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Allosteric Receptors after 30 Years

Review

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The concept of allosteric proteins was initially proposed to account for paradoxical properties exhibited by certain bacterial enzymes that catalyze strategic reactions in biosynthetic pathways. The activity of these enzymes was found to be selectively feedback inhibited by the end product of the pathway, despite its very limited structural resemblance to the substrate (Umbarger, 1956; Yates and Pardee, 1956). Subsequently, various in vitro chemical treatments or mutations were found that abolished the interactions between substrate and regulatory effector, with little or no loss of activity (Changeux, 1961; Gerhart and Pardee, 1962). These observations led to the proposal that the interactions between both classes of ligand do not result from classical mutual exclusion by steric hindrance at a common binding site, but rather occur between topographically and stereochemically distinct sites (Changeux, 1961; Monod and Jacob, 1961). The binding of the regulatory ligand to a specific allosteric site (Monod and Jacob, 1961), structurally distinct from the active site, brings about a reversible alteration of the conformation of the protein, an allosteric transition, that indirectly modifies the properties of the biologically active site (Monod et al., 1963). This indirect action via a distinct regulatory site is still the most commonly accepted meaning of the word allosteric.

On the other hand, the 1965 article by Monod, Wyman, and Changeux, "On the nature of allosteric transitions: a plausible model" (Monod et al., 1965), led to an extension of the meaning to account for another characteristic feature of these regulatory enzymes: the occurrence of cooperative interactions for both the substrate and the regulatory ligand. This property renders their function dependent upon threshold concentrations of ligand in analogy with the cooperative binding of oxygen. The three-dimensional structure of hemoglobin at 5.5 Å resolution (Muirhead and Perutz, 1963) further revealed that the molecule of hemoglobin is a symmetrical tetramer; cooperative interactions for oxygen binding take place between the four hemes separated by at least 25 Å and are thus allosteric.

In the absence of corresponding structural data for regulatory enzymes, the MWC model was first proposed to account, by a common molecular mechanism, for both the interactions between different ligands (e.g., regulatory molecules and substrates) and the positive cooperative effects observed for each category of ligand. The postulate was made that the cooperative binding properties of these proteins are determined by the presence of several identical subunits assembled into a cooperative and symmetrical quaternary structure. Moreover, the conformational transition that such protein assemblies undergo was thought to affect primarily the quaternary interactions between subunits, rather than the tertiary folding within each individual subunit. In other words, the cooperative interactions between ligand binding sites would result from the cooperative transition of the quaternary structure of the molecule. Accordingly, the symmetry properties postulated by the model would simply express a characteristic regularity of protein quaternary structure.

Specifically, the MWC model hypothesizes that: (1) regulatory proteins in general are oligomers made up of a finite number of identical subunits that occupy equivalent positions and as a consequence possess at least one axis of rotational symmetry (Figure 1A); (2) the allosteric oligomers can spontaneously exist in a minimum of two freely interconvertible and discrete conformational states ($T \rightleftharpoons R$) that differ in the energy of their intersubunit interactions (quaternary constraint), but with conserved molecular symmetry; (3) the affinity and activity of the stereospecific sites carried by the oligomers may differ between the two states, and ligand binding differentially stabilizes the particular state for which it exhibits a higher affinity; and (4) in the absence of ligand, the preexisting conformational equilibrium is characterized by an isomerization constant $L = (T)/(R)$, and modulation of the conformational equilibrium by ligand binding suffices to generate cooperative ligand binding, as well as interactions between different ligands.

The two-state concerted model contrasts sharply with the Koshland, Némethy, and Filmer (1966) sequential-type model, for which the conformational transition caused by ligand binding to an individual subunit results from an induced-fit mechanism and subsequently (and thus sequentially) propagates to the neighboring subunits within the oligomer. Hence, the KNF sequential model precludes the occurrence of any allosteric transition in the absence of ligand.

Crystallographic structure determinations of several regulatory enzymes (reviewed by Perutz, 1989) have confirmed that these proteins, like hemoglobin, are indeed oligomers and possess a symmetrical organization primarily with 2-fold symmetry axes (e.g., for threonine deaminase [Gallagher et al., 1998]) but also 3-fold axes (e.g., for the transcarbamylases [Lipscomb, 1994; Villeret et al., 1995]). Moreover, characteristic rearrangements of the quaternary structures were found associated with the allosteric transition, mostly due to relative subunit rotations (see Figure 1B) which, in all reported instances, preserve the symmetry of the assembly (Iwata et al., 1994, and references therein, for aspartate transcarbamylase, phosphorylase B, phosphofructokinase, and bacterial L-lactate dehydrogenase). Nevertheless, within these major conformational states, which impose strong quaternary constraints, local modifications of tertiary structure have been detected at the subunit level as a direct consequence of ligation, notably for hemoglobin

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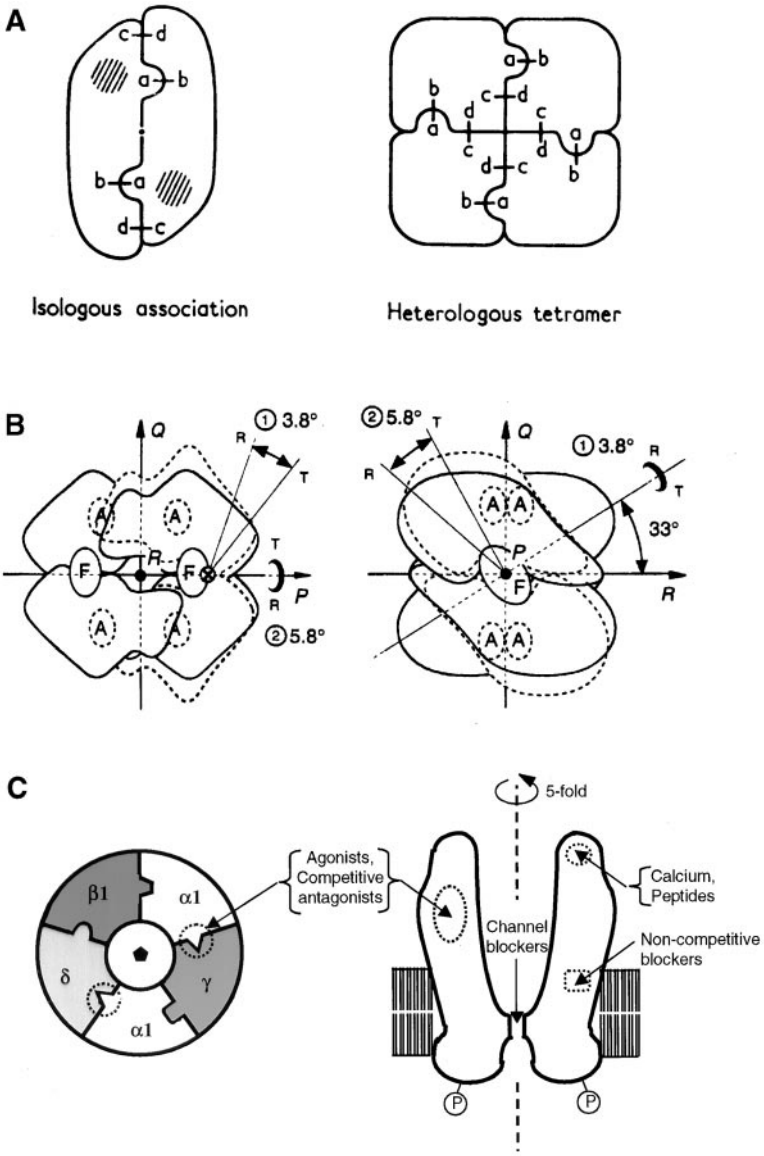

