Linear Free-Energy Relationships and the Dynamics of Gating in the Acetylcholine Receptor Channel

A Φ -value analysis of an allosteric transition at the single-molecule level

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Abstract. The muscle acetylcholine receptor channel (AChR) is a large ($M_r \cong 290K$) transmembrane protein that mediates synaptic transmission. The activation of this ion channel can be understood in the framework of a thermodynamic cycle with spontaneous gating (i.e., the closed $\rightleftharpoons$ open reaction) and ligand-binding events as the elementary steps. Because agonists bind more tightly to the open than to the closed state, gating of liganded receptors is more favorable than that of unliganded receptors. Accordingly, channel opening must involve two major conformational changes: the ACh-binding sites switch from a low-affinity to a high-affinity form, and the pore (located ~ 45 Å away from the binding sites) switches from an ion-impermeable to an ion-permeable conformation. To gain insight into the reaction mechanism of fully-liganded gating, we characterized the corresponding transition state in the context of the 'linear free-energy relationships' of physical organic chemistry (Φ -value analysis). Gating of fully-liganded AChRs was studied by recording single-channel currents using the patch-clamp technique. Perturbations to the wild-type receptor were either series of different mutations at individual positions or series of different agonists. Based on the obtained 'snapshot' of the gating reaction at the transition state, and aware of the lack of information about the rest of the energy profile, the most parsimonious mechanism seems to be one where opening proceeds asynchronously, with the low-to-high affinity change at the binding sites preceding the complete opening of the distant pore.

Key words: Brønsted plots, ion channels, nicotinic receptors, transition state

1. Introduction

The muscle acetylcholine receptor channel (AChR), also known as the muscle nicotinic receptor, is the neurotransmitter-gated ion channel that mediates synaptic transmission at the vertebrate neuromuscular junction. Under physiological conditions, the extracellular domain of this receptor is exposed to brief (~ 200 μ s) pulses of saturating (~ 1 mM) acetylcholine (ACh), which eventually leads to the transient opening of the cation-selective channel pore. The resulting inward current depolarizes the muscle and this, in turn, activates muscular sodium channels, thus initiating an action potential. These functional aspects of the AChR are common to all other

neurotransmitter-gated ion channels, the differences lying in the neurotransmitter in question (γ -aminobutyric acid, serotonin, glycine, glutamate, ATP), the ion-selectivity of the pore (cationic or anionic), the postsynaptic effects of channel activation (excitation or inhibition), and the types of cell involved in the synapse (neuron-muscle or neuron-neuron).

From a thermodynamic point of view, the activation of neurotransmitter-gated ion channels can be understood in the framework of a cyclic scheme with unliganded gating (i.e., the closed $\rightleftharpoons$ open reaction of the vacant receptor) and ligand-binding events as the elementary steps [1, 2]. Because agonists bind more tightly to the open than to the closed state, gating of liganded receptors is more favorable than that of unliganded receptors [3–5]. Accordingly, channel opening must involve two major conformational changes: the transmitter-binding sites switch from a low-affinity to a high-affinity form, and the pore (located ~ 45 Å away from the binding sites, in the case of the AChR [6]) switches from an ion-impermeable to an ion-permeable conformation. This leads to a number of questions:

- i) What is the mechanism (i.e., the sequence of molecular events) during channel gating? Does the conformational change propagate through the receptor? Does the isomerization start at the binding sites, with the low-to-high ligand affinity change, and then ‘travel’ through the protein until it reaches the gate and opens it? Or, does the conformational change start at the gate and travel to the binding site, instead? Does the conformational change travel at all, or does it occur synchronously, everywhere in the protein?
- ii) Is the mechanism of gating the same for liganded and unliganded receptors? Is it the same for different ligands?

These questions may not be important in the case of small ion channels like the KcsA or inward-rectifier K^+ channels, for example, which have few transmembrane segments and where most of the protein occurs in the transmembrane domains. In these cases, the structure of the channel in the closed and open states may be all that is needed to postulate a plausible sequence of events between the two end states. The situation is clearly different, however, in the case of neurotransmitter-gated ion channels, where the key functional domains are separated by large distances. Here, as in the case of any other large allosteric protein, observation of structural data alone would not suggest any particular reaction mechanism. This is because any two given structures could be ‘connected’ by a number of different pathways, which, in principle, may seem all equally likely. By exploring the properties of the AChR at the *transition state* of the gating reaction, we propose a specific mechanism by which the closed and open states of fully-liganded receptors interconvert.

