

# Glycine Hinges with Opposing Actions at the Acetylcholine Receptor-Channel Transmitter Binding Site<sup>§</sup>

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Received September 7, 2010; accepted November 29, 2010

## ABSTRACT

The extent to which agonists activate synaptic receptor-channels depends on both the intrinsic tendency of the unliganded receptor to open and the amount of agonist binding energy realized in the channel-opening process. We examined mutations of the nicotinic acetylcholine receptor transmitter binding site ( $\alpha$  subunit loop B) with regard to both of these parameters.  $\alpha$ Gly147 is an “activation” hinge where backbone flexibility maintains high values for intrinsic gating, the affinity of the

resting conformation for agonists and net ligand binding energy.  $\alpha$ Gly153 is a “deactivation” hinge that maintains low values for these parameters.  $\alpha$ Trp149 (between these two glycines) serves mainly to provide ligand binding energy for gating. We propose that a concerted motion of the two glycine hinges (plus other structural elements at the binding site) positions  $\alpha$ Trp149 so that it provides physiologically optimal binding and gating function at the nerve-muscle synapse.

## Introduction

At the vertebrate neuromuscular synapse, transmitter molecules released from the nerve terminal bind at two specific sites in the extracellular domain of the nicotinic acetylcholine receptor (AChR) to promote opening of a distant ion channel and depolarization of the muscle cell membrane (Edelstein and Changeux, 1998; Karlin, 2002; Auerbach, 2010). The equilibrium constant of the AChR “gating” conformational change is coupled to an affinity change for agonists at the transmitter binding sites. Acetylcholine (ACh) binds more tightly to active-open ( $R^*$ ) compared with resting-closed ( $R$ ) AChRs, hence there is a higher probability that liganded receptors will adopt the  $R^*$ , open-channel conformation compared with unliganded receptors. Each AChR transmitter binding site is composed, in part, of three loops in the  $\alpha$  subunit. Here we are concerned with the roles of residues in loop B with regard to the agonist affinity change and the global gating isomerization.

The probability of adopting  $R^*$  is reduced by mutations of aromatic residues at the binding sites (Dennis et al., 1988; Abramson et al., 1989; Galzi et al., 1990; Cohen et al., 1991; Middleton and Cohen, 1991; Aylwin and White, 1994;

O’Leary et al., 1994; Sine et al., 1994; Chen et al., 1995; Chiara et al., 1998; Akk, 2001). The mutations of  $\alpha$ Trp149 (loop B) and  $\alpha$ Tyr190 (loop C) decrease the affinity of the  $R$  conformation for ACh but, to an even greater extent, that of the  $R^*$  conformation (Purohit and Auerbach, 2010). Residues  $\alpha$ Tyr93 (loop A),  $\alpha$ Tyr198 (loop C), and  $\epsilon/\delta$  subunit position Trp55/Trp57 (loop D) show similar, but less pronounced, behaviors. Most of these mutations have relatively smaller effects on the unliganded gating equilibrium constant ( $E_0$ ), so the primary mechanism by which they decrease activation of fully liganded AChRs is by decreasing the net ligand binding energy available for gating. This energy is proportional to the natural logarithm of the  $R/R^*$  equilibrium dissociation constant ratio ( $\lambda$ , the “coupling” constant). Loop B contains two glycines at positions  $\alpha$ Gly147 and  $\alpha$ Gly153 and a conserved Trp at position  $\alpha$ Trp149. Our goal was to estimate the effects of mutations of these and other loop B amino acids with regard to both  $E_0$  and  $\lambda$ .

Some loop B residues have been studied previously.  $\alpha$ G153S is a slow channel congenital myasthenic mutation (Sine et al., 1995) that yields AChRs with a higher  $R$ -conformation affinity for the transmitter and an increased intrinsic gating isomerization constant (Zhou et al., 1999). This residue has recently been suggested to be important with regard to setting the AChR affinity specifically for the partial agonist nicotine (Xiu et al., 2009).  $\alpha$ Trp149 has long been known to play a central role in both agonist binding and channel gating (Zhong et al., 1998; Akk, 2001). Many mutations here increase  $E_0$  (Purohit and Auerbach, 2010), but, because they

This work was supported by the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant NS064969].

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.

doi:10.1124/mol.110.068767.

<sup>§</sup> The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

**ABBREVIATIONS:** AChR, nicotinic acetylcholine receptor; ACh, acetylcholine;  $R$ , resting-closed AChRs;  $R^*$ , active-open state AChRs; PBS, phosphate-buffered saline; wt, wild type;  $P_o$ , open probability;  $R/E$ , rate-equilibrium; Cho, choline.

also substantially reduce the R affinity, their effects on liganded gating have not yet been quantified. Less is known about the contributions of other loop B residues in ligand binding and channel gating.  $\alpha$ Thr148 and  $\alpha$ Thr150 mutations have little effect on agonist binding or channel gating (Lee and Sine, 2004; Cashin et al., 2007),  $\alpha$ Tyr151 is required for strong binding of imidacloprid to insect AChRs (Liu et al., 2005), and  $\alpha$ Asp152 is important for setting both the affinity of the resting binding site and the diliganded gating equilibrium constant (Sugiyama et al., 1996; Zhou, 1999). In sum, pieces of information about ligand binding and channel gating are known for loop B residues, but a full exposition of the effects of mutations here is currently not available.

We have carried out a systematic single-channel study of  $\alpha$  subunit loop B residues  $\alpha$ Gly147– $\alpha$ Gly153. For each position, we dissect the underlying cause of its influence on AChR function with regard to  $E_0$  and  $\lambda$ .

## Materials and Methods

**Mutagenesis and Expression.** Mutants were made by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by complete cDNA sequencing. Human embryonic kidney 293 cells were transiently transfected using calcium-phosphate precipitation with a mixture of cDNAs encoding mouse muscle AChRs ( $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\epsilon$ ;  $\sim 3\text{--}4\ \mu\text{g}/35\ \text{mm}$  dish, in the ratio 2:1:1:1). cDNA ( $0.1\ \mu\text{g}/\mu\text{l}$ ) encoding green fluorescent protein was added as a marker to the transfection cocktail. The cells were incubated at  $37^\circ\text{C}$ , the culture medium was washed after  $\sim 16\ \text{h}$ , and single-channel patch clamp recording was performed in the cell-attached configuration  $\sim 4$  to  $6\ \text{h}$  later at  $23^\circ\text{C}$ . The bath solution was usually Dulbecco's phosphate-buffered saline (PBS) containing 137 mM NaCl, 0.9 mM  $\text{CaCl}_2$ , 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{MgCl}_2$ , and 8.1 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4. Sometimes, NaCl was replaced with KCl (K-PBS) in the bath solution. The pipette solution was always PBS. In some experiments, an agonist (acetylcholine or choline) was added only to the pipette solution. The pipette potential was either  $+70$  (PBS) or  $-70$  mV (K-PBS), which corresponds to membrane potentials of approximately  $-100$  or exactly  $+70$  mV. Currents were sampled at 50 kHz after low-pass filtering at 20 kHz. QuB software (<http://www.qub.buffalo.edu>) was used to acquire and analyze the single-channel currents.

