Allosteric Mechanisms in the Activation of Ligand-Gated Channels

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INTRODUCTION

Protein-mediated signaling is a fundamental biological process. As a result of research in many different fields, phenomena as diverse as metabolic regulation, behavior, development, and the control of cell division have been reduced to ligand-induced changes in the states of proteins. Thus, a conceptual grasp of the basic mechanisms of protein-mediated signaling is essential to the investigation any biological system at a molecular level. As is common with research on basic questions in biology, model systems are sought in which the most meaningful and appropriate measurements can be made with the greatest precision and ease. The principals revealed by such studies can then be applied widely to other situations. A number of powerful model systems have been developed and exploited to elucidate the structural and energetic changes in proteins during signaling. One of these is the nicotinic acetylcholine receptor, a protein which has emerged as the prototypical ligand-gated channel. This chapter develops the allosteric theory of proteins (Monod et al., 1965), using the acetylcholine receptor to illustrate the principals of this theory. Allosteric analysis of this protein has provided a better understanding of neuromuscular synaptic transmission, the biological process mediated by the acetylcholine receptor. Furthermore, the insights provided by this theory into the activation of the acetylcholine receptor provide a general framework for the analysis of molecular mechanisms of signal transduction.

One of the reasons that the acetylcholine receptor is useful in studies of signal transduction is that two of the functional conformations of this protein are well defined as

open and closed states of an ion channel. Thus, the activation of the receptor involves transitions between these two states. It has made an enormous difference in research in this area that the closed and open states of the acetylcholine receptor can be detected in individual protein molecules with the patch-clamp technique. The sensitivity of this technique has permitted the detection of rare states that account for a minuscule fraction of total receptor but provide valuable clues about the transduction process. It should be noted that the receptor has at least one additional state referred to as desensitized. In the desensitized receptor the channel is closed despite the presence of acetylcholine. Some studies suggest even more conformations of the protein, but these other states complicate matters somewhat, so for the sake of keeping things simple desensitization will receive rather limited attention in this chapter. This is no loss because once the ideas are understood for transitions between closed and open states, they are readily extended to other functional states of the receptor, as well as to ligand-induced transitions in other kinds of proteins. The important thing is to understand how to develop a theory based on allosteric transitions between allosteric states. Then we can go on to understand allosteric interactions between ligands binding to distant sites on the same protein, and allosteric mutations that influence the response to ligands even though they are far away from the binding sites.

ALLOSTERIC THEORY

In the Monod-Wyman-Changeux theory of allosteric proteins, everything can be reduced to two elementary processes. The first is ligand binding to a stereospecific binding site on a protein. The second is a structural transition in the protein. In fact, an important element of allosteric theory is the clear separation of these two processes. Ligand binding and conformational transitions can then be combined in many ways to develop a rich variety of theoretical models. Binding is a bimolecular process described by an equilibrium of the following form.

$$R_0 + L \rightleftarrows R_1 \quad (1)$$

R_0 represents a protein with an empty binding site and R_1 represents the same protein with a binding site occupied by the ligand, L. At equilibrium the concentrations of the various species are related by the expression

$$\frac{[R_1]}{[L][R_0]} = K \quad (2)$$

where K is the association equilibrium constant, which is also referred to as a binding affinity. The change in free energy during binding is then given by the expression

$$\Delta G = -RT \, ln \, K \quad (3)$$

This free energy arises from noncovalent contacts such as hydrogen bonds, salt bridges, and hydrophobic interactions between the ligand and specific residues in the binding site of the protein. The noncovalent nature of these contacts makes binding, and thus receptor activation, a reversible process. The details of how these contacts influence things will be expanded upon toward the end of this chapter.

The other elementary process, a structural transition in which the protein changes its conformational state, is referred to as an allosteric transition. The allosteric transition takes place between two conformations, referred to as allosteric states. This transition is described by a simple equilibrium of the following form

$$T \rightleftarrows R \quad (4)$$

The letters R and T are used for historical reasons because Monod et al. (1965) choose to think of these two states as "tense" and "relaxed". At equilibrium the concentrations are related by the expression

$$\frac{[R]}{[T]} = K \quad (5)$$

Here the equilibrium constant, K, is that of allosteric transition rather than the association process above. (The equilibrium constants used in Eqs. 2 and 5 will be given appropriate subscripts below to keep these and other distinctions clear). The change in free energy is given by the same expression as above for binding (Eq. 3).

The physical processes underlying the allosteric transition in a protein are not well understood. We can surmise that they involve rotations around some of the bonds in the peptide backbone of the protein as well as in the amino acid side chains. We also consider that noncovalent bonds such as salt bridges, hydrogen bonds, and hydrophobic contacts break and others form during the transition between two states of a protein. It is important to bear in mind that the same basic kinds of noncovalent contacts play key stabilizing roles in both binding and conformational transitions. Ligands are held in place at binding sites by essentially the same kinds of physical interactions that are responsible for maintaining the secondary, tertiary, and quaternary structure of a protein (Weber, 1975). This means that both ligand binding and conformational transitions can have free energy changes of the same order of magnitude, and this enables the two processes to influence one another. This capacity for mutual influence is what makes signal transduction by proteins possible.

To reiterate the crucial point, allosteric theory is developed from the assumption of an allosteric transition between allosteric states, where the allosteric states are defined as having unique functional and structural properties. We will begin by working with these basic assumptions and not worry about the details of how specific contacts involving the protein and ligand make things happen. After using the concepts of allosteric transitions and states to develop the theory and to study the acetylcholine receptor, we will return to these basic assumptions and try to see what they mean in terms of the molecular details.

Allosteric Proteins with a Single Binding Site. The classical allosteric theory was designed primarily to explain the phenomenon of cooperativity exhibited by multi-subunit, multi-binding site proteins. However a more basic aspect of signaling, the interaction between the binding step and the allosteric transition, can be seen in a simpler system consisting of a protein with a single subunit and a single binding site. To develop this point consider a receptor with two allosteric states, T and R, and a single binding site.

Assume that state T is inactive from the functional standpoint, and state R is functionally active. Although we could keep the functional nature of the transition completely abstract, in keeping with the goal of this chapter of understanding how ligand-gated channels work, we will use open and closed channels to illustrate the two conformations (Fig. 1). Each of these two states binds ligand, L, with characteristic binding affinities, denoted as K_T and K_R, respectively, where each binding process is described by Eqs. 1-3 above. The two allosteric states can interconvert or undergo allosteric transitions, and the transition can occur with or without bound ligand (Fig. 1). The tightly-binding conformation is associated with the open channel and the weakly-binding conformation is associated with the closed channel. A key assumption of the theory is that the ligand binding site switches between the two affinity states concomitantly with the gating of the channel, as depicted in Fig. 1.

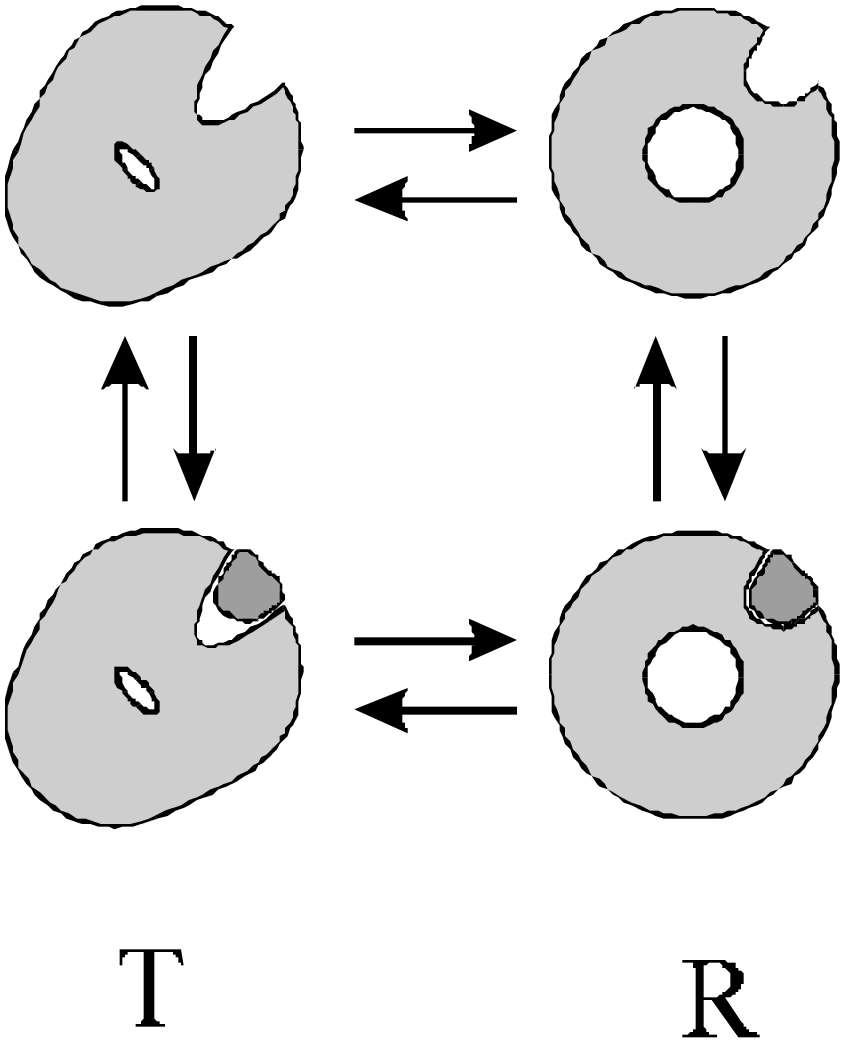


Fig. 1. An allosteric transition in a ligand-gated channel with a single binding site. In this case the "tense" and "relaxed" conformations of the protein correspond to the closed and open states of a channel. The allosteric transition can occur with the binding site empty (above) or occupied (below). Note that the channel and binding site change concomitantly. Because of the improved fit between the ligand and binding site, binding induces the conformational transition to the relaxed conformation in which the channel is open.

In the absence of ligand the protein is almost entirely in state T. A tiny fraction of state R is also present and the ratio at equilibrium is given by the equilibrium constant for the allosteric transition (Eq. 5). Here we introduce the equilibrium constant for the unligated protein, and denote this quantity as K_0, for zero receptor occupancy. K_0 is a very small number; this means that the T conformation has a low free energy and ΔG_0 is positive. To make this protein a receptor capable of being activated by ligand, the R

conformation must bind with a higher affinity. The higher affinity of R allows the binding of ligand to shift the equilibrium from favoring T to favoring R. This means that ΔG_1 and ΔG_0 have opposite signs (where ΔG_1 is the free energy difference between the two protein conformations when the binding site is occupied). Now consider the complete reaction scheme that includes all of the relevant steps.

$$\begin{array}{ccc} & K_T & \\ T_0 + L & \rightleftarrows & T_1 \\ K_0 \downarrow\uparrow & & \downarrow\uparrow \; K_1 \\ R_0 + L & \underset{K_R}{\rightleftarrows} & R_1 \end{array} \qquad \text{(A)}$$

Because the free energy is a function only of the state, and does not depend on the pathway taken from one state to another, we know that the total free energy change going from T_0 + L to R_1 will be the same whether the transition occurs via T_1 or R_0. Thus, the free energy changes for taking these two alternative routes must be equal, and this leads to the following relationship between the equilibrium constants of the four steps.

$$RT\, ln\, K_T + RT\, ln\, K_1 = RT\, ln\, K_R + RT\, ln\, K_0$$

More simply, we have

$$K_T K_1 = K_R K_0 \qquad (6)$$

This relation can be derived by connecting any pair of states in Scheme A in the two possible ways, but care must be taken to maintain a consistent sign convention. Eq. 6 makes it especially easy to see how the conformational equilibria and binding equilibria are related. If the binding affinity of one conformation exceeds that of the other by a given factor, then the equilibrium constants for the allosteric transition in the unligated and ligated protein must be related by the same factor (e.g. from Eq. 6 $K_R/K_T = K_1/K_0$). This illustrates in terms of equilibrium constants how the binding of ligand converts the protein from the T state to the R state. Eq. 6 is often referred to as a condition of detailed

balance, because it can also be derived from the assumption that the rate of clockwise cycles balances the rate of counterclockwise cycles.