2. Mechanisms, Transitions States, and Kinetics

Ideally, the elucidation of the mechanism of gating should entail the determination of the structures of all the conformations, however brief lived, that the channel

visits during its interconversion between the closed and open states. Because gating of fully-liganded muscle AChRs can be modeled as a two-state reaction, the only intermediate conformation whose structure can be inferred is that of the transition state. This can be done by combining the results of kinetic and equilibrium measurements in the context of the 'linear free-energy relationships' of physical organic chemistry [7].

2.1. LINEAR FREE-ENERGY RELATIONSHIPS

The free energies of the reactant, product, and transition state of a reaction are expected to change upon perturbation (say, a mutation in the case of the AChR; Figure 1). Is the magnitude of the transition-state's free-energy change correlated in any way with the magnitude of the free-energy changes undergone by the end states? The answer to this question has been sought from very different perspectives (organic chemistry [7, 8], enzymology [9], protein folding [10], ion-channel gating [11]) and, consistently, two types of extreme behavior have been observed: **a)** the transition-state's free energy is oblivious to the free energies of the end states and, therefore, the transition-state's free-energy change upon perturbation does not bear any correlation whatsoever with those of reactant and products. In other words, rate and equilibrium constants are not related in any predictable manner. **b)** the transition-state's free-energy change upon perturbation is intermediate between those of the end states much like the transition-state's position along the reaction coordinate is. More precisely, the transition-state's free-energy change upon perturbation is the weighted average of the free-energy changes of reactants and products, the weighting coefficients being a measure of how product-like (and how reactant-like) the perturbed region of the reacting molecule is when the transition state is reached [7]. As shown below, this implies that the logarithms of rate and equilibrium constants are linearly related (a 'rate-equilibrium linear free-energy relationship' or LFER, for short). In symbols, in the case of ion-channel gating:

$$(G^{\ddagger p} - G^{\ddagger}) = \Phi(G_{\text{OPEN}}^{op} - G_{\text{OPEN}}^o) + (1-\Phi)(G_{\text{CLOSED}}^{op} - G_{\text{CLOSED}}^o) \quad (1)$$

where $(G^{\ddagger p} - G^{\ddagger})$, $(G_{\text{OPEN}}^{op} - G_{\text{OPEN}}^o)$, and $(G_{\text{CLOSED}}^{op} - G_{\text{CLOSED}}^o)$ are the free-energy changes upon perturbation (denoted by the superscript 'p') experienced by the transition state, the open state, and the closed state, respectively, and Φ ($0 \leq \Phi \leq 1$) is a measure of the extent to which the transition state resembles the open state. Thus, the closer Φ is to 1, the more open-state-like the transition state is. Since the quantities in Equation 1 are not immediately accessible experimentally, it is desirable to rearrange Equation 1 into Equations 2 and 3, which are less intuitive but far more useful [12]:

$$\Delta G_{\text{opening}}^{\ddagger} = \Phi \Delta G_{C \rightleftharpoons O}^o + \Delta G_0^{\ddagger} \quad (2)$$

or

Figure 1. The concept of a linear free-energy relationship [11]. A hypothetical slice of the free-energy landscape of AChR gating showing the most likely (the lowest free-energy) pathway. The energy profile can be considered to be smooth and dominated by a single transition state (i.e., minimally frustrated) based on the single exponential kinetics of fully-liganded gating (Figure 3B). The solid and dashed lines represent the AChR before and after a stabilizing local perturbation (denoted by 'p'), respectively. The arrows were drawn for the case of $\Phi = 0.3$, as an example. This implies that the stabilization of the transition state is 30% like that of the open and 70% like that of the closed state. In turn, this suggests that, when the transition state is reached, the conformational change in the vicinity of the perturbed region is $\sim 30\%$ of the way to the open state. The position of the transition state along the reaction co-ordinate for the AChR as a whole, however, is approximately given by the average of Φ -values of all individual residues. In this figure, we placed it arbitrarily at ~ 0.5 .