The single-channel currents were corrected for baseline drift. Clusters of single-channel openings, which each reflect binding and gating events of an individual AChR, were selected by eye and idealized into noise-free conducting/nonconducting intervals by using the segmental- $k_-$  means algorithm (Qin, 2004). To estimate the gating rate constants, the idealized current interval durations were modeled using a maximum interval likelihood method after imposing a  $25\text{-}\mu\text{s}$  dead time and approximate missed-event correction (Qin et al., 1997).

The scheme we used for interpreting the functional properties of AChRs is shown in Fig. 1B. The diliganded gating equilibrium constant ( $E_2$ ) was calculated as the ratio of the forward/backward isomerization rate constants ( $f_2/b_2$ ). Choline was used to activate constructs in which  $E_2$  was approximately equal to or larger than that of the wild type (wt), and ACh was used to activate constructs in which  $E_2$  was smaller than that of the wt. In most experiments, the initial test agonist concentration was either 20 mM choline or 1 mM ACh.

Some loop B mutations caused a severe loss of activity so that openings were not clustered even at these high agonist concentrations. To compensate, we coexpressed these mutants with an  $\epsilon$  subunit having two transmembrane domain background mutations,  $\epsilon$ (P245L+L269F). Together, these two mutations increase  $f_2$  by  $\sim 14$ -fold and decrease  $b_2$  by  $\sim 33$ -fold (increase  $E_2$  by  $\sim 450$ -fold). The

background mutations were located far from loop B, so we assumed that their effects and those at loop B were energetically independent.

In some experiments, we fully saturated the transmitter binding sites by using 140 mM acetylcholine, a concentration that is  $\sim 1000$  times larger than the resting equilibrium dissociation constant ( $K_d$ ) of wt AChRs (Chakrapani et al., 2003). To be sure that this concentration fully saturated the binding sites, we also measured the gating rate constants at 100 mM ACh. If the effective opening rate was approximately the same at both concentrations, we considered the binding sites to be completely saturated (Supplemental Fig. S2).

At these high ACh concentrations, open-channel block by the agonist reduced the current amplitude almost to zero. To compensate, in some experiments, the pipette was held at  $-70$  mV (which corresponds to a membrane potential of  $+70$  mV) and the kinetic parameters of the outward, single-channel currents were quantified. Depolarization also changes the gating rate constants. Because we wanted to compare the measured equilibrium and rate constants with wt values obtained at a physiological membrane voltage ( $\sim -100$  mV) we used correction factors that pertain to a 170-mV depolarization, which alone causes a 1.5-fold decrease in  $f_2$  and 10-fold increase in  $b_2$  (a 15-fold decrease in  $E_2$ ) (Auerbach et al., 1996; Jha et al., 2009). We assumed that the voltage and mutation perturbations are energetically independent. The background perturbation corrected constants are reported in Supplemental Table S1. All experiments with 20 mM choline were at  $-100$  mV membrane potential. To correct for the effect of channel-block on  $b_2$ , we multiplied the observed value by 3.1 (corrected values shown in Supplemental Table S1) (Jha et al., 2009).

$K_d$  was estimated for some mutants (Fig. 3, Supplemental Table S4). For the  $\alpha$ Gly153 series, AChRs were activated by using three different choline concentrations, and interval durations within clusters were fitted together by using the kinetic scheme  $R \leftrightarrow RA \leftrightarrow A_2R \leftrightarrow A_2R^*$ , where A is the agonist. We assumed that the two binding steps are equal and independent (Akk et al., 1996; Salamone et al., 1999; Jha et al., 2009), so only four rate constants were free parameters: single-site association ( $k_+$ ) scaled by the agonist concentration, single-site dissociation ( $k_-$ ),  $f_2$  and  $b_2$ .  $K_d$  was calculated as the ratio  $k_-/k_+$ . For  $\alpha$ G147S the relationship between the [ACh] and the open probability of clusters ( $P_o$ ) was fitted by (from the scheme above):  $P_o = (c^2E_2)/(1 + 2c + c^2 + c^2E_2)$ ;  $c = [\text{ACh}]/K_d$ . At each ACh concentration,  $P_o$  was calculated from the rate constants:  $P_o = f_2/(f_2 + b_2)$ .

In wt AChRs, unliganded (spontaneous) openings are both rare and brief. To increase both the frequency and duration of such openings, we used a background construct that had multiple point mutations in both  $\alpha$  subunits ( $\alpha$ D97A+ $\alpha$ Y127F+ $\alpha$ S269I; 'DYS'). Individually, each of these mutations increases the diliganded gating equilibrium constant [ $\alpha$ D97A, 168-fold (Chakrapani et al., 2003);  $\alpha$ Y127F, 59-fold (Purohit and Auerbach, 2007); and  $\alpha$ S269I, 115-fold (Mitra et al., 2005)] by an approximately parallel change in the unliganded gating equilibrium constant (Purohit and Auerbach, 2009). None of these mutations has a significant effect on  $K_d$ . The fold changes in  $E_0$  for the loop B mutations in this study are expressed in reference to the DYS background, so the only assumption we make is that the loop B and DYS mutations have independent energetic consequences. There was no agonist-associated open-channel block in experiments without agonists in the pipette solution, and the single-channel current amplitude was in all cases  $\sim 7$  pA at a pipette potential of  $+70$  mV.

For some mutants, the spontaneous open- and closed-interval durations within clusters were well described by a single exponential component. For these,  $f_0$  and  $b_0$  were simply the inverse mean lifetimes of the nonconducting and conducting intervals. In some constructs there were multiple open/shut components, and for these the intracluster interval durations were fitted by using a kinetic model having two nonconducting and two conducting states. We tested both coupled and uncoupled kinetic schemes, with nearly equivalent results for the  $E_0$  estimate. The values reported in the