The above analysis makes the point that changes in the free energy of binding and changes in the free energy of the conformational change are intimately related. This can also be seen in the dose-response relation for a single-binding site allosteric protein. The response (defined here as V) is taken as the fraction of receptors in the functionally active R state.

$$V = \frac{[R_{total}]}{[R_{total}]+[T_{total}]}$$

$$= \frac{[R_0]+[R_1]}{[R_0]+[R_1]+[T_0]+[T_1]} \qquad (7)$$

We then use the following four relationships defined by the equilibrium constants (Eqs. 2 and 5)

$$K_T = \frac{[T_1]}{[T_0][L]} \qquad K_R = \frac{[R_1]}{[R_0][L]}$$

$$K_0 = \frac{[R_0]}{[T_0]} \qquad K_1 = \frac{[R_1]}{[T_1]}$$

Factoring $[R_0]$ out of the numerator and denominator of Eq. 7 and substituting with the expressions immediately above gives the following, where for brevity L is used for [L].

$$V = \frac{K_0(1+K_R L)}{K_0(1+K_R L)+1+K_T L} \qquad (8)$$

This is the complete dose-response relation, and V can be seen to vary from $K_0/(1+K_0)$ at $L = 0$ to $K_1/(1+K_1)$ at high L.

If the protein is a good receptor, we can obtain a simple approximation for Eq. 8. A good receptor will have very low levels of the R conformation in the absence of ligand, and the fraction of active receptor, $K_0/(1+K_0)$, will be very small. Likewise a good receptor will have a high value of K_1 and $K_1(1+K_1)$ will be almost one. Exploiting these conditions gives the following expression for the dose-response relation

$$V = \frac{K_R K_0 L}{1+K_R K_0 L} \qquad (9)$$

This is a standard saturation expression commonly seen in the analysis of enzyme kinetics and ligand binding data. It is important to realize that this dose-response behavior makes it look like the receptor has a single binding site with an apparent binding affinity of $K_R K_0$ (note that by Eq. 6 the apparent affinity can also be expressed as $K_T K_1$). This apparent binding affinity that one would measure from an experimental dose-response curve differs from the true affinity, K_R, by a factor of K_0. This provides a quantitative assessment of how the conformational equilibrium influences the observed dose-response behavior of the receptor. It is also easy to show that the probability of finding a receptor binding site occupied has the same dependence on ligand concentration as Eq. 9 (Problem 2), so that even when one measures binding, the curve does not give a true binding affinity but rather an apparent affinity equal to $K_R K_0$.

Allosteric Proteins With Many Binding Sites.

In the above analysis of a protein with a single ligand binding site, the allosteric transition of the protein is invisible in that the saturation behavior is identical to that of a protein without an allosteric transition, but with a binding affinity equal to $K_R K_0$. Thus, neither binding nor dose-response experiments would provide a clue that the protein can undergo a conformational transition. In this kind of a situation there is little incentive to develop a more complicated theory. However, things are different in an allosteric protein with multiple binding sites, because here the allosteric transition has a clear experimental manifestation of sigmoidal binding and dose-response behavior. It was chiefly to explain sigmoidal behavior that the theory was originally developed, and that is why the single-site model examined in the previous section has been neglected despite its considerable pedagogic value.

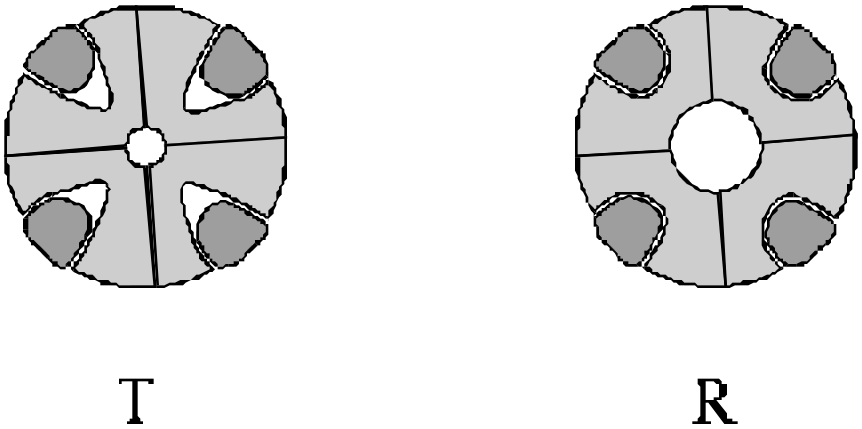


Fig. 2. An allosteric transition in a protein composed of four identical subunits, each with a binding site. As in Fig. 1, the tense and relaxed conformations correspond to closed and open channels. Channel opening is concomitant with a change in the binding site to increase affinity. Occupancy of just one site will increase the probability of the entire protein assuming the open conformation, and since the open conformation binds ligand with a higher affinity the net effect of occupying one site is an apparent increase in the affinity of the other sites. In this way, the cooperativity of the allosteric transition confers cooperativity on ligand binding.

One can generalize the above single-site model by combining an arbitrary number of identical subunits (Fig. 2 shows four). Each subunit is allowed to bind equivalently, but when it comes to the conformational change an important restriction is introduced by insisting that all of the subunits undergo the transition in unison. Thus, the allosteric transition is intrinsically cooperative; separate transitions by individual subunits are not allowed. This is a far-reaching assumption that provides a potent, albeit indirect, mechanism for the binding sites to interact with one another. An additional advantage of these assumptions is that the model becomes relatively simple because there are just a few free parameters. By assuming that all of the binding sites are equivalent, we only have to deal with two affinities, K_R or K_T, one for each allosteric state. This model gives the following response as a function of ligand concentration.

$$V=\frac{K_0(1+K_RL)^n}{K_0(1+K_RL)^n+(1+K_TL)^n} \qquad (10)$$

where n is the number of subunits. With n > 1, this dose-response relation is sigmoidal as expected for a cooperative system.

To obtain an apparent affinity for this model, we ask what value of L produces a 50% maximal response. This occurs when

$$K_0(1+K_RL)^n = (1+K_TL)^n \qquad (11)$$

If $K_R >> K_T$, then we obtain the approximate expression for the apparent affinity of $K_R(K_0)^{1/n}$. (Note that the reason K_0 is raised to the 1/n power relates to the fact that K_0 is a whole-protein quantity while K_R is a single-subunit quantity). This of course is consistent with the affinity for the single-site allosteric model above, and shows once again that the conformational equilibrium plays a role in determining the concentration dependence of an allosteric protein.

An important feature of the Monod-Wyman-Changeux model for a multisubunit protein is that binding depends on the allosteric state of the whole protein, and only indirectly on the state of occupancy of other binding sites. Thus, as more binding sites are filled, the free energy difference between the two allosteric states is incrementally reduced. This then increases the probability of conversion to the high-affinity R state. To see this, consider the following expression for the free energy change of the allosteric transition as a function of n, the number of binding sites occupied.

$$\Delta G(n) = \Delta G_0 + n(\Delta G_R - \Delta G_T) \qquad (12)$$

where ΔG_0 is the free energy of the allosteric transition in the unligated protein, and ΔG_R and ΔG_T are the binding free energies of individual sites in the open and closed states, respectively. It was noted above that ΔG_0 is a positive number. In the open state the binding site has a higher affinity, and this means that the quantity $\Delta G_R - \Delta G_T$ is negative. Thus, as more binding sites fill up the free energy change of the allosteric transition will eventually change signs. This point is illustrated below in Fig. 6.

It is important to appreciate that in allosteric theory cooperativity occurs solely through the allosteric transition of the whole multi-subunit protein. The binding sites do not interact with one another by any direct route. The real interaction takes place at the interfaces where the subunits pack together; these interfaces are such that a T - R contact

is highly unfavorable. The packing of subunits at their interfaces is assumed to force all of the subunits to undergo the allosteric transition in a concerted all-or-none fashion. Thus, occupying one of the sites in the tense state lowers the free energy barrier for the cooperative conversion of entire protein to the relaxed state. By making it easier to form the relaxed state with its higher affinity, the affinities of the other empty binding sites are effectively increased.

Other Models for Protein Signaling.

1. *The Hill Equation*. One of the earliest ways of looking at receptor activation followed from the simple assumption that binding is perfectly cooperative. This means that all of the binding sites of a single protein must be simultaneously empty or full. For n identical binding sites this leads directly to the Hill equation for the response as a function of ligand concentration

$$V = \frac{(KL)^n}{1+(KL)^n} \qquad (13)$$

where K is interpreted as an effective affinity of one site. Despite the artificial nature of the assumption of perfect cooperativity in binding, this equation is still very useful as a simple first approach to cooperative phenomena. The Hill coefficient, n, is often determined by empirical fitting of Eq. 13 to experimental data to provide a qualitative index of cooperativity (see Fig. 3 below). This quantity can reasonably be interpreted as the minimum number of binding sites on the protein.

Another related model can be developed as a variant of the Hill equation by assuming that a multisubunit protein has n binding sites with no cooperativity of binding. If it is assumed that only the fully ligated protein has biological activity (or an open channel), then the dose-response equation looks like this

$$V = \left(\frac{KL}{1+KL}\right)^n$$

In this interesting case the response will have a sigmoidal dependence on ligand concentration, but the binding-site occupancy will not.

2. *The Koshland-Nemethy-Filmer Model.* A reasonable way to extend the Monod-Wyman-Changeux model is to relax the assumption of absolute cooperativity and allow both of the allosteric conformations to coexist in the same protein complex (Koshland et al., 1966). Adjacent subunits in different conformations may be compatible to varying degrees, and this is accounted for by assigning a term for the interaction energy. One can recover the Monod-Wyman-Changeux model by letting that interaction energy become infinitely large, because this would force the transition to be perfectly concerted. This more general theory can take on many interesting forms, and a corresponding variety of dose-response relations can be generated. If the interaction energy is zero, and a subunit does not care what the conformation of its neighbor is, there will be no cooperativity even when there are many subunits. Positive cooperativity ensues when the interaction term is positive; negative cooperativity, when the interaction term is negative. Thus, as with the Monod-Wyman-Changeux model, cooperativity is still mediated by the interface between adjacent subunits.