Figure 1. Subunit Interactions in Allosteric Proteins

(A) Isologous association and a heterologous tetramer reproduced from the article of Monod et al. (1965). Isologous association arises when the domain of bonding has a 2-fold axis of rotation symmetry, as in the case of EGFR (see Figure 2B), whereas for heterologous association each bonded group pair is unique, with the domain of bonding having no element of symmetry.

(B) Symmetry-conserving transition between R and T states as represented by Iwata et al. (1994) for the two oligomeric structures coexisting in the same crystals of L-lactate dehydrogenase from *Bifidobacterium longum*. Projections of the R state tetramer (solid lines) with a superimposed T state dimer (dashed lines) viewed down the R axis on the left and down the P axis on the right. The allosteric transition is a combination of two rotations: (1) a 3.8° rotation about an axis passing through the point marked by ⊗; and (2) a 5.8° rotation about the P axis, where the axis for the 3.8° rotation is 33° away from the R axis in the plane perpendicular to the P axis, as shown on the right. The active sites are indicated by A, and the allosteric sites (to which binds the effector, fructose-1,6-bisphosphate) are indicated by F.

(C) Rotational symmetry for a heteropentameric membrane receptor. The transversal and longitudinal sections correspond to the muscle-type acetylcholine receptor; for additional details see Figure 3B.

(Perutz, 1989). Steady-state kinetic data for these molecular species in solution, however, do not significantly deviate from the predictions of the MWC model. In particular, the crucial assumption that the state function $\bar{R}$ (which describes the fraction of the protein molecules that has undergone the conformational transition) differs from the binding function $\bar{Y}$ (which describes the occupancy of the sites by the ligand) was soon verified with several systems under equilibrium conditions, such as aspartate transcarbamylase (Changeux and Rubin, 1968; Gerhart and Schachman, 1968; Schachman, 1988). Despite the claim that asymmetric hybrid conformational states arise during oxygenation of hemoglobin (Ackers et al., 1992), the presence of such states was found to be incompatible with the high degree of cooperativity observed in equilibrium oxygen binding measurements (Edelstein, 1996). Moreover, recent rapid binding experiments show that the data are compatible with only two kinetically distinct states, as predicted by the MWC model (Henry et al., 1997; see also Shibayama et al., 1998). Nevertheless, even with its success, the model was never thought to give an exhaustive description of reality and remains a mechanistic description of a defined molecular behavior of regulatory proteins.

In addition, two features unsuspected in the original model have been established by subsequent structural studies of regulatory enzymes. First, ligand recognition sites (catalytic *and* regulatory) are in many cases located at subunit interfaces, with different interfaces accommodating different categories of stereospecific ligands (see references in Iwata et al., 1994); some interfaces bind pharmacological agents despite the absence of known endogenous ligands (Perutz et al., 1986). Second, for hemoglobin, the physiological regulatory ligand 2,3-diphosphoglycerate (as well as synthetic drugs) binds at a rather unsuspected location: within the axial cavity

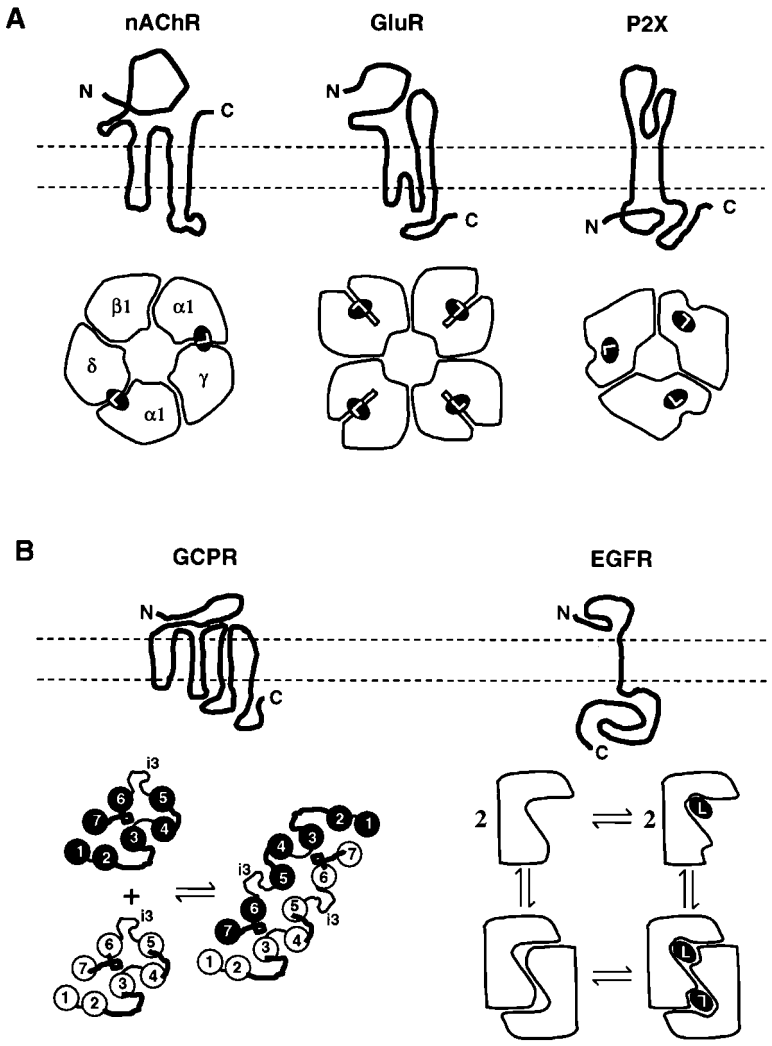

Figure 2. Topologies and Oligomeric Structures for Allosteric Membrane Receptors

(A) Stable oligomers.