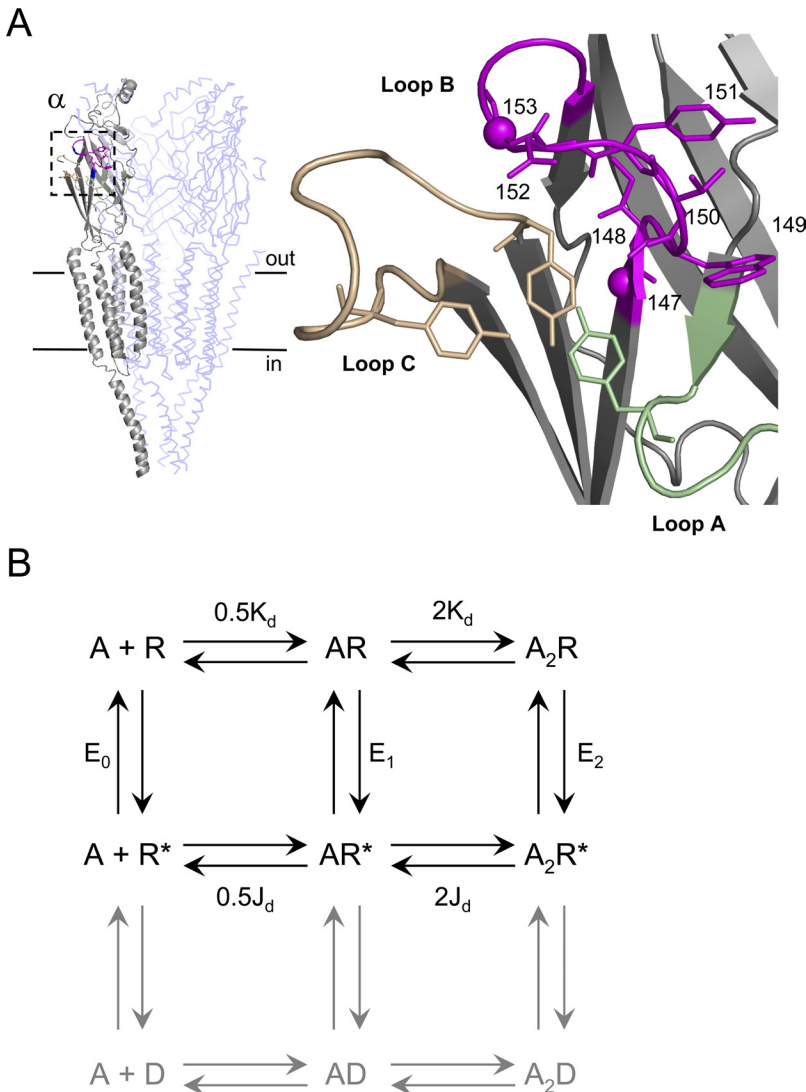
Tables were obtained by using a coupled conducting-state model (CCOO), with  $E_0$  calculated as the ratio of  $f_o/b_o$  associated with the predominant (typically, ~90%), brief open component.

For each mutant, the fold change in parameter  $X$  was estimated as  $(X^{\text{mutant}}/X^{\text{background}})$ . The change in the free energy ( $\Delta\Delta G$ , in kilocalories per mole) caused by mutation was calculated as  $-0.59\ln(\text{fold change})$ . The coupling energy between side chains was calculated as  $-0.59\ln(X^{\text{observed}}/X^{\text{predicted}})$ , where the predicted value was calculated as the sum of the  $\Delta\Delta G$  values for each individual mutation.

For all constructs, example currents are shown in Supplemental Fig. S1, and the rate constants are shown in Supplemental Tables S1 and S2. The kinetic parameters are displayed in the form of rate-equilibrium (R/E) relationships, in which the forward isomerization rate constant is plotted as a function of the gating equilibrium constant for a family of mutations of that residue on a log-log scale (Fersht, 1995). The “range-energy” is the natural logarithm of the ratio of the largest/smallest equilibrium constant for a series of mutations of one position (Jha et al., 2009).  $\Phi$  is the linear slope of the R/E relationship and provides information about the relative timing of the energy change of the perturbed residue, on a scale from 1 (early) to 0 (late).

## Results

An unliganded *Torpedo californica* AChR transmitter binding site is shown in Fig. 1, A and B (Unwin, 2005). The



model we used for quantifying AChR function is a cyclic activation scheme (Fig. 1C). In this scheme the resting R state has a low affinity for ACh and a closed channel, and the active  $R^*$  state has a high affinity for ACh and an open channel. The R and  $R^*$  shapes must be different insofar as they have different functional properties.

The cyclic scheme predicts that AChRs should open and close spontaneously, in the absence of exogenous agonists (the  $R \leftrightarrow R^*$  step). Such events are indeed present in wt AChRs. Jackson (1986) estimated that in embryonic mouse muscle, the unliganded gating equilibrium constant  $E_0 \sim 10^{-7}$ . In adult mouse muscle AChRs expressed in human embryonic kidney cells,  $E_0 \sim 6.5 \times 10^{-7}$  (Purohit and Auerbach, 2009; Jha and Auerbach, 2010). In AChRs the allosteric constant  $E_0$  is small, but it is measurably different from zero. Mutations that increase  $E_0$  in human AChRs are the fundamental cause of some congenital myasthenic syndromes (Zhou et al., 1999).

The adult mouse AChR binding sites have approximately equal affinities for ACh (Salamone et al., 1999; Jha and Auerbach, 2010), so there are only four equilibrium constants to consider in the cyclic scheme: the dissociation constant of the R conformation ( $K_d$ ), the dissociation constant of the  $R^*$

**Fig. 1.** AChR structure and function. A, the transmitter binding site. Left, unliganded *T. californica* AChR [Protein Data Bank code 2BG9 (Unwin, 2005)]. Subunits:  $\alpha_g$ , gray;  $\beta/\delta$ , light blue ( $\gamma$  and  $\alpha_d$  not displayed). Boxed area is the  $\alpha_g$  transmitter binding site. Horizontal lines approximate the location of the membrane. Right, close-up of the  $\alpha_g$  binding site. Green, loop A ( $\alpha$ Tyr93 shown); tan, loop C ( $\alpha$ Tyr190 shown); magenta, loop B (all side chains 147–153 shown, C $\alpha$  atoms of  $\alpha$ Gly147 and  $\alpha$ Gly153 as spheres). B, cyclic activation scheme for the AChR. A is the agonist and the other bold letters represent stable ground states (structural ensembles represented by wells in an energy diagram). Paired arrows represent the unstable intermediates that connect the ground states. R, resting (low affinity for the agonist and low ionic conductance);  $R^*$ , active (high affinity for the agonist and high ionic conductance); D, desensitized states (high affinity for the agonist and low ionic conductance) are gray. Next to the arrows are the salient equilibrium constants.  $E_0$ , unliganded (spontaneous) gating;  $E_1$ , monoliganded gating;  $E_2$ , diliganded gating;  $K_d$ , dissociation constant for agonist binding to R;  $J_d$ , dissociation constant for agonist binding to  $R^*$ . The two binding sites have approximately the same  $K_d$  and  $J_d$  for ACh and choline (Jha and Auerbach, 2010). Without an external energy source, the net energy change,  $R$  to  $A_2R^*$ , must be equal for the “physiological” pathway  $R \leftrightarrow AR \leftrightarrow A_2R \leftrightarrow A_2R^*$  and for the alternative pathway  $R \leftrightarrow R^* \leftrightarrow AR^* \leftrightarrow A_2R^*$ . Hence,  $E_2/J_d^2 = E_0/J_d^2$ , or  $E_2 = E_0\lambda^2$ .

conformation ( $J_d$ ), the diliganded gating constant ( $E_2$ ), and the allosteric constant ( $E_0$ ). Without an external energy source, the energy required to move from R to  $A_2R^*$  must be the same for the clockwise and counterclockwise paths, hence  $E_2 = E_0\lambda^2$ , where  $\lambda = K_d/J_d$ . The efficacy of an agonist (which is a function only of  $E_2$ ) is the product of two more-fundamental parameters: the allosteric constant ( $E_0$ ) and the coupling constant ( $\lambda$ ) at two transmitter binding sites. In our wt preparation,  $\lambda^{ACh} \approx 6600$ .