3. *The Weber Theory.* The idea of allosteric transitions was developed to explain cooperativity in proteins with multiple binding sites, but Weber questioned the need for allosteric transitions and developed a theoretical approach to cooperativity without independent conformational transitions in the protein (Weber, 1992). In this theory cooperativity is brought about by a ligand-induced distortion of the protein structure initiated at the binding site. The distortion spreads to other binding sites to alter their affinity. This is then a rapid change in the protein concomitant with ligand binding, and the attendant changes in protein functionality are not explicitly defined. One develops this theory by introducing coupling energies that are equal to the difference in binding free energy of one site when another site is either empty of full. This energetic approach has lead to the important insight that the perfect cooperativity assumed in the derivation of the Hill equation (Eq. 13) requires infinite coupling energies and is therefore unrealistic (Weber, 1992). (This is the reason that the Hill coefficient should be

interpreted as the minimum number of binding sites rather than the actual number.) However, because the interfaces between subunits in multisubunit proteins are quite large, the energies of juxtaposing subunits in different conformations could also be large, so the high energies necessary for the cooperativity of an allosteric transition are not unreasonable. In the case of ion channels, allosteric transitions are clearly evident (Jackson, 1994), so the Weber theory is inappropriate. Nevertheless, formulations in terms of these coupling energies have been very useful in the analysis of other types of signaling proteins (Weber, 1992; Forsén and Linse, 1995).

EXPERIMENTAL ANALYSIS OF LIGAND-GATED CHANNELS

Cooperative Activation.

For many years the theories of Monod et al., Koshland et al., and Weber were used to analyze binding and dose-response data. Each of these theories provided models that could be fitted to data by variation of parameters. But because such curve fitting worked well for all of the models, it was concluded that dose-response and binding data are not very sensitive to the underlying assumptions of the different theories. This made it difficult to determine the physical mechanism of cooperativity, and to evaluate the very different assumptions that went into the different theories.

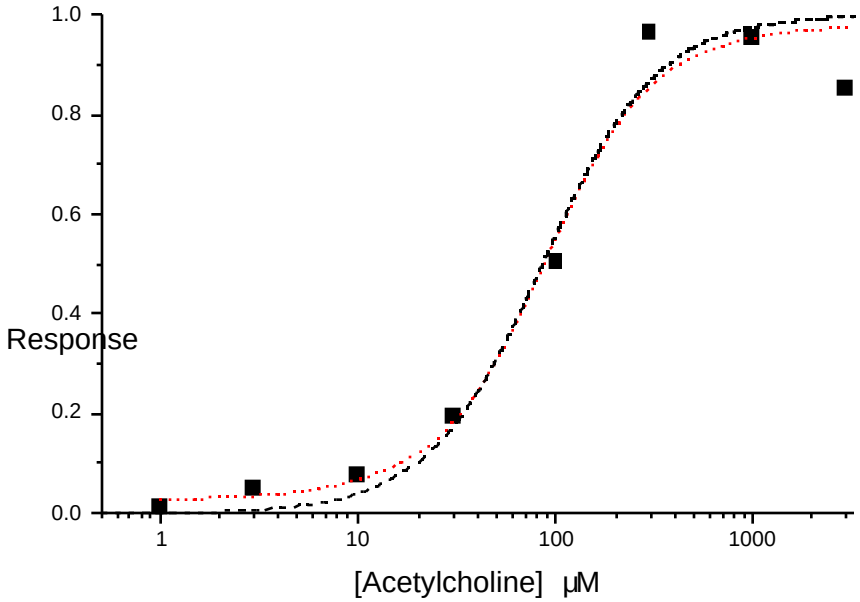


Fig. 3. A dose-response curve for the *Torpedo* acetylcholine receptor (data replotted from Forman and Miller, 1988). The two curves shown are the best-fitting Hill equation (Eq. 13; dashes), and the best-fitting version of Eq. 9 (dotted) with the number of binding sites fixed at two. The fit to the Hill equation gave a value of n = 1.5, which is less than the actual number of binding sites (two).

With ligand-gated channels, activation is generally cooperative. A dose-response plot for the acetylcholine receptor is shown in Fig. 3. Two different curves are drawn to illustrate the insensitivity of the data to different theories. One of the curves drawn is simply the Hill equation with a Hill coefficient of 1.5. The other is Eq. 10 for the Monod-Wyman-Changeux model with n = 2. It is very difficult if not impossible to decide which model is correct by comparing these kinds of fits.

Although dose-response data cannot help in distinguishing between alternative models, it is nevertheless significant when cooperativity is observed because the receptor must then have more than one binding site for the activating ligand. This has been born out by the molecular characterization of ligand-gated channels. They are all multimeric proteins formed from identical or homologous subunits. In the case of the acetylcholine receptor it has been clearly shown that the α subunit has several residues involved in

binding, and that the receptor has two copies of this subunit. The Hill coefficient of somewhat less than two is clearly consistent with this structure. However, to go beyond this to a more detailed understanding of the protein requires experiments that are more sensitive to the specific mechanism of activation.

Affinity Transitions.

An important first step in evaluating the Monod-Wyman-Changeux theory is to test for different affinities of the protein, because these can presumably be attributed to different allosteric states. However, detecting different affinities can be difficult. With binding described by expressions such as Eqs. 10 and 11, an experiment in which binding site occupancy is measured as a function of ligand concentration will give the appearance of being governed by only one binding site. An examination of the desensitized state of the receptor turned out to be of value in this situation, because the desensitized conformation forms slowly enough to allow the separate study of desensitized and undesenitized receptors. A rapid binding experiment thus reveals mostly low-affinity binding. Prolonged exposure to acetylcholine converts the receptor to a high-affinity state (Changeux, 1984). The high-affinity conformation detected by binding measurements is thought to represent desensitized receptors rather than open channels. The open state should also bind acetylcholine more tightly, but because of the rapid interconversion with the closed state, these two conformations behave like a receptor with one apparent affinity (Eq. 10). The low-affinity receptor detected in these experiments thus represents a mixture of closed and open channels. Binding experiments succeeded in demonstrating the presence of different affinity states of the receptor, and this constituted some of the earliest evidence in support of the Monod-Wyman-Changeux theory for ligand-gated channels.

Detection of Allosteric Transitions.

Another important tenet of the Monod-Wyman-Changeux theory is that the protein undergoes allosteric transitions between functionally distinct conformations, and

that this process has an equilibrium with or without ligand (Fig. 1). Binding studies provided some of the earliest evidence for allosteric transitions by showing that even when the binding measurements were made before the added ligand had time to convert receptor to the desensitized state, about 20% of *Torpedo* acetylcholine receptor already had a high affinity. This fraction was thought to represent desensitized receptors in equilibrium with receptors in the closed-channel state in the absence of ligand (Changeux, 1984). It is a remarkable footnote to this discovery that several years prior to publication of the Monod-Wyman-Changeux theory, Katz and Thesleff (1957) in what was the first quantitative description of receptor desensitization predicted that unligated desensitized receptors should be present, and that they should have a higher affinity than undesensitized receptors. This prediction was based on a careful energetic analysis of a scheme very similar to scheme A above.

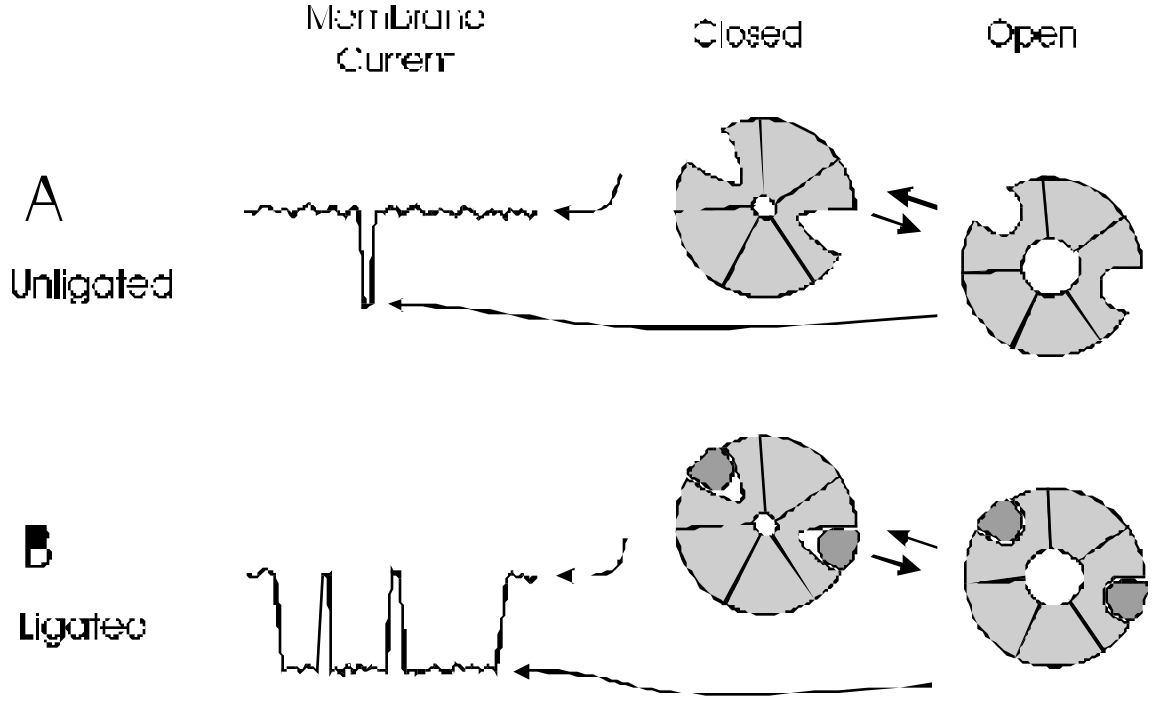


Fig. 4. Allosteric transitions are seen as channel opening and closing events in single-channel current records. Two binding sites are shown among the five subunits, and this follows the accepted pattern in the acetylcholine receptor. When the binding sites are vacant (top) openings occur only rarely and are brief in duration. When the binding sites are occupied (bottom) the channel is open most of the time, but closes intermittently.

More evidence for allosteric transitions in ligand-gated channels came from single-channel recordings with the patch clamp technique. This technique provides a direct view of conformational transitions as stepwise changes in membrane conductance. Channels can be seen to flicker rapidly between the open and closed states during bursts of activity. The closures during a burst represent ligated receptors with closed channels (Colquhoun and Sakmann, 1981). This observation thus demonstrated discrete transitions between two states for a fixed state of receptor occupancy. On the other hand, in the absence of ligand channels can open spontaneously, showing that the two conformations are still in equilibrium even when the binding sites are empty (Jackson, 1984; 1994). In the ligated receptor the channel opens about 10^5 times more rapidly than in the unligated receptor. These transitions are depicted in Fig. 4, and experiments of this kind have been used to measure the conformational equilibrium constants, K_0 and K_n. For the acetylcholine receptor in the absence of acetylcholine the equilibrium constant between the open and closed states of the channel is only about 10^{-6}. In the presence of acetylcholine the equilibrium constant is about 50. Thus, binding of acetylcholine changes the equilibrium constant for the allosteric transition by a factor of $5x10^7$ (Jackson, 1989; Jackson, 1993b).