(B) Transient dimers.

In each case, a transmembrane topology of a single subunit is shown, with a cross-sectional view (below) at the level of the extracellular ligand binding site, except for GPCR, which is viewed from an extracellular perspective (hence, extracellular loops are represented by thick lines, intracellular loops by thin lines).

of the molecule (Perutz et al., 1986). As we shall see, these new features are of importance for ligand-gated ion channels.

At the time the MWC model was accepted for publication, the possibility was mentioned that the "membrane phenomena that together give rise to the recognition of stereospecific metabolic signals and to their transmission (e.g., at synapses) might involve mechanisms analogous to those described for allosteric proteins" (Changeux, 1965). This additional hypothesis may have been considered somewhat farfetched and unnecessary, since in the 1960's no synaptic receptors had been identified and only pharmacological (Dale, 1953; Nachmansohn, 1959) or electrophysiological (Katz, 1966) data were available. However, the progressive extension of the MWC model to various features of membrane receptors for neurotransmitters (Changeux et al., 1967; Karlin, 1967; Changeux, 1969; Edelstein, 1972; Colquhoun, 1973; Jackson et al., 1990; Edelstein et al., 1996) has provided new concepts (and some reevaluations) of theoretical and experimental importance often in relation with their transmembrane orientation (Figures 1C and 2). First, the receptor proteins exhibit transmembrane polarity; the regulatory site to which the neurotransmitter binds is exposed to the synaptic side of the membrane (or the cytoplasmic side, in the case of cyclic GMP [cGMP]); the biologically active site is either a transmembrane ion channel, a G protein binding site, or a kinase catalytic site facing the cytoplasmic side of the membrane; and both sites are assumed to be topographically distinct, such that the interactions between the two classes of sites are indirect and thus mediated by a transmembrane allosteric transition. Second, as a consequence of the transmembrane polarity, only symmetry axes perpendicular to the membrane plane are acceptable (Changeux, 1969), allowing for the formation of heterooligomers and thus resulting in a partial breakdown of symmetry. Third, signal transduction or activation would be mediated by a "concerted" cooperative transition between a silent resting state and an active (open channel, G protein binding, or enzymatically proficient) state, with the intrinsic conformational equilibrium between oligomeric states (defined by the L value) established prior to ligand binding; agonists stabilize the active state and competitive antagonists the silent state, and partial agonists may nonexclusively

bind to both (Rubin and Changeux, 1966; Edelstein, 1972). Fourth, membrane receptors undergo, in general, a cascade of slower, discrete allosteric transitions, which include refractory regulatory states resulting in the desensitization or "potentiation" of the physiological response (Katz and Thesleff, 1957; Heidmann and Changeux, 1978, 1979; Neubig and Cohen, 1980; Schlessinger, 1988; Lefkowitz et al., 1993, 1998; Edelstein et al., 1996; Weiss and Schlessinger, 1998).

In the following sections various aspects of this updated model will be critically examined.

Transmembrane Oligomers: from Perfect Symmetry to Pseudosymmetry

Whereas globular allosteric enzymes generally possess perfect symmetry involving, in general, several axes (e.g., three dyad axes for L-lactate dehydrogenase) (Figure 1B), for membrane receptors the exclusive occurrence of a single rotational axis perpendicular to the membrane (Figure 1C) reduces the symmetry properties. Nevertheless, several different oligomeric structures, all with rotational symmetry, have been recognized in membrane receptors, from pentamers down to dimers.

Pentamers

The pentameric organization of the nicotinic acetylcholine receptor (nAChR), a ~300 kDa protein first identified from fish electric organ using snake venom α toxins and solubilization in nondenaturing detergent (Changeux et al., 1970; reviewed by Changeux, 1981), involves four different subunits, α1, β1, γ, and δ, with a rather baroque stoichiometry of 2:1:1:1 (Reynolds and Karlin, 1978). Microsequencing analysis and cloning of the different subunits soon revealed extensive sequence identities, thus pointing to a possible pseudosymmetrical organization of the molecule and further suggesting a divergence from a common ancestral gene by 4-fold duplication (Raftery et al., 1980; Noda et al., 1983). This interpretation was subsequently supported by the identification of a multigene family of neuronal subunits, α2–α9 and β2–β4 (Boulter et al., 1986), and the discovery that a single class of subunits from the ancestral-type genes (α7–α9) may give rise, in reconstituted systems, to functional homooligomers (Le Novère and Changeux, 1995). Ancestral-type neuronal nAChR may thus possess a perfect rotational symmetry (see Figure 3A).

For the receptors with a heteropentameric structure, two modes of association can then be distinguished. In the first mode, the different subunits occupy a fixed and unique position within the heteropentamer (Figure 3B), as in the case of *Torpedo* (and most probably muscle) receptor where the clockwise order of the subunits, as viewed from the synaptic cleft, is α_H–γ–α_L–δ–β, with α_H and α_L indicating the respective contributions of each α subunit to the high- and low-affinity sites for d-tubocurarine (Machold et al., 1995). A specific sequence of subunit interactions and binding-site formation may also prevail during assembly (Green and Wanamaker, 1998). Alternatively, in the second mode, one subunit (or several) may be shared between different heterooligomers, as in the case of the neuronal oligomers composed of α4, "α"5, and β2 subunits; under these conditions, the same subunit may occupy several equivalent positions, with different partners (Figure 3C).