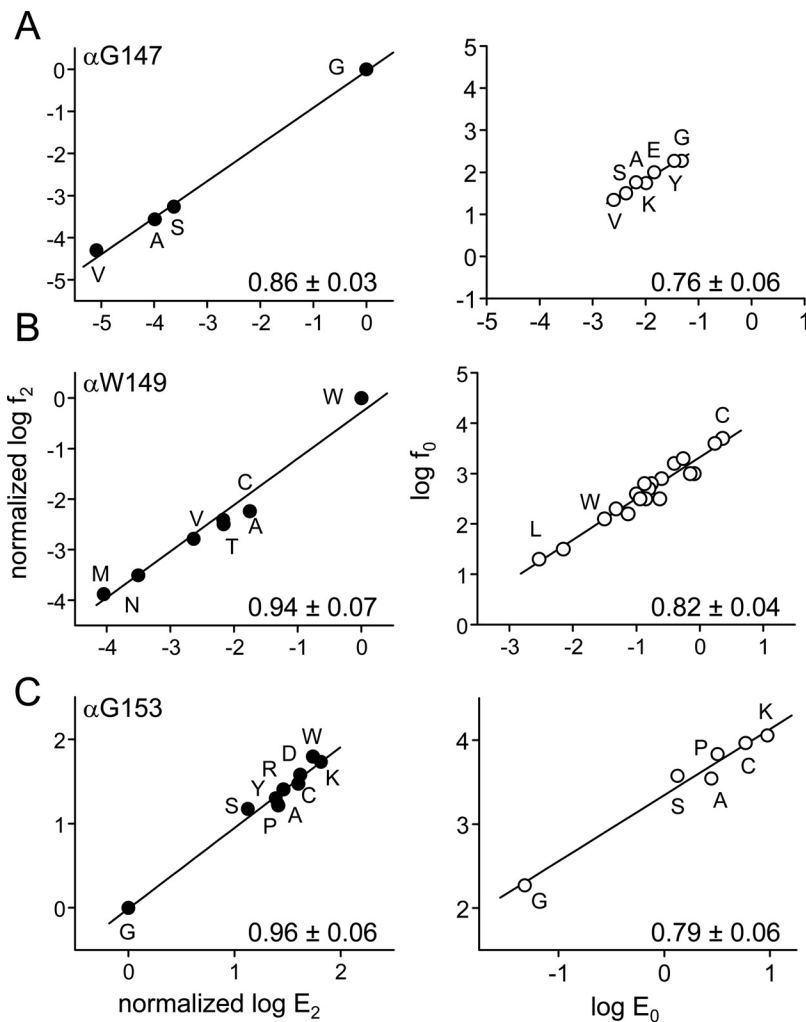
Our objective was to measure  $E_2$  and  $E_0$  for different loop B mutations and calculate  $\lambda [\sqrt{(E_2/E_0)}]$ . For each mutation, the energy (kilocalories per mole) from the ligand affinity change is  $\Delta G^{bind} = -0.59\ln(\lambda)$ , and the energy change for unliganded gating is  $\Delta\Delta G^{gate} = -0.59\ln(E_0^{mut}/E_0^{wt})$ . In wt AChRs, we estimate that each ACh molecule is  $\Delta G^{bind} \approx -5.2$  kcal/mol more stably bound in  $R^*$  versus R. We also calculated the change in binding energy caused by the mutation,  $\Delta\Delta G^{bind} = \Delta G^{bind, mut} - \Delta G^{bind, wt}$ .

**$\alpha$ Gly147.** The results for  $\alpha$ Gly147 are summarized in Fig. 2. This residue is conserved in mouse AChR  $\alpha$ -subunits and is also present in the acetylcholine binding protein. We examined 10 different side chain substitutions of this amino acid (Ala, Ser, Val, Asp, Glu, Arg, Lys, Trp, Tyr, and Pro), but single-channel currents activated by 1 mM ACh were apparent only with the Ser, Ala, and Val mutants. Some of the silent  $\alpha$ Gly147 mutations express functional AChRs. The

$\alpha$ G147S, Ala and Val single-channel currents were apparent but did not occur in clusters when activated by 1 mM ACh. The effective opening rate at this concentration ( $R \rightarrow A_2R^*$ ) is small because either  $K_d$  was large,  $E_2$  was small, or both.

To enhance cluster formation and allow the estimation of gating rate and equilibrium constants, we expressed these mutants on a background that increased  $E_2$  by  $\sim 450$ -fold (see Supplemental Fig. S1 for example current traces). This background “boosts”  $E_0$  to allow cluster formation and rate constant estimation but has no effect on the loop B mutation’s effects on  $E_0$  or  $\lambda$ . Using this background, and by fully saturating the transmitter binding sites by using 140 mM ACh (Supplemental Fig. S2), we estimate that the  $\alpha$ G147S, Ala and Val mutations indeed decrease  $E_2^{ACh}$  substantially (Supplemental Table S1).

Our next objective was to measure  $E_0$  for the  $\alpha$ Gly147 mutants. For these experiments, we used a background construct that was active spontaneously so that the effect of the loop B mutation could be estimated directly from the durations of intervals within spontaneous clusters (Purohit and Auerbach, 2009). With this background, 6 of the 10 substitutions at  $\alpha$ Gly147 (Ala, Ser, Val, Glu, Lys, Tyr) produced spontaneous single-channel current clusters. Asp, Arg, Pro, and Trp side-chain substitutions did not result in the expression of functional AChRs. All of the expressing mutants reduced  $E_0$  relative to the background (Supplemental Table



**Fig. 2.** R/E relationships for three loop B residues. Left, diliganded gating; right, unliganded (spontaneous) gating. The x-axis is the logarithm of the gating equilibrium constant ( $E_2$  or  $E_0$ ) and the y-axis is the logarithm of the forward, channel-opening rate constant ( $f_2$  or  $f_0$ ). A,  $\alpha$ Gly147 mutations reduce  $E_2$  but mainly increase  $E_0$  [values from Purohit and Auerbach (2010)]. B,  $\alpha$ Trp149 mutations reduce  $E_2$  but mainly increase  $E_0$  to similar extents (Fig. S3). C,  $\alpha$ Gly153 mutations increase  $E_2$  and  $E_0$  to similar extents (Fig. S3). The kinetic parameters are shown in Supplemental Tables S1 and S2. The number in each panel is the R/E slope ( $\Phi$ )  $\pm$  S.D. of the linear fit, which gives the relative timing of the residue’s gating motion (larger is earlier). All three residues move early, but the diliganded  $\Phi$  values are larger than the unliganded ones.

S2). However, even the largest reduction ( $\alpha$ G147V) was modest, only  $\sim 20$ -fold less than the background.

With these estimates of  $E_0$  and  $E_2$  for the Ser, Ala, and Val mutants of  $\alpha$ Gly147, we calculated  $\lambda$  and  $\Delta G^{\text{bind}}$  for ACh (Supplemental Table S3). On average, for these three  $\alpha$ Gly147 mutants  $\lambda^{\text{ACh}} \sim 194$  and  $\Delta \Delta G^{\text{bind}} \sim +2.1$  kcal/mol. Compared with the wt values for ACh, these mutations decreased the net ACh binding energy by  $\sim 60\%$ . In contrast, the average effect of these mutations on the allosteric constant was smaller ( $\Delta \Delta G^{\text{gate}} \sim +1.4$  kcal/mol). The main effect of mutating  $\alpha$ Gly147 is to reduce the amount of energy made available by the affinity change at the transmitter binding site.