$$\Delta G_{\text{closing}}^{\ddagger} = (\Phi - 1) \Delta G_{C \rightleftharpoons O}^o + \Delta G_0^{\ddagger} \quad (3)$$

where the left-hand side values are the activation free energies of opening and closing, $\Delta G_{C \rightleftharpoons O}^o$ is the standard-free-energy change associated with gating, and $\Delta G_0^{\ddagger}$ is the intrinsic activation barrier (i.e., $\Delta G_{\text{opening}}^{\ddagger}$ and $\Delta G_{\text{closing}}^{\ddagger}$ when $\Delta G_{C \rightleftharpoons O}^o = 0$). Finally, expressing the ΔG -values in terms of the logarithms of rate or equilibrium constants gives what is known as the Brønsted equation:

$$\log \beta = \Phi \log \beta/\alpha + \text{constant} \quad (4)$$

or

$$\log \alpha = (\Phi - 1) \log \beta/\alpha + \text{constant} \quad (5)$$

where β and α are the channel's opening and closing rate constants, respectively, and β/α is the gating equilibrium constant. If an LFER held for every position in the protein, then the slopes of Brønsted plots constructed for all possible 'perturbation series' (where a perturbation series is a set of wild-type and mutant receptors differing only in the amino acid at a given position) would provide a very detailed, residue-by-residue description of the transition-state in terms of its resemblance to the closed and open states.

Depending on the mutated position, one or the other types of behavior (**a** or **b**, see above) can be observed for the gating reaction of fully-liganded muscle AChRs (and this is also the case of enzyme-catalyzed reactions and two-state protein folding). Here, we will only focus on those perturbation series that follow an LFER and, thus, that allow us to gain insight into the transition state of gating. As mentioned above, the Φ -value at a given position in the protein is a measure of how open-state-like (or closed-state-like) that region is at the transition state. Although this resemblance is, admittedly, an 'energetic' one, it is generally accepted that a structural correlate must lie behind. Thus, it seems appropriate to state that '... the *structure* of the transition state was inferred from an LFER analysis ...' although, strictly speaking, we are not really dealing with structural data.

2.2. LFER ANALYSIS OF PERTURBATION SERIES

Series of point mutations were engineered at several positions of the muscle AChR (Figure 2), and the corresponding Brønsted plots were constructed. Patch-clamp recordings of single-channel currents were performed on HEK-293 cells expressing wild-type or mutant mouse muscle AChRs [11, 13]. The opening and closing rate constants were estimated from the duration of closed and open dwell times by applying a full-likelihood algorithm [14] to a two-state kinetic model. Because ACh, the physiological neurotransmitter acting on the AChR, opens the wild-type receptor at rates that are close to the time resolution of the recording apparatus, and because most mutations increase this rate, gating of fully-liganded AChRs was studied with choline as the ligand (a weak agonist that supports a much slower opening rate).

Figure 2. Current view of the AChR-subunits' topology. The AChR is a pentamer of homologous subunits with stoichiometry $\alpha_2\beta\delta\epsilon$ in adult muscle. Two agonist binding sites per pentamer are formed by the extracellular N-terminal domains. The channel pore is lined, for the most part, by the five M2 segments. M2 residues are typically numbered 1' to 19' from the intracellular to the extracellular end.

Figures 3 and 4 [13] show data corresponding to the mutation series at position 12' of the second transmembrane segment of the AChR's δ subunit (M2 δ 12'; Figure 2). Figure 5 shows the corresponding Brønsted plot, which extends over a three-order-of-magnitude change (~ 4.0 kcal/mole) in gating equilibrium constant, and has a Φ -value of 0.275 and a correlation coefficient of 0.966 (●). The figure also shows the Brønsted plot that corresponds to fully-liganded gating in the presence of acetylthiocholine, another weak agonist, as the ligand (○). In this case, Φ is 0.346, which is reasonably close to that estimated in the presence of choline.

Figure 3. Single-channel currents through the 12' δ S $\rightarrow$ T AChR mutant [13]. The 12' position of the M2 segment of the δ subunit is occupied by a Ser in the wild-type receptor. The S $\rightarrow$ T mutation increases the opening rate constant and decreases the closing rate constant. **A:** Continuous single channel traces recorded in the cell-attached configuration in the presence of saturating, 20-mM choline in the patch pipette, at $\sim$ 100 mV. For display purposes, the current traces were low-pass filtered at $\sim$ 6 kHz. Openings are downward deflections. The solid horizontal bars indicate clusters of consecutive openings separated by monoexponentially distributed closed intervals. All closed and open intervals within clusters are interpreted as representing the interconversion of individual channels between the closed and open states (i.e., gating). The closed intervals between clusters are interpreted as sojourns in desensitized states. **B:** Monoexponential dwell-time histograms and superimposed density functions.