Nonequivalence of Binding Sites.

The cooperativity of the acetylcholine receptor dose-response curve indicates that this receptor has multiple binding sites for acetylcholine (Fig. 3), and the molecular structure indicates that the number is two. It is tempting to assume that these binding sites are equivalent, because one then has fewer parameters to worry about and the mathematical derivation produces a relatively simple dose-response equation. However, it turns out that the binding sites on the acetylcholine receptor are not equivalent. Chemical modification experiments showed that disulfide bonds near the two binding sites have different chemical reactivities (Damle and Karlin, 1980). Furthermore, the binding sites have different affinities for the antagonist curare (Sine and Taylor, 1981).

The demonstration that the binding sites differed in affinity for agonist came from single channel studies which can detect singly-ligated receptors as a distinct component of brief-duration channel openings. Doubly-ligated receptors produce channel openings with longer durations. As the concentration of an agonist is increased, the relative frequency of these long-duration openings increases. The true binding affinity of the closed state for agonist can be measured from these kinds of experiments because the rate of opening is proportional to the fraction of receptors in a ligated closed state. The frequency of brief-duration singly-ligated channel openings of the mouse acetylcholine receptor saturated at about 5 μM carbachol (an acetylcholine receptor agonist); the frequency of long-duration doubly-ligated openings continued to increase, showing no sign of saturation. This indicated that the second binding site had a much lower affinity than the first binding site (Jackson, 1988). Single channel recordings also showed a difference in binding affinity for the *Torpedo* acetylcholine receptor, where the rates of binding and dissociation were measured as a function of acetylcholine concentration (Sine et al., 1990). The strategy employed in this case was to solve a complicated mathematics problem that relates the rate constants of the binding steps to the three exponentials of the closed-time distribution. The on and off rates obtained in this way show the dependence on acetylcholine concentration expected for a binding process (Fig. 5). From these data the affinities of the two binding sites were shown to differ by a factor of about 100 (Sine et al., 1990).

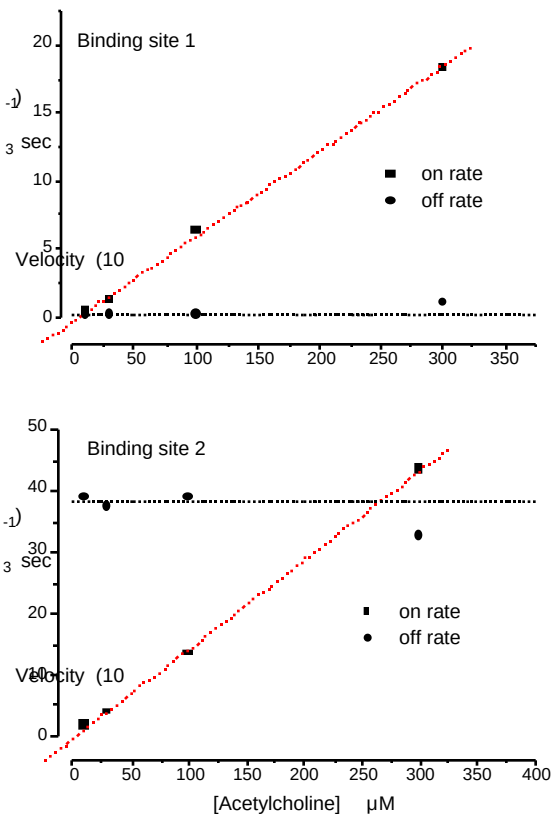


Fig. 5. On and off rates for acetylcholine at the two sites of the acetylcholine receptor in the closed-channel conformation. Based on single-channel closed times the on and off rates were determined for four different acetylcholine concentrations. The on rates increased linearly with acetylcholine concentration while the off rates were concentration independent. These data reveal differences between the two binding sites in both the on and the off rates. The affinities are also clearly different as the two lines cross at very different acetylcholine concentrations in the two plots. The rates of binding to sites one and two are 0.6 x 10^8 and 1 x 10^8 M^{-1} s^{-1}, respectively. The off rate for site 1 was too small to read off the graph and was 250 s^{-1}. (Data from Sine et al. (1990); analysis according to Jackson (1997b)).

Additivity of Binding Energies.

The single-channel analysis that permitted measurements to be made of the affinities of the two binding sites had the important advantage of providing rates for binding specifically to the closed conformation of the receptor. This is an important distinction because, as stressed above, binding and dose-response experiments on allosteric proteins give the appearance of a single site with an effective affinity that is the product of a true affinity and a conformational equilibrium constant (Eqs. 9 and 10). With measurements of true binding affinities of receptors in the closed-channel state, we can address another important distinction between the different models of receptor activation. Because in the Monod-Wyman-Changeux theory cooperativity depends on the allosteric transition, the occupation of successive binding sites of the *same conformation* should exhibit no cooperativity. In other words, the binding energies should be perfectly additive

(Eq. 12). On the other hand, in the Koshland-Nemethy-Filmer theory occupying one site induces a transition in that subunit, leading to an additional energy change at the interface between neighboring subunits. With the Weber theory one expects cooperativity to appear even within a given conformation. Thus, the question of additivity of binding energies is central to the different assumptions underlying the various theories.

With the binding affinity measurements just discussed we can address this issue. Converting binding affinities to free energy, we can make a plot of the free energy versus the number of binding sites occupied (Fig. 6). The Monod-Wyman-Changeux theory predicts linear plots for each conformation (Eq. 12), whereas the subunit interaction terms of the Koshland-Nemethy-Filmer theory and coupling energies of the Weber theory predict deviations from linearity reflecting these additional energy terms. The plot in Fig. 6 shows some nonlinearity for the closed state, but much less for binding to the open state. Even in the closed state the nonequivalence discussed above appears as a rather small deviation from linearity, because the binding energies themselves are large compared to their difference. If we interpret the nonlinearity in terms of interfacial energies or coupling energies, this plot suggests that these energies are small.

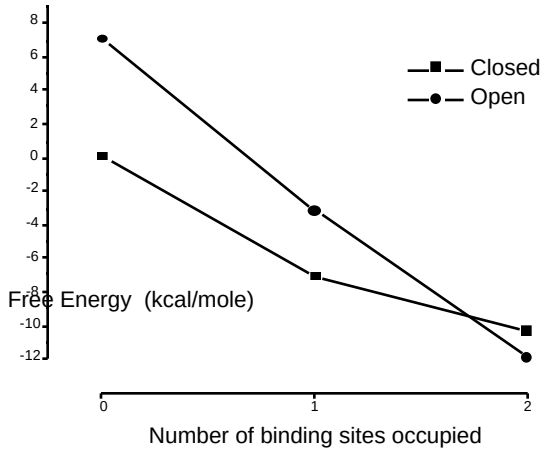


Fig. 6. The free energy of binding is plotted versus the number of binding sites occupied. The steeper slope for binding to the open state reflects the higher binding affinity of this conformation. The curves cross to the right of n = 1, indicating that binding a single ligand is insufficient to gate the channel (see Eq. 12). The nonlinearity in the plot for the closed state is another reflection of nonequivalent binding affinities for this conformation of the protein.

Independence of binding sites

The weak nonlinearity in Fig. 6 puts the nonequivalence of the two binding sites in an interesting perspective, but an important basic question remains unanswered. Is the appearance of different binding affinities due to intrinsic differences between the two sites, or is there negative cooperativity such that occupation of one of the sites reduces the affinity of the other? Negative cooperativity in binding to the closed conformation would mean that the binding sites can interact without the aid of an allosteric transition. This would be inconsistent with the Monod-Wyman-Changeux theory, which requires all cooperativity to be manifest through allosteric transitions. Thus, even if Fig. 6 plays down the nonequivalence as being relatively small, the question remains as to whether the binding sites are capable of interacting without an allosteric transition. The alternative explanation is that the binding sites are intrinsically different. The two binding sites would then have different structures even when both are empty. This would necessitate dropping the symmetry of the Monod-Wyman-Changeux model. The dose-response relation (Eq. 10) would lose its simplicity, but the underlying mechanism of cooperativity would remain. Thus, the question of why the affinities of the two binding sites are different impinges on a fundamental question of how the subunits interact during receptor activation.

Kinetic techniques such as single-channel analysis could not answer this question because it is not easy to reverse the order in which the two sites are occupied in an experiment. Molecular analysis of receptor structure turned out to be a more effective way of determining the basis for different binding affinities. An intrinsic difference between the two sites is in excellent accord with what is known about the structure of the acetylcholine receptor. Although the binding sites are primarily on the two α subunits of the receptor, the pentameric arrangement of the subunits forces the α subunit into nonequivalent environments (Fig. 7). The δ subunit is adjacent to one of the α subunits, and the γ subunit is adjacent to the other α subunit. Each of these adjacent subunits

contributes structurally to its respective binding site (Czajkowski et al., 1993; Karlin and Akabas, 1995).