The slope of the R/E plot ( $\Phi$ ) for the  $\alpha$ Gly147 diliganded gating was 0.86 (Fig. 2A). This indicates that with ACh present in the binding sites, this residue experiences a change in energy near the onset of the channel-opening process, at approximately the same time as the energy change of the agonist molecule itself (Grosman et al., 2000). Interestingly, the  $\Phi$  value for the unliganded reaction (0.76), although high, was smaller than that for the diliganded reaction.

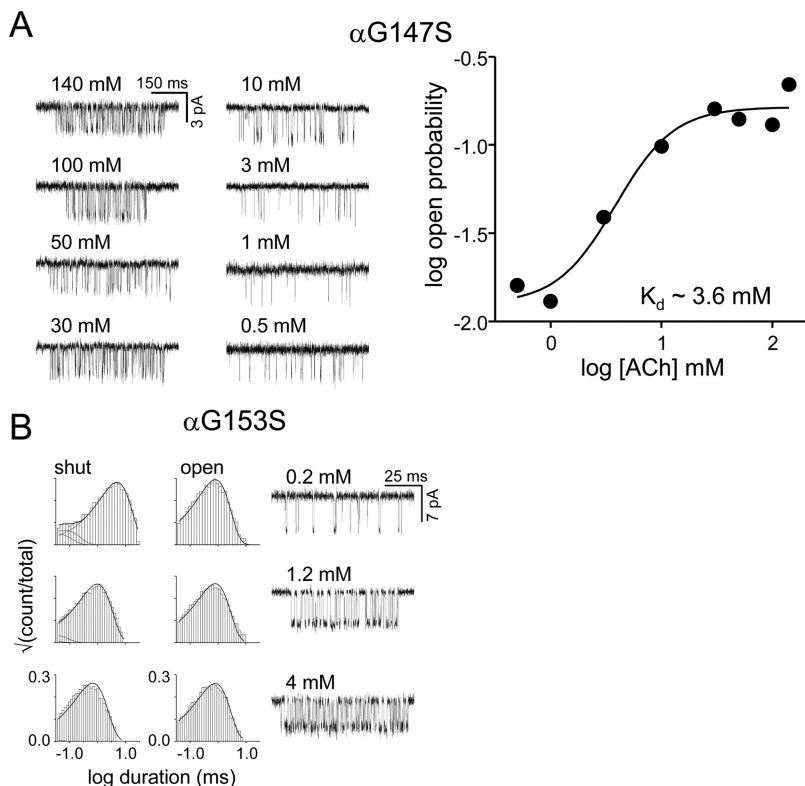
The reduction in  $\lambda^{\text{ACh}}$  in  $\alpha$ G147S could be caused by an increase in the R affinity (a decrease in  $K_d$ ), a decrease in the R\* affinity (an increase in  $J_d$ ), or both. We estimated  $K_d$  by examining the  $\alpha$ G147S mutant activated by different concentrations of ACh (Fig. 3A). We estimate that  $K_d$  for ACh in this construct is  $\sim 3.6$  mM, which is  $\sim 25$  times higher than that of the wt (Chakrapani et al., 2003). Using the  $E_2$  and  $E_0$  values for this mutant and the equation  $E_2 = E_0 \lambda^2$ , we calculate that  $J_d$  for ACh in this construct is  $12.1 \mu\text{M}$ , which is  $\sim 550$  times higher than that of the wt (Jha and Auerbach, 2010). Thus, the reduction in the amount of energy made available by the affinity change at the transmitter binding site can be attrib-

uted mainly to a decreased affinity for ACh in the R\* conformation of the binding site.

**$\alpha$ Trp149.**  $\alpha$ Trp149 is an important binding site residue that provides binding energy to the ligand in both the R and R\* conformations, probably by cation- $\pi$  interactions (Zhong et al., 1998). We have shown previously that mutations of  $\alpha$ Trp149 also influence  $E_0$  (Purohit and Auerbach, 2010). Some of these mutations caused a substantial increase in  $E_0$  ( $\Delta \Delta G^{\text{gate}} = -2.3$  kcal/mol for  $\alpha$ W149C), but most had more modest or negligible effects ( $\Delta \Delta G^{\text{gate}} = +0.3$  kcal/mol for  $\alpha$ W149M).

We measured the effects of  $\alpha$ Trp149 mutations on diliganded gating to estimate  $\lambda^{\text{ACh}}$  (Fig. 2B and Supplemental Fig. 1S). All of the tested mutations reduced  $E_2$ , the largest effect being a  $\sim 10,000$ -fold reduction for  $\alpha$ W149M (Supplemental Table S1). For all mutants, the predominant effect was to reduce the net agonist binding energy. The clearest example is  $\alpha$ W149M, for which  $E_0$  was almost unchanged but  $\lambda^{\text{ACh}}$  decreased by  $\sim 80$ -fold ( $\Delta \Delta G^{\text{bind}} = +2.6$  kcal/mol). On average, the  $\alpha$ Trp149 mutations reduced the R versus R\* ACh binding energy by  $\sim 50\%$ . As was the case with  $\alpha$ Gly147, the diliganded  $\Phi$  value for  $\alpha$ Trp149 was higher than the corresponding value for unliganded gating (0.94 versus 0.82) (Fig. 2B).

**$\alpha$ Gly153.**  $\alpha$ Gly153 is an interesting loop B residue, because a Ser here reduces  $K_d$  and causes a myasthenic syndrome (Sine et al., 1995). In addition, AChRs that have a high affinity for nicotine (with  $\alpha 2$ ,  $\alpha 3$ , or  $\alpha 4$  subunits) have Lys here, whereas those that do not ( $\alpha 1$  or  $\alpha 7$ ) have a Gly. We measured  $E_0$  and  $E_2$  for several  $\alpha$ Gly153 mutants. In these experiments, we used the partial agonist choline (Cho) because  $\alpha$ Gly153 mutations increased the opening rate constant to such an extent that it was not possible to obtain estimates using ACh.



**Fig. 3.** Equilibrium dissociation constants. A, the affinity of the resting  $\alpha$ G147S AChR for ACh was estimated from the  $P_o$  dose-response profile. B,  $K_d$  values for choline were estimated for  $\alpha$ G153S by fitting interval durations across concentrations. The rate constants and  $K_d$  values for all  $\alpha$ Gly153 mutants were similar and are given in Supplemental Table S4.

All of the tested  $\alpha$ Gly153 side-chain substitutions (Lys, Trp, Asp, Cys, Arg, Tyr, Ala, Pro, and Ser) produced functional AChRs that had a higher  $E_2$  values compared with wt (Fig. 2C and Supplemental Table S1). We also measured  $E_0$  for all nine of these  $\alpha$ Gly153 mutants. AChRs with Trp, Asp, Arg, or Tyr substitutions produced single-channel currents having multiple open/closed components. The fractional contribution of each of these components was variable between patches, to an extent that  $E_0$  could not be estimated reliably. The other substitutions had more simple kinetic behavior and exhibited clearly predominant open and closed components. All of these substitutions increased  $E_0$  relative to the background, from 206-fold for Lys to 28-fold for Ser (Supplemental Table S2).