Electron microscopy of negatively stained, purified, or membrane-bound receptor from *Electrophorus* or *Torpedo* accordingly reveals ring-like (~8 nm diameter) pseudosymmetrical assemblies (Cartaud et al., 1973) with a 5-fold rotational axis normal to the plane of the membrane (Unwin, 1995, 1996, and references therein). Furthermore, cross-sections in the intramembrane portion reveal five "rods" symmetrically organized around the central axis and interpreted as the channel-forming α helices (Unwin, 1995) (see The Ion Channel in the Axis of Symmetry). The electron microscopy data are thus consistent with a pseudosymmetrical oligomeric organization of the quaternary structure.

Integration into the membrane favors a "transverse polarity" in the tertiary organization of each receptor subunit with several distinct structural and functional domains (Figure 2A): (1) a large hydrophilic extracellular N-terminal domain that carries the neurotransmitter binding sites, the main immunogenic region, and the glycosylation sites; (2) four membrane-spanning segments (M1–M4), among which at least M2 lines the ion channel; and (3) another hydrophilic domain of variable length (between M3 and M4) exposed to the cytoplasm and susceptible to phosphorylation at a variety of sites (reviewed by Devillers-Thiéry et al., 1993). Evidence for the functional specificity of the extracellular, transmembrane, and cytoplasmic domains is provided, in addition, by the construction of chimeric subunits from the N-terminal extracellular domain of the α7 nAChR and the complementary portion of the 5-HT_3 receptor subunits, yielding functional homooligomeric channels that are gated by nicotinic ligands (Eiselé et al., 1993). The same basic rules of oligomerization of subunits with homologous transverse polarity may plausibly be extended to other members of the nAChR family such as the γ-aminobutyric acid (type A) and glycine receptors (Betz, 1990). Accordingly, the most ancestral oligomeric forms would be perfectly symmetric pentamers, and the more evolved forms would incorporate different, though homologous, subunits resulting in structural and functional diversification (Role and Berg, 1996).

Tetramers

The tetrameric organization of the glutamate receptor oligomers has only recently been recognized, first on biochemical grounds (Wu et al., 1996) and then with functional assays based on electrophysiological recordings of either coexpression of wild-type and mutant subunits (Laube et al., 1998; Mano and Teichberg, 1998) or graded antagonist occupancy (Rosenmund et al., 1998). The tetrameric quaternary structure further strengthens the analogy with potassium channels for which 4-fold symmetry has been established by X-ray crystallography at 3.2 Å resolution (Doyle et al., 1998). Functional homooligomers may arise from single subunits of glutamate receptors (GluRs), although for NMDA receptors heterooligomeric assemblies (NR1 and NR2) are required to obtain joint activation by glutamate and the coagonist glycine (Laube et al., 1997).

The transmembrane topology of the glutamate receptor subunits was initially thought to resemble that of nAChR on the basis of hydrophobicity plots (Hollmann and Heinemann, 1994) but in fact differs dramatically. The currently accepted disposition involves a two-lobed

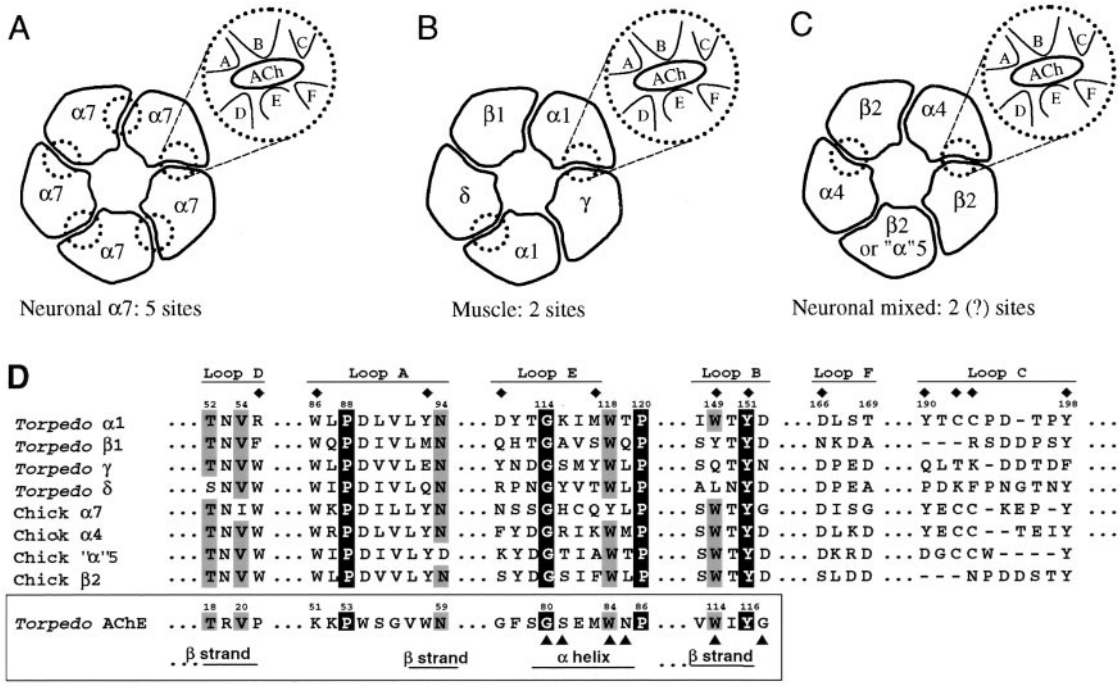

Figure 3. Subunit Organization and Ligand Binding Loops for Nicotinic Receptors

(A) Subunit arrangement of homopentameric receptors. Agonist binding sites are indicated by the dashed circles; all five are equivalent, but one is enlarged to show the contributions of loops A–F.

(B) Subunit arrangement of muscle-type receptors. Two agonist binding sites are indicated by the dashed circles.

(C) Subunit arrangement of heteropentameric neuronal receptors with 2 or 3 subunit types. Two agonist binding sites are indicated by the dashed circles. The α in quotation marks indicates that, unlike other α subunits, α5 may not directly contribute to agonist binding.