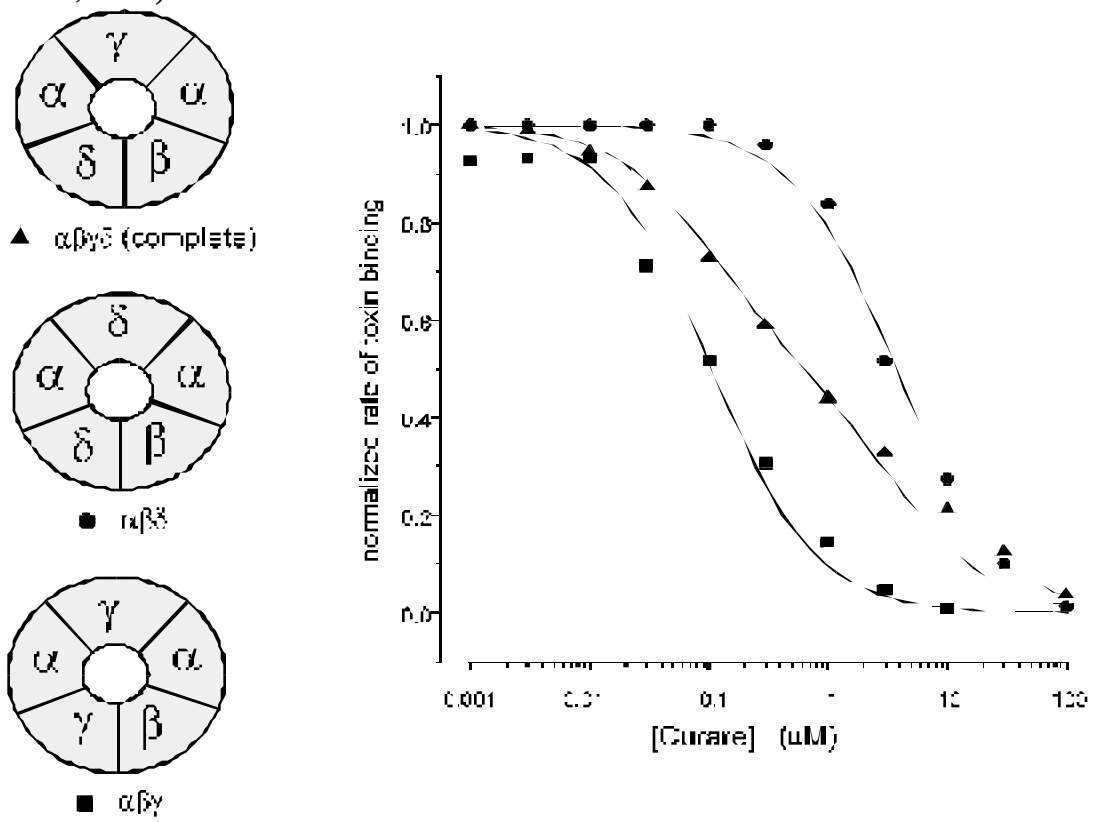


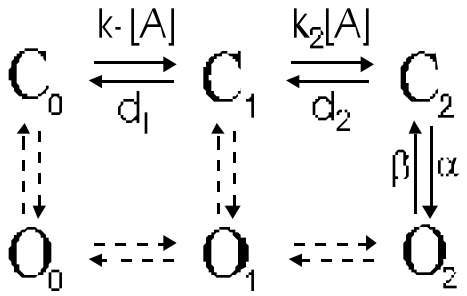
Fig. 7. Curare binding to acetylcholine receptors formed from different subunit combinations. The receptor formed from the $\alpha_2\beta\gamma_2$ subunit combination was well fitted by a single-site saturation expression with an apparent affinity of 0.1 μM (squares). For the $\alpha_2\beta\delta_2$ receptor the apparent affinity was 3.7 μM (circles). The curve drawn through the data for the complete $\alpha_2\beta\gamma\delta$ receptor was the average of the two saturation curves for the two incomplete receptors (triangles). The structural schematics to the left of the plot indicate the arrangement of the subunits in each experiment. Binding sites are thought to form at the interfaces between the α subunit and one of the adjacent subunits, which in this scheme is the subunit in the counterclockwise direction. These data show that the behavior of the binding sites are determined by the adjacent subunit. Data were replotted from Sine and Claudio (1991).

To test the hypothesis that the adjacent γ and δ subunits account for the differences between the binding sites, receptors were made with the α and β subunits, together with either the δ or the γ subunits. The native receptor with its stoichiometry of $\alpha_2\beta\gamma\delta$ could then be compared to receptors with a stoichiometry of $\alpha_2\beta\gamma_2$ and $\alpha_2\beta\delta_2$. In

studies of curare binding, the results were especially clear. The native $\alpha_2\beta\gamma\delta$ receptor has a binding curve that shows saturation of two different sites with different affinities (Fig. 7). If the occupation of the first site altered the affinity of the second site, then receptors constituted as $\alpha_2\beta\gamma_2$ or $\alpha_2\beta\delta_2$ should still appear to have dual affinities. However, each of these receptors had binding curves showing a single site, and the sum of these two binding curves completely reproduced the binding behavior of the native $\alpha_2\beta\gamma\delta$ receptor. These results indicate that the differences between the binding sites are intrinsic and are determined by the adjacent γ and δ subunits. The existence of intrinsic differences is further supported by structural analysis showing differences in the electron density profiles of the parts of the protein thought to make up the binding sites (Unwin, 1993). The difference does not depend on the state of occupancy of another binding site. This leads to the conclusion that the binding sites have intrinsic differences prior to binding, and that they are not capable of influencing one another except through an allosteric transition.

PHYSIOLOGICAL FUNCTION OF THE ACETYLCHOLINE RECEPTOR

The various experiments described above provide the basis for a fairly complete allosteric model of acetylcholine receptor. The experiments that lead to this model were conducted on channels in the presence of constant concentrations of acetylcholine, but if the model is correct it should describe the activation of the acetylcholine receptor by the rapidly changing acetylcholine concentrations that occur during the physiological process of conveying signals from motoneurons to skeletal muscle fibers. With two binding sites the allosteric scheme takes the following form.



(B)

The receptor binding sites can be empty, or one or two binding sites can be occupied; in each state of receptor occupancy the protein can undergo the allosteric transition between the closed (C) and open (O) states. This scheme can be seen as an extension of the single-site model (Scheme A above) to include a second binding site. Note that the rates of the binding transitions are proportional to the concentration of acetylcholine, [A] (Fig. 5). The work of Sine et al. (1990) has given us rate constants for the steps indicated by solid arrows; the rates of the steps indicated by dashed arrows in Scheme B are unknown for the *Torpedo* receptor, but see Jackson (1993b) for a more complete tabulation of known rate and equilibrium constants. It turns out that during normal receptor function the unligated and singly-ligated open states play no significant role so they can be neglected in a simulation of synaptic function. Based on mass balance between the three closed states and the doubly-ligated open state, we can derive a set of kinetic equations for the rate of change of the concentration of receptor in each state. According to Scheme B, the concentration of C_0 is decreased by ligand binding with a rate $k_1[A][C_0]$. $[C_0]$ is increased by dissociation of ligand from C_1 with a rate $d_1[C_1]$. This gives the relation

$$\frac{d[C_0]}{dt} = - k_1[A][C_0] + d_1[C_1] \qquad (14a)$$

Note that k_1 is an association rate constant with units $M^{-1}s^{-1}$ and d_1 is a dissociation rate with units of s^{-1}. Similar arguments give the following expressions for the rates of change of concentrations of the other relevant species.

$$\frac{d[C_1]}{dt} = k_1[A][C_0] - (d_1 + k_2[A])[C_1] + d_2[C_2] \qquad (14b)$$

$$\frac{d[C_2]}{dt} = k_2[A][C_1] - (d_2 + \alpha)[C_2] + \beta[O_2] \qquad (14c)$$

$$\frac{d[O_2]}{dt} = \alpha[C_2] - \beta[O_2] \qquad (14d)$$

If [A] were dependent on time it would be hard to solve these equations, but acetylcholine is usually present in considerable excess over receptor so that we can neglect the changes in [A] due to binding. With constant [A] the equations are linear and the mathematical methods of linear algebra can be used. With an initial condition of all receptors in an unligated state, the solution to this equation for [A] = 1 mM (a reasonable estimate during synaptic transmission) simulates the response to the sudden increase that occurs when the nerve terminal is stimulated. This solution then shows that a synaptic current will be activated within about 100 μsec (Fig. 8A). In fact, excitatory postsynaptic currents at neuromuscular junctions are activated with roughly this speed (Land et al., 1980; Wathey et al., 1979). Desensitization was neglected in this calculation, but if it were included it would cause a decay that is very slow compared to the activation time course, and not visible with the time axis used in Fig. 8A. The situation is quite different for synaptic receptors activated by glutamate (Trussell and Fischbach, 1989). In these receptors desensitization is much faster.

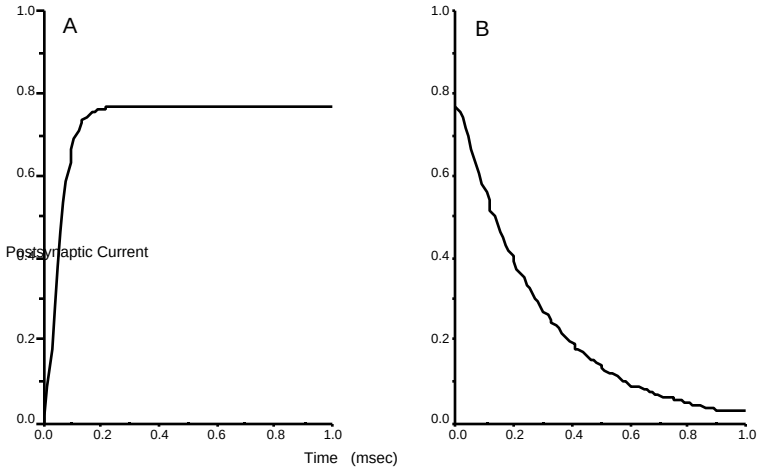


Fig. 8. Solution of the kinetic equations for the acetylcholine receptor simulate the time course of the rise and fall of a postsynaptic current. Scheme B was used to derive the kinetic equations (Eqs. 14a-d). The rate constants were from Sine et al. (1990), with α = 45,000 s^{-1} and β = 8,000 s^{-1} (Fig. 5). The solution was obtained by using Eqs. 14a-d to set up a rate matrix. The computer program MATHCAD (Mathsoft, Cambridge, MA) was then used to determine the eigenvalues and eigenvectors. The eigenvalues are the decay constants; the eigenvectors were used to compute the weights in the four-exponential

solution. For the rise of postsynaptic current (**A**), the initial state vector was (1,0,0,0) and the acetylcholine concentration was 1 mM. For the decay (**B**), the initial state vector was the distribution of states at the end of the rising simulation, and the acetylcholine concentration was zero.

In the neuromuscular junction diffusion and enzymatic hydrolysis reduce the acetylcholine concentration to nearly zero (actually about 10^{-8} M) very rapidly after release from the nerve terminal. This means that we can simulate the decay of the postsynaptic current by starting with the receptor at equilibrium with 1 mM acetylcholine (the steady state solution to the equations with this concentration). The time course obtained by solving the same set of equations with this initial condition and with zero acetylcholine is shown in Fig. 8B. This again approximately follows the time course of decay of the postsynaptic current at the neuromuscular junction (Anderson and Stevens, 1973). These simulations thus show that the allosteric model gives a good kinetic description of synaptic transmission at the neuromuscular junction.

Beyond a rapidly rising and falling postsynaptic current, a number of other physiological demands are made of the receptor, and examination of each of these in terms of an allosteric model shows that the protein is well adapted for its function in synaptic transmission. Spontaneous channel openings when acetylcholine is not being released from the nerve terminal would clearly be harmful if enough current were generated to collapse the membrane potential of the postsynaptic cell. However, in an unligated receptor the equilibrium constant for the allosteric transition is so low that the current from spontaneous openings is somewhat smaller than the ambient leak current of a muscle cell membrane (Jackson, 1989). Thus, spontaneous openings do no harm. By contrast, when acetylcholine is bound, the equilibrium constant for the allosteric transition of doubly-ligated receptors is high enough for the channel to be open more than 90% of the time to generate a near maximal postsynaptic current from each receptor that binds acetylcholine.

Consideration of the binding process also shows a well-adapted protein. Adding up the energies of all the possible contacts that could possibly form between a protein and

one molecule of acetylcholine gives an upper bound to the binding energy of 13.4 kcal/mole, which converts to a maximum possible binding affinity of about 10^{10} M^{-1} (Jackson, 1989). This estimate was based on an atom-by-atom tally of every possible interaction between the ligand and putative partner atoms in a receptor with a binding site that exploits every possible interaction. The highest affinity should be found in the receptor with an open channel, and the experimentally estimated affinity of this conformation is within a factor of fifty of the theoretical maximum. By contrast, the affinity of the receptor in the closed-channel state has a minimum; it must be more than 10^3 M^{-1}, because otherwise the receptor would not be activated rapidly by the concentrations seen by the receptor during synaptic transmission.