The fold-changes in  $E_2$  versus  $E_0$  for the  $\alpha$ Gly153 mutations are shown in Supplemental Fig. S3. The maximum effect on both  $E_2$  and  $E_0$  was for the Lys substitution. Overall, the fold changes in  $E_2$  and  $E_0$  were linearly correlated ( $r^2 = 0.99$ ) (Supplemental Fig. S3). This result indicates that  $\alpha$ Gly153 mutations alter diliganded gating by an approximately parallel change  $E_0$  and have almost no effect on  $\lambda^{\text{Cho}}$  (Supplemental Table S3). Indeed, the average  $\Delta\Delta G^{\text{bind}}$  for the Lys, Cys Ala, Pro, and Ser mutants was only +0.3 kcal/mol. Although  $\alpha$ Gly153 mutations increase the affinity of the resting binding site, they have the same quantitative effect on the affinity of the active binding site. We conclude that  $\alpha$ Gly153 makes essentially no contribution to the binding energy difference between R and R\*. As was the case for  $\alpha$ Gly147 and  $\alpha$ Trp149, the  $\alpha$ Gly153 diliganded  $\Phi$ -value was higher than that for unliganded gating (0.96 versus 0.79) (Fig. 2C).

We also measured the single-site association and dissociation rate constants and equilibrium dissociation constant for Cho binding to the resting conformation for four different  $\alpha$ Gly153 mutants (Ser, Ala, Asp, and Lys) (Fig. 3B and Supplemental Table S4). All of the mutations reduced  $K_d$  (increased the resting affinity) compared with the wt (Supplemental Table S4) to approximately the same extent (mean,  $\sim 14$ -fold).

**Other Loop B Mutations.** We measured  $E_2$  and  $E_0$  for Ala side-chain substitutions at the five residues between the two loop B glycines (Thr148, Trp149, Thr150, Tyr151, and Asp152). All of these constructs except  $\alpha$ W149A resulted in only small ( $<2$ -fold) changes in both  $E_2$  and  $E_0$  compared with the wt (Tables S1 and S2 and Supplemental Fig. S1F). In  $\alpha$ W149A,  $E_0$  increased by  $\sim 8$ -fold, but  $E_2$  decreased by  $\sim 134$ -fold. Using the equation  $E_2 = E_0\lambda^2$ , we estimate that for this mutant,  $\lambda^{\text{ACh}} \sim 200$  and  $\Delta\Delta G^{\text{bind}} = +2.0$  kcal/mol.

The  $E_0$  values were estimated for a few additional mutants of these positions (Supplemental Table S2 and Supplemental Fig. S1). At residue  $\alpha$ Asp152, only the Ala substitution resulted in functional AChRs (no currents for Trp, Lys, Arg, or Tyr). At positions  $\alpha$ Thr148,  $\alpha$ Thr150, and  $\alpha$ Tyr151, the largest fold decrease in  $E_0$  was for  $\alpha$ T150Y ( $\sim 25$ -fold).

Global estimates for the energetic consequences of loop B perturbations from R/E plots for all the mutants are shown in Supplemental Fig. S4. The  $\Phi$ -values for diliganded and unliganded gating were 0.92 and 0.77, respectively. The largest fold increase in  $E_0$  was for  $\alpha$ G153K and the largest fold decrease was for  $\alpha$ T150Y. This range (for both  $\alpha$ -subunits combined) corresponds to an energy of  $\sim 5.5$  kcal/mol.

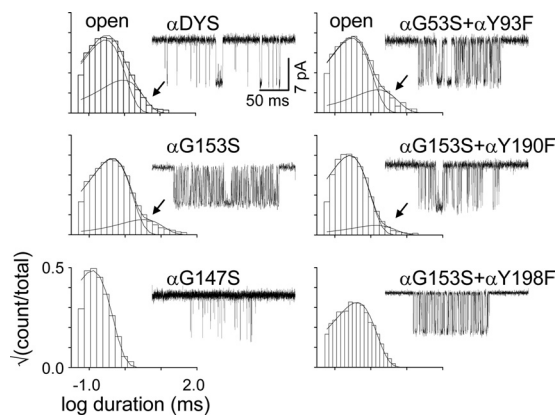
**Multiple Open-Channel Lifetimes.** We now turn to the description of multiple open-time components, which are common in unliganded AChRs (Jackson, 1986; Grosman, 2003; Mukhtasimova et al., 2009; Purohit and Auerbach, 2009). These events are worth examining, in part, because they have been interpreted as reflecting an intermediate state in the channel-opening conformational cascade (Mukhtasimova et al., 2009). We reported previously that many binding site mutations eliminate this long-lived component [ $\alpha$ Tyr93 (loop A),  $\alpha$ Trp149 (loop B), and  $\alpha$ Tyr190/ $\alpha$ Tyr198 (loop C)], even when combined with distant mutations in the transmembrane domain that alone make it more prevalent simply because these have low  $\Phi$  values and therefore slow all closures (Purohit and Auerbach, 2010). This observation led us to conclude that the long openings arise from local structural fluctuations of binding site residues, primarily of  $\alpha$ Trp149.

$\alpha$ Gly153 mutations did not eliminate the long-open component and, in fact, enhanced them in some cases. We therefore tested whether or not substitutions at the abovementioned positions that eliminate spontaneous long openings do so when a  $\alpha$ G153S mutation is also present (Fig. 4). We examined five double-mutant pairs,  $\alpha$ G153S+[ $\alpha$ W149F,  $\alpha$ Y93F,  $\alpha$ Y190F,  $\alpha$ Y198F, or  $\alpha$ G147S] where the bracketed mutation was previously shown to eliminate long openings. The  $\alpha$ W149F,  $\alpha$ Y198F, and  $\alpha$ G147S mutations again eliminated the long-open component, but the  $\alpha$ Y93F and  $\alpha$ Y190F substitutions no longer did.

Inter-residue coupling energies for  $\alpha$ G153S paired with a Phe substitution at four  $\alpha$  subunit binding site aromatic residues ( $\alpha$ Tyr93,  $\alpha$ Trp149,  $\alpha$ Tyr190, and  $\alpha$ Tyr198) and  $\alpha$ Gly147 are shown in Supplemental Table S5. In all cases, the Ser-Phe side-chain pairs behaved nearly independently in unliganded gating.