(D) Alignments corresponding to the ligand binding loops for a selection of *Torpedo* and chick receptor subunits and for *Torpedo* acetylcholinesterase. Complete identity for aligned residues in all sequences shown is indicated by a black bar; extensive but incomplete identity is indicated by a gray bar. Residues that interact with ligands for at least one of the subunit types of the acetylcholine receptor are indicated by black diamonds above the alignments, and residues implicated in substrate binding for acetylcholinesterase are indicated by black triangles below the alignments, along with the secondary structure motifs observed for these regions (Sussman et al., 1991).

synaptic domain carrying the neurotransmitter binding site (see Paas et al., 1996; Armstrong et al., 1998), three transmembrane segments (M1, M3, and M4), a cytoplasmic reentrant loop (M2) assumed to form the ion selectivity filter of the channel (as in the case of the extracellular P region of the K^+ channel), and a cytoplasmic C-terminal domain (Figure 2A). As was noted for nAChR, functional chimeras between several of these subunits possessing such transverse polarity can assemble into functional homooligomers, supporting a common transmembrane organization for all members of the GluR family (Stern-Bach et al., 1994; Wo and Oswald, 1994; Kuner et al., 1996; reviewed by Paas, 1998).

A tetrameric organization most probably extends to the cyclic nucleotide receptors, but with a transmembrane topology that includes three additional transmembrane domains and a nucleotide binding site facing the cytoplasm (Zagotta and Siegelbaum, 1996; see also Liu et al., 1998).

Trimers

An unexpected trimeric organization of the P2X cation channels gated by extracellular ATP has been recently established by cross-linking experiments (Nicke et al., 1998). This receptor family, as represented in Figure 2A, has two transmembrane regions, M1 and M2 (Newbolt et al., 1998). The ion channel would be contributed mainly by M2, which would posses some β structure (Rassendren et al., 1997), in contrast to the mainly α-helical structure of M2 in the nAChR family.

Dimers

The G protein–coupled receptors (GPCRs) have generally been viewed as monomeric allosteric proteins. Although the atomic structure of a GPCR has not been determined, the similarity with bacteriorhodopsin (Henderson et al., 1990) and the low-resolution structure of rhodopsin (Unger et al., 1997) supports a tertiary organization of the molecule with seven transmembrane α helices. The ligand binding site would either be located between transmembrane helices or within the extracellular domain (Bockaert and Pin, 1998). On the other hand, the intracellular loop i3 and C-terminal segment would interact with the G protein. Such transmembrane "allosteric" interactions were classically assumed to be mediated by a single receptor unit (Leff, 1995).

Recent experimental evidence, however, suggests that active forms of the GPCRs can occur as transmembrane oligomers (dimers or higher oligomers). For example, functional complementation has been reported between two inactive chimeras of muscarinic and adrenergic receptors (Maggio et al., 1993), as well as for two inactive point mutations of the type 1 angiotensin II

receptor (Monnot et al., 1996). Dimers have also been directly observed in several GPCR systems (Hebert et al., 1996, 1998; Cvejic and Devi, 1997). Moreover, studies on "split" GPCRs show that portions of the structure composed of two or more helices can constitute independent interlocking folding units (Gudermann et al., 1997) (see Figure 2B). Such a status of allosteric dimer (and, in certain cases, heterodimer) for GPCRs is possibly associated with a diversification in their interactions with different G proteins and kinases, as well as in their desensitization and sequestration properties (Lefkowitz et al., 1998).

The cell surface receptors with tyrosine kinase activity have long been reported to behave as transmembrane allosteric dimers resulting from the isologous association of two receptor units with an axis of dyad symmetry perpendicular to the membrane (Schlessinger, 1988). Activation in response to growth factors is indeed associated with the presence of dimers that catalyze autophosphorylation and activation of the kinase domains for phosphorylation of other substrates on their cytoplasmic face (Schlessinger and Ullrich, 1992). The transmembrane organization includes, in this case as well, distinct domains for ligand binding, spanning of the bilayer, and kinase activity on the cytoplasmic side (Figure 2B).

Dimerization was suggested by in vitro measurements (Lemmon et al., 1997) to occur exclusively upon ligand binding (Weiss and Schlessinger, 1998). On the other hand, the ligand may stabilize a preexisting population of unliganded dimers, as suggested by fluorescence resonance energy transfer microscopy on A431 human epidermoid carcinoma cells (Gadella and Jovin, 1995).

In conclusion, the perfect symmetry found with ancestral-type receptors is replaced by pseudosymmetrical organizations associated with heterooligomerization and diversification of their functional properties.

The Neurotransmitter Binding Site at Protein Interfaces

As found for classical allosteric enzymes, the binding sites for regulatory ligands are often located at the boundary between individual polypeptide chains. In the case of the nAChR, labeling studies with native or reduced *Torpedo* receptor and affinity ligands with broad side-chain reactivity (e.g., α toxins, p-N,N-[dimethylamino]-benzenediazonium fluoroborate [DDF], and other compounds), as well as site-directed mutagenesis experiments, have all revealed the contribution of the two α subunits present within the receptor oligomer (see Figure 3B) together with the adjacent γ or δ subunits (Oswald and Changeux, 1982; reviewed by Devillers-Thiéry et al., 1993; Karlin and Akabas, 1995; Hucho et al., 1996; Taylor et al., 1998). The large N-terminal extracellular domain contributes to the acetylcholine binding pocket by two distinct components that bridge the boundary between subunits: the principal component that consists of three loops designated A, B, and C on the α subunit; and the complementary component that consists of loops designated D, E, and F (as summarized in Figure 3D) present on the other side of the subunit interface (reviewed by Corringer et al., 1995; Chiara and Cohen, 1997; Martin and Karlin, 1997). The labeled residues from loops A, B, and C are conserved from α1 to α8 subunits (except for α5); those from loops D, E, and F may vary.

For the homooligomeric receptors such as α7 or α8, there are five identical sites per receptor molecule (Couturier et al., 1990; Palma et al., 1996), and each subunit contributes to both a principal and a complementary component (Figure 3B). A homolog of loop D Trp is indeed present in the α7 sequence and has been shown by mutagenesis to contribute to the binding of nicotinic agonists (Corringer et al., 1995). For the heterooligomeric receptors such as *Torpedo* and muscle receptor, the association of the α subunits with the γ and δ subunits introduces nonidentical contacts, and only two of the five possible interfaces (see Figure 3B) participate in the high-affinity binding of nicotinic ligands. These features represent a significant departure from classical allosteric enzymes, for which the number of binding sites generally equals the number of identical subunits or protomers. Furthermore, differences in the complementary component account for nonequivalent functional properties of the two binding sites. The site at the α/δ interface, for instance, binds α-conotoxin with a 10^4-fold preference over the site at the α/γ interface (Sine et al., 1995a), whereas the site at the α/γ interface preferentially binds d-tubocurarine relative to the site at the α/δ interface (Chiara and Cohen, 1997).

