receptor function.

Several years ago structural changes in the ECD were detected following exposure of *Torpedo* receptors to a brief spray of ACh (Unwin, 1995). However, recent images at higher resolution (4.6 Å), interpreted in light of the AChBP structure, revealed larger structural changes localized to regions within the α subunits (Unwin et al., 2002). For receptors not exposed to ACh, the structure of the AChBP protomer could be superimposed over the electron densities of the β-, γ-, and δ-subunits of the *Torpedo* receptor, but not over the α-subunits. However, upon brief, millisecond exposure to ACh, the AChBP structure could be superimposed over electron densities of all five subunits. Thus, the α-subunits undergo a major conformational change upon activation that converts them from the resting α- to the active non-α-subunit conformation. This conformational change corresponded to twisting of the inner set of β-strands by some 15 degrees in a clockwise direction about an axis running through the channel (Fig. 4). Additionally upon activation, the outer set of β-strands tilted outward about 11 degrees about an axis normal to the axis through the channel. Thus, upon activation of the receptor, conformational changes in the inner and outer β-sheets convert the α to the non-α conformation, and these changes likely underlie the state change from low to high affinity for ACh.

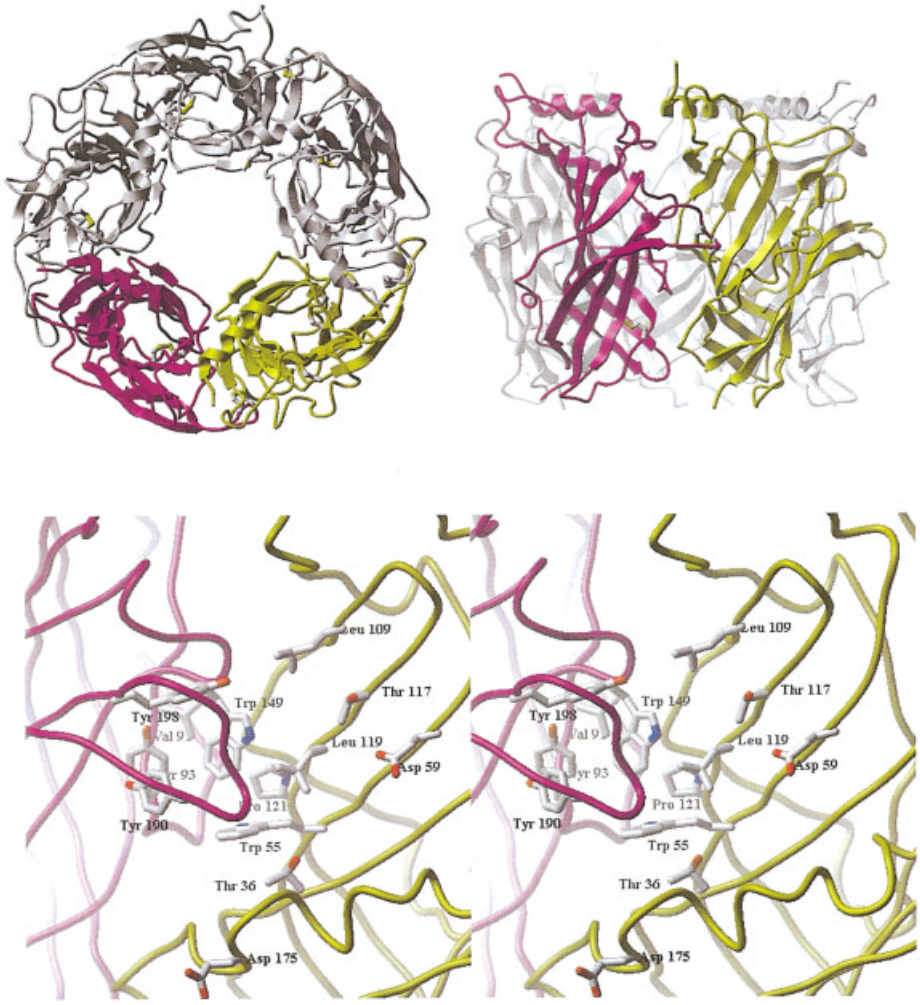


Figure 3 Structural model of the LBD from the adult human muscle receptor (Sine et al., 2002). Upper left panel views the structure from the top with the α-subunit highlighted in magenta, the ϵ-subunit in yellow, and the remaining three subunits in gray. Upper right panel shows the structure from the side. Lower panel shows a stereo view of the ACh binding site at the α–ϵ-subunit interface. Main chains are magenta for the α-subunit and yellow for the ϵ-subunit. Side chains of key conserved aromatic and hydrophobic residues are displayed, along with three nonconserved residues above and to the right of the binding site center (see text).