## Discussion

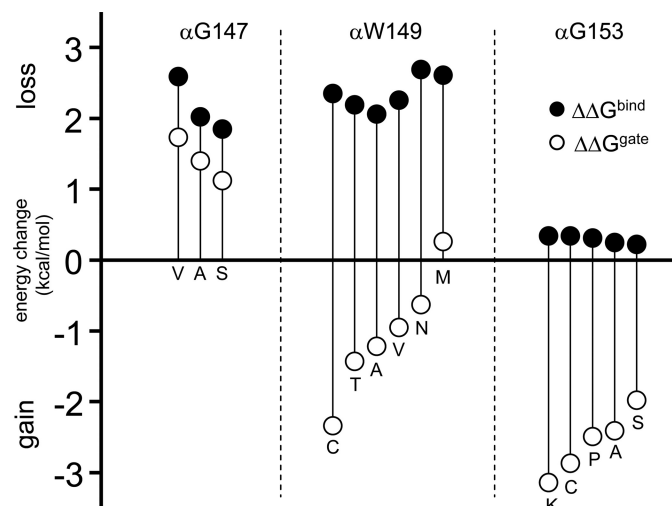
The results (summarized in Fig. 5 and Table 1) provide insight into the nature of the energy changes at the AChR transmitter binding site when it changes conformation be-



**Fig. 4.** Multiple open components. Example histograms and single-channel current traces for different loop B mutants. Arrow indicates the long-opening component. There was no agonist present (spontaneous currents). In all constructs, the background was  $\alpha$ DYS (Supplemental Data). Long openings are apparent in the background (no binding site mutations) and with the binding site mutations  $\alpha$ G153S, ( $\alpha$ G153S+ $\alpha$ Y93F), and ( $\alpha$ G153S+ $\alpha$ Y190F). They were absent with the mutations ( $\alpha$ G153S+ $\alpha$ G147S) and ( $\alpha$ G153S+ $\alpha$ Y198F). The long openings possibly arise from a local isomerization of  $\alpha$ Trp149 (Purohit and Auerbach, 2010).

tween its low-affinity R and high-affinity R\* conformation. It is this change in conformation that triggers the gating conformational cascade and determines ligand efficacy.

In brief,  $\alpha$ Gly147 and  $\alpha$ Gly153 seem to act as hinges that influence the allosteric constant ( $E_0$ ), the resting affinity ( $K_d$ ), and the agonist affinity ratio ( $\lambda$ ). However, these hinges have opposite actions. Mutations of  $\alpha$ Gly147 change the above parameters so that there is a decrease in  $P_o$ , whereas  $\alpha$ Gly153 mutations change them to cause an increase in  $P_o$ . Below, we associate a change in energy with a local change in structure (a “strain” or “movement” of the perturbed position



**Fig. 5.** Binding site mutations change the energy of intrinsic gating and agonist binding. Closed symbols, the gating free energy change [ $\Delta\Delta G^{\text{gate}} = -0.59\ln(E_0^{\text{mut}}/E_0^{\text{wt}})$ , in kilocalories/mole]. Open symbols, the binding free energy change or [ $\Delta\Delta G^{\text{bind}} = -0.59\ln(\lambda^{\text{mut}}/\lambda^{\text{wt}})$ ]. Favorable mutations (that produce a negative  $\Delta\Delta G$ ) increase the  $P_o$ . Left,  $\alpha$ Gly147 mutations are unfavorable with regard to both gating and binding, but the effect is larger for binding. Center,  $\alpha$ Trp149 mutations mostly are favorable with regard to gating but have larger and unfavorable effects on binding. Right,  $\alpha$ Gly153 mutations have a large and favorable effect on gating but little effect on binding. The values are listed in Table 1.

TABLE 1

Energy changes of unliganded gating ( $E_0$ ) and agonist binding ( $\lambda$ ) for different loop B mutants

The units are kilocalories per mole. The values are shown in graphical form for  $\alpha$ Gly147,  $\alpha$ Trp149, and  $\alpha$ Gly153 in Fig. 5.  $\lambda$  is the R/R\* affinity ratio ( $K_d/J_d$ ) for the appropriate agonist.  $\Delta\Delta G^{\text{bind}} = -0.59\ln(\lambda^{\text{mut}}/\lambda^{\text{wt}})$  and  $\Delta\Delta G^{\text{gate}} = -0.59\ln(E_0^{\text{mut}}/E_0^{\text{wt}})$ . See Supplemental Tables S1 to S3.

Construct	Ligand	$\lambda$ ( $K_d/J_d$ Ratio)	Net Binding ( $\Delta\Delta G^{\text{bind}}$ )	Gating ( $\Delta\Delta G^{\text{gate}}$ )
wt <sup>ACh</sup>	ACh	6587	0.00	0.00
wt <sup>cho</sup>	Cho	266	0.00	0.00
G147V	ACh	82	2.59	1.73
G147A	ACh	215	2.02	1.40
G147S	ACh	287	1.85	1.12
W149C	ACh	123	2.35	-2.34
W149T	ACh	161	2.19	-1.43
W149A	ACh	202	2.06	-1.22
W149V	ACh	143	2.26	-0.95
W149M	ACh	69	2.69	-0.63
W149M	ACh	79	2.61	0.26
G153K	Cho	151	0.34	-3.14
G153C	Cho	151	0.34	-2.87
G153P	Cho	159	0.31	-2.49
G153A	Cho	175	0.25	-2.41
G153S	Cho	184	0.22	-1.98
T148A	Cho	196	0.18	-0.28
T150A	Cho	161	0.00	-0.64
Y151A	Cho	521	-0.39	1.04
D152A	Cho	339	-0.14	0.13

relative to its immediate environment), and use the word “favorable” to describe a molecular motion that increases  $P_o$ .

$\alpha$ Gly147 mutations reduced  $E_0$ , which is to say that in the absence of agonists, the energy change associated with the gating motion of this residue is unfavorable for all side-chain substitutions. Although we cannot with certainty identify the structural correlate(s) of this unfavorable energy change, there are some clues. Because all tested side chains substitutions were unfavorable, we speculate that in the absence of ligands, there is a favorable gating motion in the wt that requires flexibility of the backbone (an “activation” hinge). However, different amino acid substitutions caused modestly different degrees of energy change, so side-chain interactions, too, are likely to be involved. Because the larger Val side chain experienced the largest gating energy change, it is possible that steric hindrance is a factor that sets the magnitude of  $E_0$ .

Using the cyclic activation scheme and the equation  $E_2 = E_0\lambda^2$  (see below), we conclude that the  $\alpha$ Gly147S, -A, and -V mutants reduce  $\lambda^{\text{ACh}}$  from  $\sim 6600$  in the wt to  $\sim 194$ . This corresponds to a net loss in ACh binding energy of  $\Delta\Delta G^{\text{bind}} \sim +2.1$  kcal/mol per site. The  $\alpha$ Gly147S mutation reduced the affinity of both the resting and active binding sites, but the effect was larger for R\* compared with R. Hence, the main effect of this mutation seems to be to reduce the stability of the bound transmitter in the R\* conformation. This implies that with ligand present in wt AChRs, at the onset of the forward isomerization the structural change at  $\alpha$ Gly147 results in a net favorable energetic interaction of the protein with the transmitter molecule.

There are some hints regarding the structural basis of this event. Ser and Ala are both small and have a similar effect on  $\lambda^{\text{ACh}}$ , whereas the larger Val side chain had a somewhat larger effect. This suggests that  $\Delta\Delta G^{\text{bind}}$  is influenced by side-chain size as well as by the elimination of the abovementioned intrinsic favorable backbone motion.  $\alpha$ Gly147 is far from the ligand (Celie et al., 2004), so this binding energy change is likely to be indirect and involve additional structural elements at the binding site. Regardless, without the activation hinge at position  $\alpha$ Gly147 the residual net ACh binding energy is reduced from  $-5.2$  to  $-3.1$  kcal/mol/site. Interfering with the gating motion of  $\alpha$ Gly147 decreases the binding energy used to promote gating by  $\sim 60\%$ .