These two nonidentical but homologous binding sites may nevertheless establish cooperative homotropic interactions, although with a relatively low Hill coefficient ($n_H \approx 1.5$). Homopentameric α7 receptor displays a cooperativity even (relatively) lower (with $n_H \approx 1.3$ for five active sites), as anticipated for a very high value for L (Edelstein and Bardsley, 1997). The mutation L254Q, which in homomeric α7 causes, among other effects, a decrease of L, results in a value of $n_H = 4.6$ (Bertrand et al., 1993).

A distance of $\sim$6.7 nm has been estimated by energy transfer between two fluorescent labeled α toxins bound to the two acetylcholine binding sites of *Torpedo* receptor, thus demonstrating the allosteric mode of their interaction and further indicating that a portion of the toxin resides near the outer perimeter of the receptor molecule (Johnson et al., 1984; see Hucho et al., 1996, for α toxin binding).

By analogy, the concept that neurotransmitter binding sites occupy subunit boundaries has been extended to $GABA_A$ receptor homo- and heterooligomers, with the additional hypothesis that synthetic drugs such as the benzodiazepines bind at the "free" α/γ interface, which does not accommodate the neurotransmitter (Galzi and Changeux, 1995; Sigel and Buhr, 1997).

In the case of the tetrameric glutamate receptors, no evidence exists for the presence of a glutamate binding site at the boundary between subunits (Paas, 1998). To date, the available data support a location of the glutamate binding pocket at the interface between two distinct protein domains or "lobes" (Kuusinen et al., 1995; Paas et al., 1996; Chen and Gouaux, 1997; Laube et al., 1997; Armstrong et al., 1998; Lampinen et al., 1998) from the same subunits, although some contributions from the neighboring subunit cannot be excluded

(Honer et al., 1998). Considerable flexibility may thus exist within each subunit by shearing and/or pivot movements of one lobe with respect to the other (Paas, 1998). Concerning allosteric effectors such as cyclothiazide, it has been suggested that residues implicated in their binding (Partin et al., 1996) may be at subunit interfaces, since they lie over 25 Å from the ligand binding site (Armstrong et al., 1998).

For G protein–linked receptors, the ligand binding domain does not necessarily occur at the boundary between subunits. On the other hand, this may be the case for tyrosine kinase receptors. For example, reaction of epidermal growth factor (EGF) receptors with two EGF molecules causes dimerization by a mechanism that has been interpreted within an allosteric framework (Schlessinger, 1988; Weiss and Schlessinger, 1998). Ligands may bind to sites located at the interface between subunits, thus stabilizing dimer formation, as supported for human growth hormone receptor (Wells, 1996).

In conclusion, the location of neurotransmitter binding sites at protein interfaces, between subunits (or between well-defined lobes within subunits), can be viewed as a general property of membrane receptors, which, as in the case of classical regulatory enzymes, renders these sites particularly sensitive to changes in the three-dimensional structure of the molecule. The restriction of the symmetry to rotational axes perpendicular to the membrane, together with the ability to form pseudosymmetrical heterooligomers accompanied by a decrease in the number of functional binding sites, may account for the observed low cooperativity between sites, yet with the possible benefit of an increase of intrinsic affinity or gain of physiological properties (see Corringer et al., 1998).

The Ion Channel in the Axis of Symmetry

Early structural studies of hemoglobin led to the idea that the axial cavity of the molecule could serve as a model system for an ion channel that would open and close upon ligation of oxygen, with 2,3-diphosphoglycerate behaving as a channel blocker. Convergent experimental evidence from several different approaches indeed supports the view that the ion path, which is the analog of the catalytic site in regulatory enzymes, coincides with this axial cavity of the nicotinic receptor oligomer, the pore domain serving the quadruple function of selectivity filter for ion permeability, "catalyst" for ion transport, gate, and specific site for pharmacological agents.

The first identification of amino acids belonging to the ion channel (Giraudat et al., 1986, 1987; Hucho et al., 1986; Revah et al., 1990; reviewed by Changeux, 1990; Hucho et al., 1996) was provided by the affinity labeling of the native protein in situ with chlorpromazine and other noncompetitive "channel blockers." The amino acids labeled by four structurally different channel blockers in *Torpedo* receptor all belong to the second transmembrane segment (M2) in each of the subunits, with a quasisymmetrical organization of the channel comprising "rings" of homologous amino acids labeled by these blockers on *all* subunits (Hucho et al., 1986; Giraudat et al., 1987; Devillers-Thiéry et al., 1993). Mutagenesis experiments provide additional support for such an axial disposition. Mutations within M2 indeed reduce the residence time, or the open channel block, caused by the lidocaine derivative QX222 (Leonard et al., 1988) and alter the intrinsic conductance of the channel (Imoto et al., 1988), as well as the selectivity of the pore for nonphysiological ions (Villarroel and Sakmann, 1992) or for Ca^{2+}, while preserving Na^{+} and K^{+} permeability (Bertrand et al., 1993). Even more remarkable is the conversion of α7 from a cation-selective to an anion-selective channel by two mutations in the intermediate (Glu → Ala) and upper valine (Val → Thr) rings, together with the insertion of a Pro (or Ala) at the N-terminal end of M2 (Galzi et al., 1992).

The pattern of labeled amino acids supports the conclusion that M2 is at least partially an α helix, an organization that the cysteine substitution method largely confirms (Akabas et al., 1994). Consistent with the electron microscopy observations on the transmembrane domain (Unwin, 1996), the channel lumen would thus appear to be lined by the five homologous M2 α helices, with the exception of a short segment at the cytoplasmic end possibly contributing to the gating (Wilson and Karlin, 1998) and to the selectivity filter of the channel (Corringer et al., submitted). Asymmetries do exist at the level of the channel lumen in the labeling patterns by affinity reagents (Galzi et al., 1991; Blanton et al., 1998), and mutations to prolines of homologous residues on different subunits have differential effects on the functional properties of the receptor (Chen and Auerbach, 1998). Additional contributions from M1 or other membrane-spanning domains (Li et al., 1994; Akabas and Karlin, 1995; Campos-Caro et al., 1997; Ortiz-Miranda et al., 1997) may originate from the funnel shape of the channel (Hucho et al., 1996).