With these limiting values for the affinities of the two allosteric states of the protein, we can use the detailed balance constraint (Eq. 6) to estimate how much the equilibrium constant of the allosteric transition can change as a result of a single binding event. The result is that occupation of a single binding site can change the equilibrium between the two conformations by at most a factor of about 10^7 ($K_O/K_C = 10^{10} \div 10^3$). The equilibrium constant must change by about a factor of 10^7 or more during activation; it must be below 10^{-6} for unligated receptors to prevent unwanted current flow in the absence of acetylcholine, and it must be more than 10 to give a respectable current during activation. This indicates that occupation of a single perfect acetylcholine binding site has just enough energy to produce the needed change in the open-closed conformational equilibrium. However, the calculation for a perfect binding site leaves no margin of error. If the upper bound estimated above cannot actually be achieved in nature, then one binding site would be insufficient for receptor activation. On the other hand, two binding sites can give a change of up to 10^{14}, and that is more than enough. According to this analysis, the receptor has two binding sites rather than one in order to guarantee enough of a change in binding energy to gate the channel.

From this standpoint, the presence of two binding sites can be seen as an adaptation of the protein to gain the additional binding energy needed to gate the channel. This makes the biological function of multiple binding sites in the acetylcholine receptor very different from that in other classical multi-site proteins such as hemoglobin. In hemoglobin the multiple oxygen binding sites confer a steep sigmoidicity on the binding curve of the protein. As a result, small changes in oxygen tension can trigger large changes in binding site saturation. In this way hemoglobin can fill up with oxygen in the lungs and release most of it in the tissues where the oxygen tension is about 50% lower. Thus, in hemoglobin multiple binding sites are adaptive in making binding cooperative. By contrast, the acetylcholine receptor witnesses changes in acetylcholine concentration from 10^{-8} M to 10^{-3} M, so that sigmoidal behavior hardly seems necessary (Jackson, 1989). From this perspective, the cooperative activation shown in Fig. 3 is an epiphenomenon; the receptor would function well without cooperativity, but exhibits cooperativity because it has two binding sites to perform another function of providing sufficient energy for channel gating.

A final example of tailoring the activity of the acetylcholine receptor to neuromuscular transmission can be seen in the nonequivalence of the two acetylcholine binding sites, which was discussed above because of its bearing on some of the basic assumptions in the Monod-Wyman-Changeux theory. In considering the physiological function of the acetylcholine receptor it is important to bear in mind that both activation and termination of a synaptic response must be fast. This creates a bit of a dilemma for the receptor, and the nonequivalence can be shown to be a sort of compromise solution (Jackson, 1989). Since, a singly-ligated receptor has an equilibrium constant for the allosteric transition of well below one, dissociation of only one of the two bound molecules of acetylcholine will reduce the response considerably. In this way making the dissociation rate constant of one of the two binding sites very rapid (on the order of 10^4

s^{-1}; Fig. 5) provides for rapid deactivation upon acetylcholine removal, without sacrificing high sensitivity and rapid activation by acetylcholine.

A MICROSCOPIC BASIS FOR ALLOSTERIC THEORY

Seeing how well the Monod-Wyman-Changeux theory works in describing the functional activity of the acetylcholine receptor, we should be more curious about what this theory means at a detailed physical level. The assumptions regarding allosteric states and transitions have implications for the atomic interactions within the protein molecule as well as the interactions between the protein and ligand. It should be noted that the idea of an allosteric transition between two conformations with different binding affinities had been criticized as overly simplistic and unrealistic (Weber, 1975). The argument here is that the protein has approximately 2,000 amino acids. This gives a total of about 32,000 atoms, and with six degrees of freedom per atom, the protein would then have a total of roughly 192,000 internal degrees of freedom. There would be even more if we considered the energy levels of the electrons. Why then, should such a complex system exhibit only two or three global conformations when examined at the level of function?

From this perspective it might seem that a satisfactory explanation requires a complete mathematical expression for the potential energy of the protein as a function of the positions of all the atoms. Then we could see if this equation for the acetylcholine receptor had energy minima corresponding to the open, closed, and desensitized states. Computer programs have been written that are in principal capable of doing this, but with such a complicated equation it is not easy to decide what to look for, and in any case the structure of the acetylcholine receptor is not known with enough accuracy to implement this approach effectively. Furthermore, there are a number of practical problems that make it very difficult to estimate the free energies of different conformational states even when the structure of a protein is known. Accumulating errors and difficulties in

estimating the entropy have made it very difficult to use computers to predict the relative stabilities of different conformations.

We can however get some idea of what underlies the conformational plasticity of a protein by thinking about some of the basic properties of the potential energy function. Consider the potential energy function as quadratic around a central minimum for most of the internal degrees of freedom. The covalent bonds between atoms have precise bond lengths about which there is some stretching. Noncovalent interactions such as hydrogen bonds and van der Waals interactions also have potential energy minima at specific interatomic distances, and the forces between the connected atoms that maintain these minima are weaker than those of covalent bonds so that more flexibility is possible. Bond angles and bond dihedral angles also have potential energy minima with varying degrees of flexibility. One would expect the protein to assume a structure in which a large fraction of the interatomic distances and bond angles are near their potential energy minima. Such a structure could correspond to an allosteric state. However, it is hard to imagine that for such a complicated molecule any conformation could exist for which all of the interatomic distances were simultaneously at or even near their energy minima. Bringing an atom to the right distance with respect to one contact would pull the atom away from the position of minimum potential energy vis-a-vis another neighboring atom. Such a system is said to be "frustrated", such that no conformation can simultaneously satisfy all of the energetic constraints. With this idea in mind, it is easy to envisage the T and R allosteric states as two distinct conformations with different sets of internal contacts that are either near their potential energy minima or frustrated. It is an extremely difficult problem in statistical mechanics to estimate the number of such conformations in a protein and determine their potential energy, but the concept that they exist can still be put to good use.

To illustrate how such sets of contacts might operate we return to the acetylcholine receptor. Each subunit of the acetylcholine receptor has four hydrophobic

segments thought to span the membrane as α-helices. Two hypothetical arrangements of these segments are shown in Fig. 9A, again corresponding to closed and open channels. Clearly, contacts between segments M1 and M3 cannot form if segments M2 and M4 are close enough to form contacts of their own (Fig. 9B). Thus, the structure is frustrated; either segments M1 and M3 form contacts or segments M2 and M4 form contacts. There would then be two distinct energy minima corresponding to these two arrangements, and the depth of each energy minimum would depend on how the residues on each segment complement one another to form interacting pairs. From this perspective, natural selection has produced a protein with contact-forming residues at complementary sites on adjacent segments to adjust the energy differences between conformations according to specific signaling requirements (e.g. low spontaneous opening).

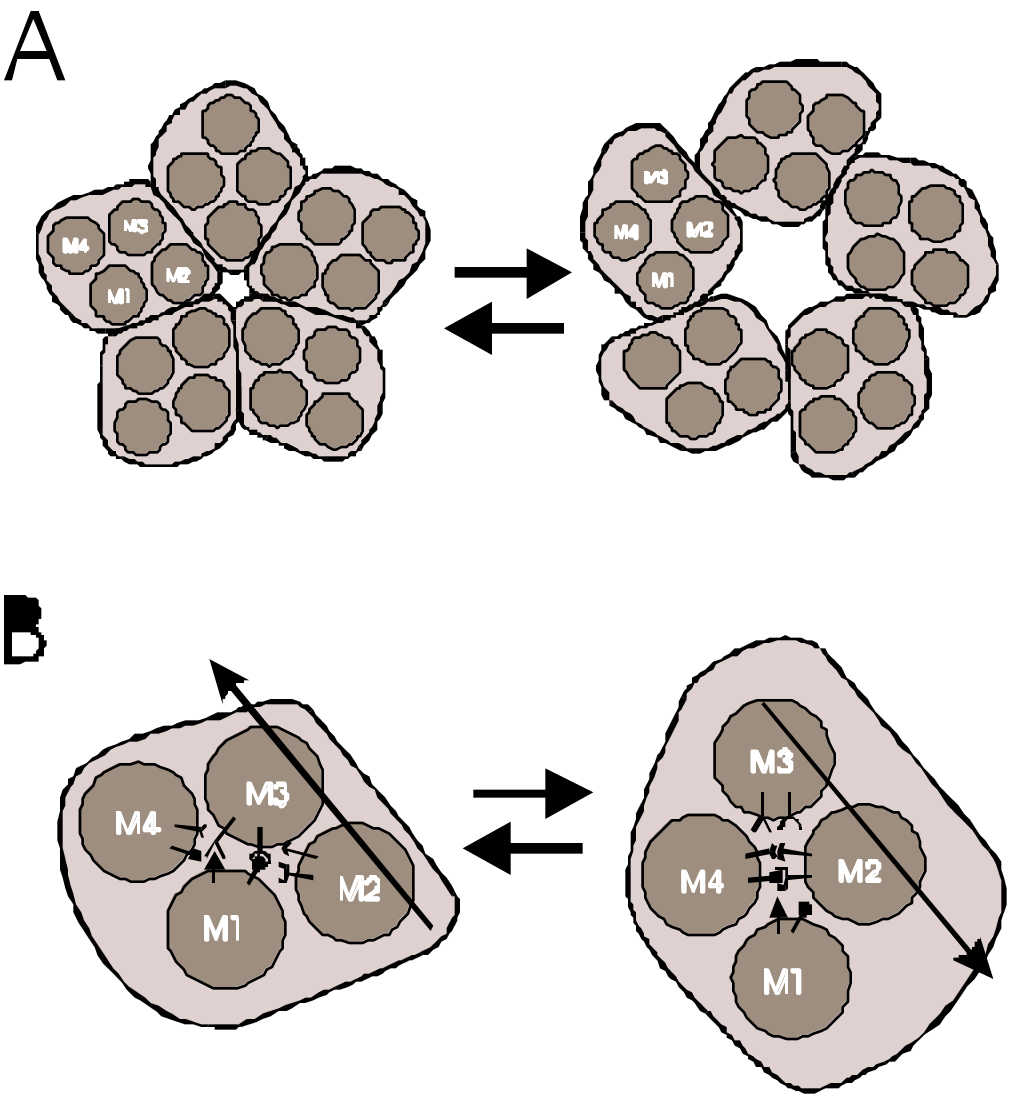


Fig. 9. A hypothetical model for the structural rearrangement of the acetylcholine receptor during channel gating. **A.** All five subunits are shown; segments M1 - M4 were labeled in one subunit. During gating these segments rearrange. The pore enlarges and fills with water to allow the passage of ions. **B.** A magnified view of one subunit (the one with the labels) shows the four membrane spanning segments in more detail. During the rearrangement M2 and M3 are shown to slide outward past M1 and M4 (in the direction indicated by the arrows). Contacts between M1 and M3 break and contacts between M2 and M4 form. One can relate the free energy change during channel gating to sums over such contacts (Eqs. 15).