FUTURE PROSPECTS

Investigators are now well poised to significantly advance understanding of how the nicotinic receptor recognizes ACh and other ligands and how the LBD triggers activation of the receptor ion channel. Although current structural models are well-constrained by the availability of the close structural homolog AChBP, the major pressing need is an experimentally determined atomic structure of the LBD of the nicotinic receptor. The most clear-cut approach is to determine the X-ray structure of the entire receptor, or of the LBD derived from a truncated receptor, but these approaches have so far met with limited success. A realistic goal, however, might be to combine a mutagenesis-based homology model of the *Torpedo* receptor with the corresponding electron microscopic images from tubular crystals. By merging residues in the model with particular electron densities from the images, a highly reliable structural model could be generated. Given atomic coordinates of the LBD of the *Torpedo* receptor, the closer homology to other receptors of the superfamily would allow construction of better constrained structural models. Computational docking of agonists or competitive antagonists could then be done to understand the nature of mo-

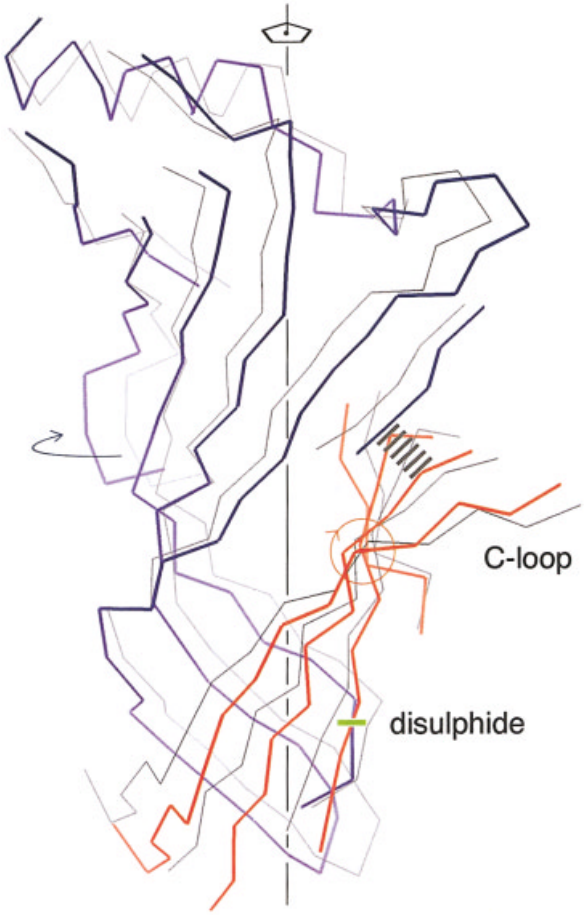


Figure 4 Conformational changes in the α-subunit upon activation by ACh. The main chain of the AChBP protomer was altered to fit three-dimensional densities of either α- or non-α-subunits from *Torpedo* AChR (generously provided by Nigel Unwin; see Unwin et al., 2002). Two sets of main chains are shown; the thick blue and red chains correspond to fitting to α-subunit densities, whereas the thin gray chains correspond to fitting to non-α-subunit densities. Arrows indicate rotations of the inner and outer β-strands of the subunit upon activation by ACh, which converts the subunit from the resting α- to the active non-α-configuration.

lecular recognition, as well as allow design of more effective and specific antagonists of the receptor. The current structural models may be good enough to do molecular docking studies (Le Novere et al., 2002), but the results would need to be tested by mutation of residues predicted to interact with the docked ligand. A good structural model also provides a basis for identifying new residues critical for function of the LBD; residues in close proximity in the three-dimensional structure provide powerful starting points for mutagenesis combined with functional measurements. Availability of atomic coordinates of residue side chains allows testing of the idea that conserved structural elements critical for function may not be apparent from sequence alignments. For example, a network of nonconserved residues might create an essential structural motif that is conserved, but more than one combination of residue side chains could generate the motif. Understanding the second function of the nicotinic receptor LBD, triggering of the biologic response, may benefit from knowing the atomic structure of the LBD. Molecular dynamics simulations can predict molecular motions of the LBD, but the practical time scale is 10^{-8} s, which falls far short of the time scale of physiologic state transitions of about 10^{-6} s. However, once the structures of the LBD in closed and open states are known, application of force using steered molecular dynamics may reduce the time required to simulate the triggering event to within practical simulation times (Israelewitz et al., 2001). Testing the results with mutagenesis and functional measurements will likely provide new insights into the molecular basis of agonist recognition and triggering of the biological response by the nicotinic receptor LBD.

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