The axial cavity thus possesses a strategic role in both ion transport and quaternary architecture through the combined effects of its pseudosymmetrical and stratified organization, including a pore–helix arrangement that participates in intersubunit contacts. A few amino acid changes, or even the replacement of an isoleucine by an alloleucine (Kearney et al., 1996), indeed suffice to modify its conformational transitions (see below), in addition to the characteristic transport properties of the channel.

The spatial relationship between the nicotinic agonist binding sites and the ion channel explored by fluorescence energy transfer measurements gives distances ranging between 2 and 4 nm, when using ethidium as a ligand of the high-affinity site for channel blockers (Herz et al., 1989). The cooperative interactions between acetylcholine binding sites, as well as their interaction with the ion channel, thus take place between topographically distinct (or distant) sites and constitute typical allosteric interactions.

While extension of the functional organization of the nAChR channel to the other members of the family (Betz, 1990; Eiselé et al., 1993) is likely to be correct, it may not necessarily hold for the other known ligand-gated ion channels built from rather different transmembrane domains (Figure 2A). The similarity found between the glutamate receptor channel and the three-dimensional organization of a K^{+} channel (Doyle et al., 1998) suggests that a common structural scheme, with a large water-filled cavity lined by α helices with exposed hydrophobic

amino acids and a selectivity filter in the pore loop, might adequately fit the channel for receptors in the glutamate family (Kuner et al., 1996), but with the pore region on the intracellular side compared to the extracellular side for the K^+ channel. The possibility exists that such a scheme, which applies as well to the cyclic nucleotide–gated ion channels, with the pore loop on the extracellular side (Sun et al., 1996), also fits the nAChR data but with the pore region on the intracellular side (Corringer et al., submitted).

A Constellation of Allosteric Sites

The concept of allosteric site is not restricted to the binding of a single category of regulatory ligand. In hemoglobin, for example, in addition to the axial site for 2,3-diphosphoglycerate, multiple binding sites for protons regulate oxygen binding and release (Edelstein, 1975). In the case of the nAChR, a large number of effectors control the functional properties at the level of the N-terminal extracellular domain, of the transmembrane segment, and of the cytoplasmic loop (Figure 1C) (reviewed by Changeux, 1990; Hucho et al., 1996). Concerning the N-terminal domain, bound Ca^{2+} ions behave as positive effectors of neuronal (but not muscular) nicotinic receptors with a major Ca^{2+} binding site involving part of loop F of the acetylcholine binding site (α7 residues 161–172; see Figure 3D) (Galzi et al., 1996a). Additional regulatory sites for ATP, substance P, physostigmine, and steroids have been recognized, although their physiological significance is far from clear (see references in Hucho et al., 1996). Ivermectin, a powerful antihelminthic drug, behaves as a strong positive allosteric effector of neuronal α7 receptor by interacting at a site that remains unidentified (Krause et al., 1998). Mutagenesis and photolabeling experiments have shown that the various transmembrane segments interact with the membrane bilayer, thus forming a "lipid belt" (Giraudat et al., 1985; Blanton and Cohen, 1994), with a specific regulatory role noted for phosphatidic acid and cholesterol (Bhushan and McNamee, 1993). Finally, the cytoplasmic domain is the potential target for a distinct group of ligands, notably protein kinases (Huganir and Greengard, 1990; Raymond et al., 1993).

A similar global picture, though with many differences in detail, accounts for other ligand-gated ion channels and other membrane receptors, which may thus "integrate" signals from the outside of the cell and from the cytoplasm, in particular through phosphorylation (Sheng and Wysznyski, 1997), as well as from the lipid bilayer (Schwartz and Rosenkilde, 1996; Paas, 1998). It can be extended to the recognition of pharmacological agents that may occupy binding sites for physiological ligands but also interact with protein domains, interfaces, or crevices that do not match any recognized regulatory signal, as noted above (see The Neurotransmitter Binding Site at Protein Interfaces) for benzodiazepines or cyclothiazides at subunit interfaces in $GABA_A$ or glutamate receptors. Models for such interactions are the various anti-sickling drug complexes revealed by Perutz et al. (1986) on the hemoglobin molecule.

In addition, receptors may interact with proteins that lead to clustering or immobilization from either the synaptic or cytoplasmic side. Examples are the 43K-rapsyn that anchors nAChR at the neuromuscular junction (see references in Duclert and Changeux, 1995), gephyrin that stabilizes glycine receptors at neuronal postsynaptic sites (Kirsch and Betz, 1998), and various PDZ-domain proteins in the PSD-95/SAP90 family that interact with glutamate receptors (Kornau et al., 1995; Kennedy, 1997; Sheng, 1997) and other PDZ binding proteins (Kim et al., 1998; Niethammer et al., 1998, and references therein). These interactions maintain the various receptor proteins immobilized at a cellular location where they are directly accessible to the high local concentrations of neurotransmitter released by the nerve ending. The phosphorylation reactions of specific sites on cytoplasmic domains are of particular interest, since they may selectively affect such interactions with cytoskeletal functions (Sheng and Wysznyski, 1997) and also play a role in synaptic plasticity (Raymond et al., 1993; Levitan, 1994; Kirsch and Betz, 1998), particularly in postsynaptic aspects of long-term potentiation (LTP) (McHugh et al., 1996; Rotenberg et al., 1996; Barria et al., 1997) or long-term depression (LTD) (Nakazawa et al., 1995).

Allosteric Transitions of Membrane Receptors Revealed by Physicochemical Techniques

The structural changes that mediate the "indirect" interactions responsible for the activation and desensitization of membrane receptors by neurotransmitters have been explored in vitro by various physicochemical techniques under rapid mixing or static conditions. In the case of the much studied nAChR, these methods exploited the initial observation that membrane fragments (forming closed "microsacs") purified from electric organs in their native form (or reconstituted from purified receptor protein and lipids) respond to nicotinic agonists by an "activation" of ionic fluxes (Kasai and Changeux, 1970) that were subsequently correlated with the binding of fluorescent agonists (Heidmann et al., 1983).

Contributions from many research groups as reviewed in detail elsewhere (see references in Changeux, 1990) demonstrate that the system can be represented by rapid chemical kinetics of channel opening (in the millisecond range), as well as fast (in the 0.1 second range) and slow (in the minute range) desensitization processes. The slow phase leads to complete desensitization, and, at very low concentrations of agonist, desensitization can occur without significant activation. The kinetic data are consistent with a minimal allosteric model (Heidmann and Changeux, 1980; Neubig and Cohen, 1980) involving discrete B, A, I, and D states, where B is the resting low-affinity state, A is the active open channel state, and I and D are desensitized states with high and very high affinity for agonists, respectively. In addition, a significant fraction ($\sim$20%) of the high-affinity, desensitized state is present in the absence of agonist (Heidmann and Changeux, 1979).