The idea of different sets of contacts stabilizing one conformation of a protein or another leads to a fairly simple way to represent the free energy difference between two allosteric states. Denote the energies of the set of contacts that stabilize the closed channel conformation as a sum Σg_C and the energies of the set of contacts that stabilize

the open conformation as a sum Σg_O. Note that contacts that remain intact during the conformational transition but move substantially within their potential energy minima can be included in both of the sums, so that the sets g_O and g_C can be quite large and overlapping. If we then assume that the contacts are all independent and additive we can write the free energy change for the allosteric transition between the open and closed states in terms of these two sums.

$$\Delta G_0 = \Sigma\, g_O - \Sigma\, g_C \qquad (15)$$

This free energy difference can then be used to estimate K_0, the equilibrium constant of the allosteric transition in the unligated protein. Eq. 15 thus provides a framework for relating changes in individual intramolecular contacts to observed changes in conformational stability.

We can also view ligand binding as the formation of contacts, this time between the ligand and the protein. The contacts would have different energies depending on the conformational state of the protein, but we can still sum over these contacts to obtain binding energies of the form

$$\Delta G_{bc} = \Sigma\, g_{bc} \qquad (16a)$$

$$\Delta G_{bo} = \Sigma\, g_{bo} \qquad (16b)$$

for binding to the closed conformation and open conformation, respectively. (This kind of sum was used to estimate the binding energy for a perfect binding site (Jackson, 1989), as discussed above). If these contact energies are also independent and additive, then we can recover the basic elements of the Monod-Wyman-Changeux theory by summing these two distinct contributions to the free energy of the protein and the protein-ligand complex. This gives the free energy difference versus the number of binding sites occupied as

$$\Delta G(n) = \Sigma\, g_O - \Sigma\, g_C + n(\Sigma\, g_{bo} - \Sigma\, g_{bc}) \qquad (17)$$

This expression has the same form as Eq. 12 above. Recall that Eq. 12 was fundamental to the Monod-Wyman-Changeux theory. Thus, we can conclude that the

condition of additivity of the atomic interactions involved in stabilizing conformations and ligand binding provides a rigorous basis for this theory. If we consider the summation of binding energies in Eq. 12 as a manifestation of macroscopic additivity and the summation of contacts in Eq. 17 as a manifestation of microscopic additivity, then this reasoning can be summarized succinctly with the statement that microscopic additivity implies macroscopic additivity. This may or may not seem like a simple deduction, but the converse of this statement is not true: macroscopic additivity does not imply, or depend on, microscopic additivity. One can easily imagine that the internal contacts of the protein very close to the binding site will be perturbed by ligand binding, but this nonadditivity could have a relatively short range. As long as these distortions are localized to the vicinity of the binding site and do no spread to other binding sites, macroscopic additivity will still hold. Although the connection is far from absolute, understanding the relationship between microscopic additivity and macroscopic additivity is still very helpful in interpreting experiments on allosteric proteins, and this will be illustrated in the following section.

The forces that stabilize higher order protein structure include hydrogen bonds, van der Waals forces in hydrophobic contacts, salt bridges, and bond stretching and bending potentials. These interactions are discussed in the standard texts on biophysics and proteins. However, the question of whether the energies of these contacts are additive is usually not covered in these discussions. Most of the potential energy functions used in computational studies of proteins treat these energies as additive. This can lead to significant errors because for each of these kinds of interactions there are departures from additivity (Jackson, 1997a). For example, the energy of a hydrogen bond will vary depending on whether other hydrogen bonds are formed with neighboring atoms (Weinhold, 1997). Some possible influences of hydrogen bond nonadditivity will be examined below. In the present context the assumption of additivity of contact energies is

especially valuable in providing a simple and workable theoretical framework for the interpretation of experiments on receptors with single amino acid substitutions.

ALLOSTERIC MUTATIONS AND MICROSCOPIC ADDITIVITY

In ligand-gated channels, mutations can alter the apparent sensitivity of the receptor for a ligand, even though the site of the mutation is thought to be quite distant from the ligand binding site. This phenomenon of mutations appearing to act across large distances resembles the long-range interactions between different ligand binding sites, which allosteric theory was originally designed to explain. When a mutation far from the binding site changes the sensitivity of a receptor (i.e. alters the apparent affinity), it need not be due to a far-reaching influence of the mutation directly on the binding site. In these situations, the form of the apparent affinity as a product ($K_R K_0$ in Eq. 9 or $K_R(K_0)^{1/n}$ in Eq. 10) suggests that the shift could reflect a change in the conformational equilibrium constant, K_0, rather than the affinity of the R conformation, K_R. The results could therefore be interpreted in terms of perturbations of specialized contacts that stabilize the allosteric states. To distinguish between the two possibilities of altering K_0 or altering K_R one can look at shifts in the sensitivity to ligands that are structurally similar, and presumably bind to the same site. If the shifts in sensitivity are the same for different ligands, then the most parsimonious explanation is a change in K_0 (Jackson, 1993a; 1993b; Zhang et al., 1994).

The domain of the acetylcholine receptor in which such allosteric effects have been most clearly demonstrated is the M2 segment (Fig. 9), where a host of mutations have been introduced and found to change both the activation and desensitization transitions. Mutations in the M2 segment have allosteric consequences in the structurally related $GABA_A$ and $5\text{-}HT_3$ receptors as well (Jackson, 1997a). The M2 segment lines the inner walls of the channel, and thus must undergo some form of movement during gating to allow the channel to open. It is therefore not surprising that residues in the M2 segment form and break contacts during channel gating transitions. A prediction of allosteric theory is that increasing the sensitivity of the receptor by increasing K_0 will also increase the rate of spontaneous channel opening, and this has been confirmed (Auerbach et al.,

1996). Parallel shifts in spontaneous openings and acetylcholine sensitivity have also been seen in a receptor formed from the α, β, and γ subunits of the cow acetylcholine receptor (with the δ subunit omitted; Jackson et al., 1991). In some instances a mutation can influence one allosteric transition but not another. For example, mutation of an alanine residue in the M2 segment of the *Drosophila* GABA receptor produces a large reduction in the rate and extent of desensitization but leaves the sensitivity of the receptor to activation by GABA virtually unchanged (Zhang et al., 1994).

An important finding from mutagenesis studies of the M2 segment of the acetylcholine receptor is that the effects of multiple mutations are additive. The ninth residue of the M2 segment is a leucine in all of the different subunits of the acetylcholine receptor. Replacing this leucine by a serine (Labarca et al., 1995) or threonine (Filatov and White, 1995) increased the sensitivity of the receptor, and when multiple substitutions were made the effects were additive. If these residues were producing a long range structural perturbation of the binding site, then one would expect that mutations in the M2 segment of the α subunit would exert greater effects because the α subunit contains most of the acetylcholine binding site. The similar effect of this replacement in each of the subunits, together with the additivity of these changes, argues for an allosteric mechanism which can be represented quantitatively as a change in the equilibrium constant of the allosteric transition, K_0.

Are there any situations where nonadditivity of contact energies might be important? So far there is no clear evidence, but based on the short range of most nonadditive microscopic interactions one should look for nonadditivity in contacts in close proximity. For example, the contacts that stabilize ligand binding are close to one another and some interesting ideas emerge when we consider the acetylcholine binding site and the possible roles of hydrogen bonds. Although hydrogen bonds were once thought to be primarily electrostatic, recent *ab initio* quantum mechanical calculations have shown that they have substantial covalent character, and covalent bonds interact

with one another through molecular orbitals. This means that hydrogen bond formation can exhibit positive or negative cooperativity; the energy of a hydrogen bond will depend on whether another part of the molecule has also formed a hydrogen bond (Weinhold, 1996).

Acetylcholine has a carbonyl oxygen and an ester-link oxygen, both of which are capable of acting as hydrogen bond acceptors. To see some of the potential implications of nonadditivity we consider a hypothetical network of hydrogen bonds at the acetylcholine binding site (Fig. 10A; see also Jackson, 1997a). This diagram draws on the fact that two tyrosine residues are thought to participate in acetylcholine binding (Tomaselli et al., 1991; Chen et al., 1995). This model thus includes a chain of hydrogen bonds, starting with a serine hydroxyl, and continuing on to a tyrosine hydroxyl, a bound water molecule, another tyrosine, and a finally a glutamate hydrogen bond acceptor. This chain of hydrogen bonds will have strong positive cooperativity according to Weinhold (1996), so that the bonds will be stronger than they would be in isolation. If the structure maintained by these contacts is important to the overall integrity of the closed state of the channel, then the channel will open only rarely. If during binding a hydrogen bond forms between acetylcholine and the water molecule in the binding site, then the arrangement of hydrogen bonds to the water becomes anticooperative. The existing hydrogen bond between the water and the second tyrosine (bond 3, Fig. 10B) will be weakened and the newly formed hydrogen bond between the acetylcholine and water molecule (indicated by the symbol $*$) will be relatively weak. The weakening of bond 3 may allow it to break easily, and if, as mentioned above, the chain is important to the integrity of the closed state, the channel will open more readily (Fig. 10C). In fact, in doubly-ligated receptors the rate of channel opening is accelerated over the rate of opening in unligated receptors by six orders of magnitude (see Detection of Allosteric Transitions, above), and this is consistent with a weakening of contacts that stabilize the closed state.