Mutations of  $\alpha$ Trp149 had approximately the same magnitude of effect on  $E_0$  as those at position  $\alpha$ Gly147; in contrast, without agonists, most  $\alpha$ Trp149 side-chain substitutions were *more* stable in R\* compared with R. The order of effect (C > T > A > V > N > M) does not immediately suggest a chemical basis of the more-favorable R\* environment around  $\alpha$ Trp149 in the unliganded site, except to note that the energy changes varied substantially with different side chains and that steric hindrance does not seem to be a factor. We speculate that the gating motion of  $\alpha$ Trp149 in the unliganded binding site involves a change in the positions of side-chain atoms in a spatially unrestricted environment.

The  $\alpha$ Trp149 mutant binding energy changes were large and unfavorable for all substitutions. There was only a small range or energies for the binding energy loss ( $\sim 0.6$  kcal/mol, Ala to Asn), which suggests that for the residues we examined, only Trp provides the extra stabilization energy to the bound ligand, probably via a cation- $\pi$  interaction (Zhong et al., 1998). This energy constitutes approximately half of the

total net ACh binding energy change. We speculate that the residual binding energy ( $\sim -2.7$  kcal/mol per site) comes from interactions between the ligand and other binding site entities, particularly  $\alpha$ Tyr190 (Purohit and Auerbach, 2010).

$\alpha$ Gly153 is quite different from both  $\alpha$ Gly147 and  $\alpha$ Trp149. Essentially all of the energetic effects of mutations here were with regard to  $E_0$ , with very little with regard to  $\lambda^{\text{cho}}$ . In the unliganded binding site,  $\alpha$ Gly153 moves and, as was the case with  $\alpha$ Trp149, with side-chain substitutions here, the AChR was in all cases *more* stable in R\* compared with R. The fact that the  $E_0$  range of gating energy change here was small ( $\sim 1$  kcal/mol) suggests that side-chain size does not play a significant role. We speculate that in both the presence and absence of agonists this glycine adopts an unfavorable R\* backbone configuration (a “deactivation” hinge) and that all mutations here prevent this from happening.

Although all  $\alpha$ Gly153 mutations reduced  $K_d$  (see below), they also reduced  $J_d$  to approximately the same extent (Supplemental Table S4). We conclude that the  $\alpha$ Gly153 does not provide agonist binding energy for the gating isomerization, either directly or indirectly. This conclusion bears directly on the recent report proposing that the  $\alpha$ Gly153 side chain is the basis for differences in the  $EC_{50}$  value for the partial agonist nicotine in different AChRs (Xiu et al., 2009). The observations that led to this hypothesis were that the  $\alpha$ G153K mutation decreases the neuromuscular  $EC_{50}$  for nicotine and restores the fingerprint of a cation- $\pi$  interaction between  $\alpha$ Trp149 and this agonist.  $EC_{50}$  is a complex parameter that reflects both binding and gating. Our results suggest that for  $\alpha$ G153K, the 14-fold reduction in  $EC_{50}$  can be attributed almost completely to the 206-fold increase in  $E_0$ . Our evidence suggests that a Lys side chain at this position increases the efficacy of all agonists equally and not specifically that of nicotine.

Previously we reported that for seven different locations (all far from the binding sites),  $\Phi$  values were the same for diliganded and unliganded gating (Purohit and Auerbach, 2009). Here, we found that the  $\Phi$  values for  $\alpha$ Gly147,  $\alpha$ Trp149, and  $\alpha$ Gly153 were all higher in the liganded versus unliganded condition. This implies that these residues experience their gating energy change relatively earlier when an agonist molecule is nearby. This could be because the residue itself moves earlier, the peak transition state for the overall protein isomerization occurs later, or both. Regardless, the shift in  $\Phi$  is an indication of an “induced fit,” whereby the agonist molecule perturbs (rearranges) the binding site so as to alter the timing, pathway, or ground states of the isomerization. The effect we have observed is small, and other results suggest that the cyclic scheme is still a valid approximation even with a perturbed binding site (i.e., that many different agonists have the same  $\Phi$  value) (Grosman et al., 2000) and that the effect of  $\alpha$ Gly153 mutations can be explained simply by a change in  $E_0$ . Nonetheless, the shift in  $\Phi$  values with versus without ligand suggests that the cyclic scheme and the equation  $E_2 = E_0\lambda^2$  may not pertain in detail to all AChRs that have binding site perturbations.

An ancillary part of this study was to measure  $K_d$  and calculate  $J_d$  for a few mutant constructs of the two loop B Gly residues. These equilibrium constants pertain to ligand binding, and the underlying molecular movements for this separate process may or may not be distinct from those for gating. Mutations of  $\alpha$ Gly147 and  $\alpha$ Gly153 had opposite effects on

$K_d$ . The  $\alpha$ G147S mutation reduced the affinity of the resting binding site for ACh, whereas four different substitutions of  $\alpha$ Gly153 increased this affinity. Apparently, backbone flexibility at these positions, perhaps in combination, is important for allowing agonists to bind with the physiologically appropriate resting affinity.

The long openings in spontaneous currents appear to arise from a local isomerization of the  $\alpha$ Trp149 side chain (Purohit and Auerbach, 2010). This fluctuation in structure is delicate because it can be eliminated by a variety of binding site perturbations, including the presence of an agonist molecule, all  $\alpha$ Trp149 side-chain substitutions, and most substitutions of  $\alpha$ Gly147,  $\alpha$ Tyr93,  $\alpha$ Tyr190, and  $\alpha$ Tyr198 (but not of  $\alpha$ Gly153 and  $\epsilon/\delta$ W55/57). We speculate that in the absence of a ligand there is a network of interactions, local to the binding site that permits  $\alpha$ Trp149 to adopt alternative conformations. One of these is associated with the relatively rare, long openings.

In summary, loop B has two glycine hinges that have opposing actions in binding and gating of wt AChRs. Removal of the activation hinge at  $\alpha$ Gly147 decreases  $P_0$  by reducing  $K_d$ ,  $E_0$ , and  $\lambda$ . The main effect of interfering with this hinge is to reduce the affinity of the active conformation of the binding site and, hence, the amount of binding energy available to promote gating. In addition, mutation  $\alpha$ Gly147 eliminates long openings that likely arise from alternative conformations of  $\alpha$ Trp149. The deactivation hinge at  $\alpha$ Gly153 plays an inverse role. Interfering with the operation of this hinge increases  $P_0$  by increasing both  $K_d$  and  $E_0$  but has essentially no consequence with regard to  $\lambda$ . Mutations of  $\alpha$ Gly153 also increase the probability of long openings. Between these two hinges is  $\alpha$ Trp149, a residue that provides approximately half of the binding energy toward the gating isomerization. It is possible that the two opposing glycine hinges act in concert (along with other elements at the binding site) to determine  $E_0$  and  $K_d$  and to position the central tryptophan so that it provides the physiologically appropriate binding energy to generate the synaptic response.

#### Acknowledgments

We thank M. Shero, M. Merritt, and M. Teeling for technical assistance.

#### Authorship Contributions

Participated in research design: Purohit.

Conducted experiments: Purohit.

Performed data analysis: Purohit.

Wrote or contributed to the writing of the manuscript: Purohit and Auerbach.

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