Together, these results suggest that, at the transition state of gating, the M2 δ 12' position adopts a conformation that is $\sim$ 30% like the one it adopts in the open state, and $\sim$ 70% like the one it adopts in the closed state. The comparable Φ -values obtained in the presence of choline and acetylthiocholine also suggest that, at least for structurally similar ligands, Φ -values are independent of the nature of the agonist. Hence, in what follows, we will assume that the estimated Φ -values reflect a property of ACh-liganded AChRs, even though a different ligand was actually used during the experiments.

Table I summarizes the Φ -values obtained for several regions of the muscle AChR. The perturbations used were point mutations (analyzed in the presence of choline) and different agonists (analyzed on the wild-type receptor), the latter being interpreted as a probe of the transition state at the transmitter binding sites. The values in the Table suggest that, at the transition state of gating, the binding sites

Figure 4. A mutation series at position M2 $\delta 12'$ [13]. The displayed clusters were excised from continuous single-channel recordings of wild-type and mutant receptors in the presence of saturating, 20-mM choline. The mutations were engineered at position 12' of the M2 segment of the δ subunit. The letter above each cluster denotes the amino-acid residue occupying this position. Ser is wild-type. The opening and closing rate constants of each construct in the series were estimated from the distribution of closed- and open-interval durations, and were used to construct the corresponding Brønsted plots. Cell-attached configuration. Membrane potential $\cong -100$ mV. Display cut-off frequency $\cong 6$ kHz. Openings are downwards.

Table I. A snapshot of the fully-liganded AChR gating reaction at the transition state

AChR REGION	Φ -VALUE RANGE
Transmitter binding sites	0.85–1.16
M2-M3 linker	0.43–0.69
Upper half of M2	0.28–0.40
Lower half of M2	–0.09–0.03

Figure 5. Brønsted plots [11]. ●: Plots of $\log \beta$ (or $\log \alpha$) versus $\log \beta/\alpha$ corresponding to the M2 $\delta 12'$ mutation series in the presence of choline (Figure 4) with the exception of the Pro mutant (▲), which was deemed an outlier. ○: Plots of $\log \beta$ (or $\log \alpha$) versus $\log \beta/\alpha$ corresponding to the M2 $\delta 12'$ Ser (wild-type), Thr, and Gln constructs in the presence of saturating, 2-mM acetylthiocholine at +50 mV.

are in the high-affinity form (i.e., open-state-like), the lower half of M2 is in the closed-state conformation, and the M2-M3 linker and the upper half of M2 have structures that are intermediate between the conformations these domains adopt in the closed and open states. It can be gathered that there is a decreasing gradient of 'openness', from the transmitter binding sites to the intracellular half of M2, at the transition state of gating [11].

3. Conclusions

Based on the obtained 'snapshot' of the transition state, and aware of the lack of information about the rest of the energy profile, the most parsimonious mechanism of muscle-AChR gating seems to be one where opening of the fully liganded channel proceeds asynchronously, with the low-to-high affinity change at the binding sites preceding the complete opening of the pore. Because the lowest free-energy path between both end states is the same in either direction, the reverse sequence of events would take place during closing [11].

It may not seem surprising that the conformational change associated with opening of a *ligand-gated* ion channel starts at the binding sites but it is important to emphasize that the agonist is actually not needed for the channel to open [3, 5]. Whether, and how, the gating mechanisms of fully-liganded and unliganded gating differ represents one of the next interesting questions.

The data in Table I also suggest that the conformational change associated with gating does not propagate, exclusively, through the protein backbone (Figure 2). The fact that the Φ -values decrease from the M2-M3 linker to the intracellular half of M2 strongly suggests that the conformational change initiated at the binding sites (in the opening direction) does not reach M2 through the intervening M1 segment but through the M2-M3 linker, to which the binding sites are not directly connected.

4. Final Remark

It is not at all obvious why such simple, almost naive, relationships hold but the fact that most perturbation series result in linear Brønsted plots can hardly be accidental [7, 8, 15]. For the time being, far from trying to understand the physicochemical underpinnings of this finding, we will keep making use of LFERs as an indirect approach to transition-state structure and, hence, mechanisms.

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