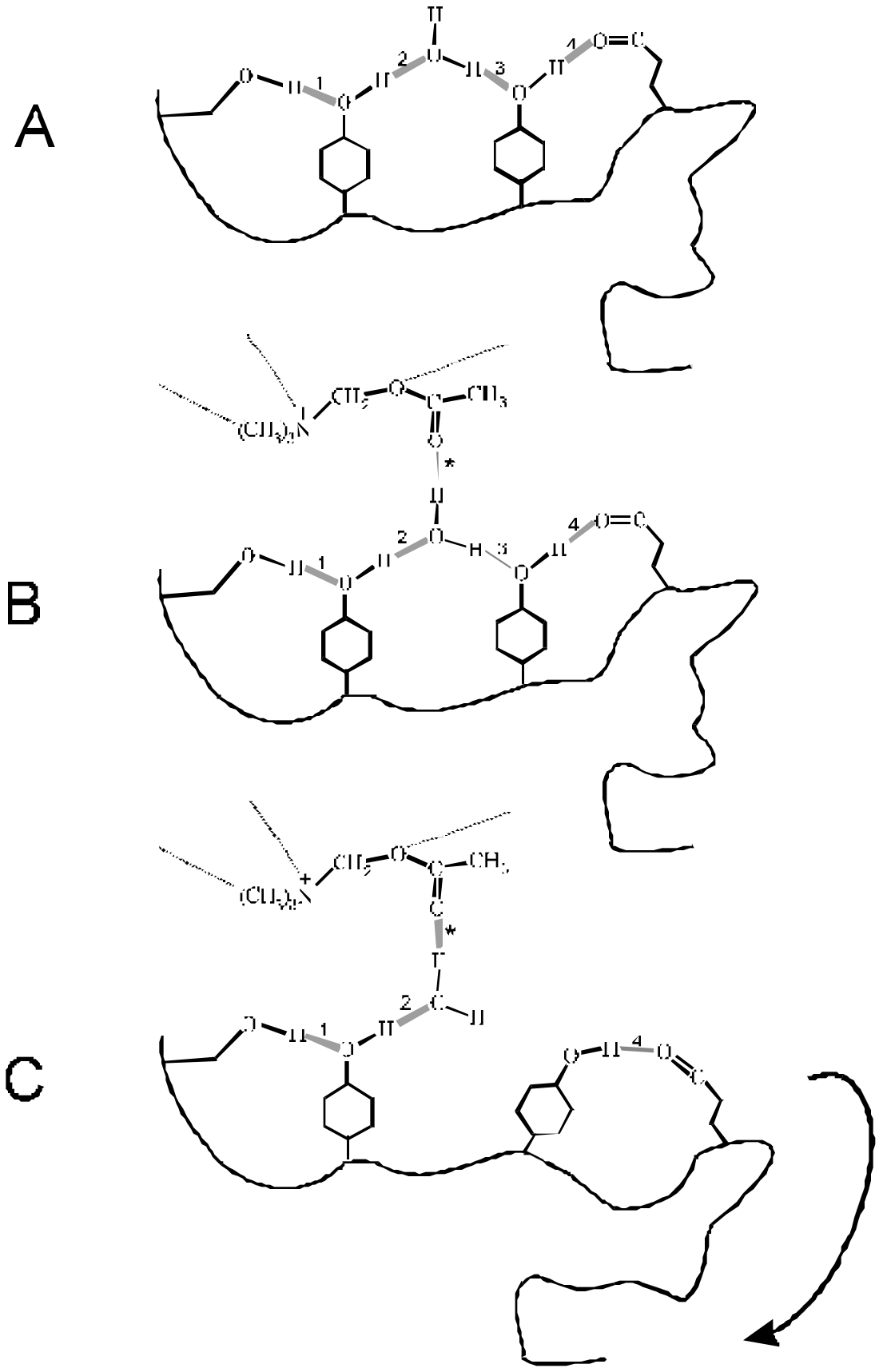

A
B
C

Fig. 10. Nonadditive interactions in the acetylcholine binding site. A possible role for hydrogen bond cooperativity. **A**. A diagram of a hypothetical structure in the acetylcholine receptor binding site shows a chain of four hydrogen bonds starting with a serine hydroxyl, to a tyrosine hydroxyl (bond #1), to a bound water molecule (bond #2), to a tyrosine hydroxyl (bond #3), to a glutamate carbonyl (bond #4). This chain of hydrogen bonds should form a cooperative unit, such that the bonds are stronger than they would be in isolation (greater strength is indicated by thicker shaded lines). **B**. During binding acetylcholine is shown forming a new hydrogen bond with the water in the binding site (indicated by the symbol $*$). After forming this additional hydrogen bond, the water becomes part of a star-shaped hydrogen bond network that should show negative cooperativity between the two bonds in which the water is a Lewis acid. **C**. This negative cooperativity will weaken hydrogen bond #3, allowing it to break more easily so that the protein can undergo the conformational transition. After bond #3 has broken the anticooperative arrangement of hydrogen bonds is lost and the remaining hydrogen bond between the acetylcholine carbonyl and the tyrosine hydroxyl will be part of a chain in which hydrogen bonding exhibits positive cooperativity. The bonds will then become stronger and strengthen the attachment of acetylcholine to the binding site. The evaluation of cooperativity in different arrangements of hydrogen bonds is based on the work of Weinhold (1997)

A further consequence of hydrogen bond cooperativity in this scheme is that after bond 3 within the binding site is broken the hydrogen bond formed between the binding site water molecule and acetylcholine will become part of a new chain with positive cooperativity (Fig. 10C). Thus, channel opening will strengthen a hydrogen bond that holds acetylcholine in its binding site, and this provides a physical basis for the stronger binding to the open state dictated by allosteric theory. Thus, although nonadditivity can lead to nonallosteric behavior if the effects are long range, nonadditive interactions over short ranges not only are consistent with allosteric theory, but lead to predictions that are a fundamental part of allosteric processes (Jackson, 1997a).

VOLTAGE-GATED CHANNELS

At the beginning of this chapter the claim was made that by learning how to apply allosteric theory to the acetylcholine receptor we would learn something more general that would help guide our thinking about other forms of biological signaling. To illustrate this a final discussion will be presented on voltage-gated channels. Here too, the process of channel gating can be thought of as an allosteric transition, but now the transition involves the movement of charge through a portion of the transmembrane field rather

than a change in the shape of a binding site. Once again sets of contacts break and form within the protein as the switching takes place, and a sum can be taken over the energies of these contacts to obtain the ΔG of the transition (Eq. 15). However, because there is also charge movement through the transmembrane field ΔG will contain an additional term (Fig. 11). Each moving charge will make a contribution to ΔG equal to $zF\delta V$, where z is the valence of the charge, F is Faraday's constant, δ is the fraction of the membrane potential traversed, and V is the membrane potential. Adding all of these terms together then gives an expression for ΔG of the allosteric transition in a voltage-gated channel

$$\Delta G = \sum g_o - \sum g_c + \sum z_i F \delta_i V \qquad (18)$$

where the final sum was performed over any number of charged groups that move during the transition. This equation can be inserted into the Boltzmann distribution and then rearranged to give the probability of the channel being open

$$P_o = \frac{1}{1+e^{(V - V_o)/\kappa}} \qquad (19)$$

with $1/\kappa$ replacing $\sum z_i F \delta_i V/RT$ and $-V_o/\kappa$ replacing $\left(\sum g_o - \sum g_c\right)/RT$. V_o is the voltage at which half of the channels are open and κ is the steepness of the voltage dependence of the transition. Thus, we see that the contacts that stabilize each conformation set the voltage of the transition and the charges that move during gating determine the steepness. The parallel with ligand-gated channels extends further when one considers the subunit composition of a voltage-gated channel. Then one has to make some assumptions about how the subunits influence one another, and whether that influence depends on differences in allosteric state. The choice of assumptions will lead to models with a wide range of behaviors, and should therefore be useful in the interpretation of experiments on voltage-gated channels. Progress is being made in evaluating how these assumptions match up with detailed analysis of experimental data (Zagotta et al., 1993).

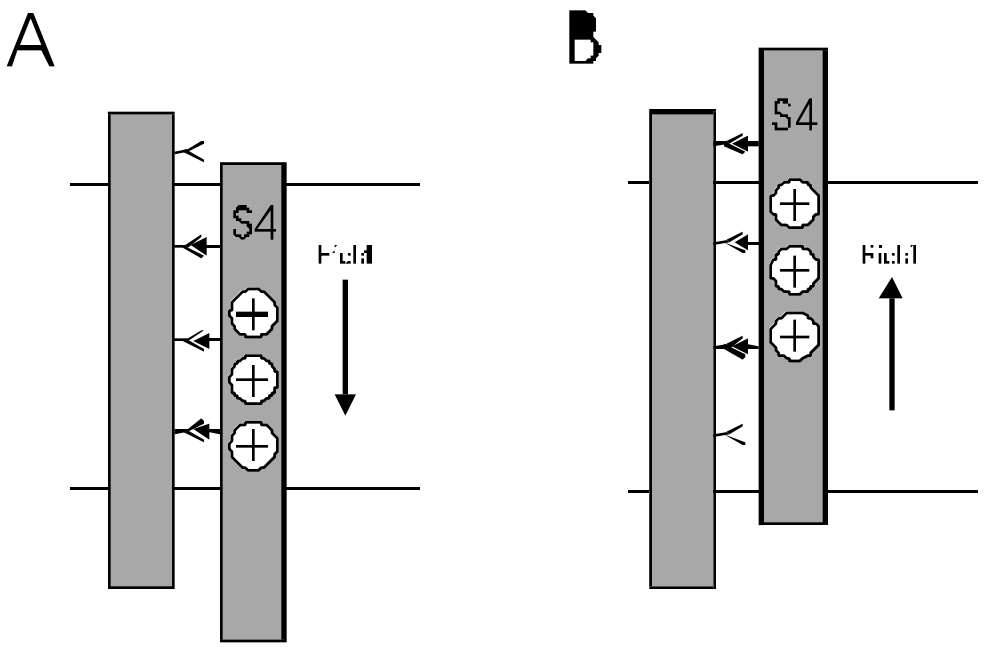


Fig. 11. A hypothetical model for the structural rearrangement in a voltage-gated channel. It has been proposed that the S4 segment performs a voltage sensing function, and that voltage-dependent transitions in the channel involve a sliding motion of the S4 segment within the field of the membrane (Guy and Conti, 1990). The positions of the charged groups enter into the energetics of a particular conformation through terms of the form $qF\delta V$ (Eq. 18). These terms appear in the part of the Boltzmann equation that reflects the voltage sensitivity (κ of Eq. 19). The contacts drawn between the S4 segment and an adjacent segment contribute terms of the form g_O and g_C to the free energy of a conformation. These terms appear in the part of the Boltzmann equation that reflects the voltage midpoint of the transition (V_O of Eq. 19).

Many of the mutagenesis studies in voltage-gated channels give results that fit with this functional distinction (Sigworth, 1994). Mutations all over the protein can produce a shift in the voltage dependence mimicked by a change in V_O. In contrast, mutations of just a few specific charged residues in the S4 transmembrane segment (a motif seen in most of voltage-gated channels) produce a change in the steepness reflecting a change in κ in Eq. 19. Thus, viewing functional changes in proteins as allosteric transitions involving global changes in structure, with internal contacts forming and breaking during transitions, provides a conceptual framework that can be applied to a variety of biological transduction systems.

PROBLEMS

1. Show that Eq. 8 goes to the limit of Eq. 9 for small K_0 and large K_1.
2. Derive the exact expression for the fraction of binding sites occupied for the single-site allosteric model corresponding to the dose-response expression (Eq. 8). Determine the limiting approximate expression analogous to Eq. 9.
3. Derive Eq. 10. Derive the fraction of ligand binding sites occupied as a function of ligand concentration for this model. (Hint: Terms can be collected into sums that can be expressed as binomial expansions.)
4. Derive the equilibrium distribution of states C_0, C_1, C_2, and O_2 in Scheme B in the presence of 1 mM acetylcholine, using affinities calculated from the rate constants given in Figs. 5 and 8. Neglect states O_0 and O_1. What fraction of the receptors have open channels, and does this concur with the maximal responses shown in Fig. 8?
5. For Scheme B derive expressions for the equilibrium frequency of channel opening transitions into each of the open states O_0, O_1, and O_2 as a function of acetylcholine concentration. Express the answer symbolically using the $k_1[A]$, $k_2[A]$, d_1, d_2, and α from Scheme B. Introduce the symbols β and γ for the rates of opening of C_0 and C_1. Assume that there is only one channel.
6. A mutation is introduced into a single-subunit allosteric protein that removes a single hydrogen bond. This hydrogen bond is in no way involved in ligand binding, but contributes 3.5 kcal/mole to the stability of the closed state. Further, this hydrogen bond is broken during the gating transition so that it contributes zero free energy to the stability of the open state. By what factor will this mutation alter the apparent affinity of the receptor for an agonist? If such a mutation were made in a voltage-gated channel, how would the voltage dependence of the gating transition be altered?

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