Numerous changes in the physicochemical properties of the receptor proteins accompany these functional transitions, as reflected by measurements of intrinsic fluorescence (Bonner et al., 1976; Grünhagen and Changeux, 1976), chemical reactivity toward a variety of probes including bifunctional cross-linking reagents (Watty et al., 1997) and Raman spectroscopic properties (Aslanian et al., 1993), all of which are selectively modified in the

presence of nicotinic agonists or antagonists. Cryoelectron microscopy reveals symmetrical changes in the shape and orientation of the five transmembrane rods, interpreted as M2 α helices and viewed as representing the ion channel in its open and closed conformations (Unwin, 1995). On the other hand, prolonged exposure of *Torpedo* receptors to agonist promotes a nonsymmetrical quaternary reorganization of the entire molecule, with one subunit more tangentially inclined and another subunit displaced away from the axis of symmetry of the molecule (these subunits, initially interpreted as γ and δ, respectively [Unwin et al., 1988], most likely correspond to δ and β [Machold et al., 1995]). In any case, it is clear that desensitization involves extensive changes in quaternary organization that may require longer times for the transition to take place than for channel opening.

Consistent with nonsymmetrical quaternary transitions, photoaffinity labeling of the acetylcholine binding area with DDF shows an increase in the contribution of the δ subunit to the "intersubunit" contact under conditions of stabilization of the desensitized state (by a noncompetitive blocker, meproadifen), while that of the γ subunit decreases (Galzi et al., 1991). In addition, the relative contributions of loops A and B in the α subunit increase up to 6-fold, a finding consistent with a tighter binding of the nicotinic ligands to the D state (Galzi et al., 1991). Also, the pattern and reaction kinetics of photolabeling of the M2 channel domain by several noncompetitive blockers may change upon equilibration with carbamylcholine (White and Cohen, 1992; Blanton et al., 1998). Major changes of tertiary and quaternary structure in the intersubunit binding domain for nicotinic agonists, as well as in the channel crevice, take place in the course of activation and desensitization, thus providing molecular correlates for the cascade of allosteric transitions affecting the receptor protein.

Despite more limited in vitro structural information, the above scheme offers a plausible representation of the conformational transitions mediated by other members of the nAChR family (Betz, 1990), as well as by other families of ligand-gated ion channels. It was speculated above (see The Neurotransmitter Binding Site at Protein Interfaces) that changes in the relative position of the lobes of the glutamate binding site of ionotropic glutamate receptors move en bloc with channel elements within a given subunit (Paas, 1998; see also Krupp et al., 1998), but this mechanism needs confirmation. It is premature to mention a possible reorganization of the quaternary structure of GPCR upon ligand binding. On the other hand, in the case of light activation of rhodopsin, a rigid body movement of the helices M3 and M6 relative to one another would take place upon light activation, mediating the interaction à distance between retinal and transducin (Farrens et al., 1996).

All-or-None Channel Gating and the MWC Model

Our knowledge of the dynamics of ligand binding and enzyme activation by classical allosteric proteins arises primarily from studies of molecular populations. A unique opportunity is offered by ligand-gated ion channels to follow the behavior of single molecular species directly and with high resolution by patch-clamp recordings of channel openings (Neher and Sakmann, 1976). The MWC formalism can thus be extended to ligand-gated ion channels by implementing kinetic mechanisms that cover a wide time range and involve multiple transitions, including activation as a rapid (millisecond) but transient process and desensitization occurring on a time scale (0.1 second to minute) that is much slower than generally observed for the conformational changes in allosteric enzymes (Hammes and Wu, 1974).

First, the identification of at least four distinct conformational states requires a minimal scheme with conformational transitions between the B, A, I, and D states interconverting in a "tetrahedral" pattern (Heidmann and Changeux, 1980) (Figure 4A). The dynamics of the interconversions ranging from milliseconds to minutes essentially lead to the selection of a predominant kinetic pathway (Edelstein et al., 1996) that simplifies to the linear cascade $B_i \rightleftharpoons A_i \rightleftharpoons I_i \rightleftharpoons D_i$ (Figure 4B).

Second, within the linear cascade, numerous individual rate constants must be assigned, since interconversion rates for the transitions between each pair of states vary with the number of ligands bound. However, according to linear free energy relations, the variations of these sets of rate constants obey a scaling factor that may be fixed by a transition state position parameter (Figure 4C). The resulting values are fully consistent with the experimental observations, and in all cases channel opening is predicted for nonliganded receptors. Such "spontaneous" all-or-none openings have been recorded at a low frequency (Jackson, 1986; Jackson et al., 1990), and, in agreement with the model, their frequency dramatically increases for certain mutants (as described in Extremely Pleitropic Mutations and Pathology). On the other hand, Auerbach et al. (1996) have noted minimal voltage dependence of spontaneous openings compared to the openings of liganded receptors.

Third, the complete set of rate constants obtained using the linear free energy relations yields an adequate fit of the various kinetic properties of activation and desensitization for muscle and neuronal receptors (Edelstein et al., 1996). Moreover, this novel formalism suggests new experimental tests based upon measuring single binding events (Edelstein et al., 1997b). Single channel measurements on muscle receptors contributed valuable information about ion channel properties and their ligand gating (Sakmann et al., 1980; Colquhoun and Sakmann, 1985). However, the linked events of ligand binding have so far only been inferred indirectly from single channel recordings, since parallel observations on the relevant binding steps have not been possible. Developments in the field of fluorescence correlation spectroscopy (Eigen and Rigler, 1994; Edman et al., 1996; Rauer et al., 1996; Schwille et al., 1997) now place such measurements in the realm of possibilities for the near future and offer new tests for the assumptions of the allosteric model.

Fourth, measurements of single binding events could in principle resolve conflicts concerning the degree of equivalence of the two ligand binding sites in single channel recordings of muscle nicotinic receptors (Edmonds et al., 1995). For example, wild-type human muscle receptors expressed in HEK cells have been interpreted in terms of ligand binding affinities varying from a 350-fold difference for the affinity of the two sites in the

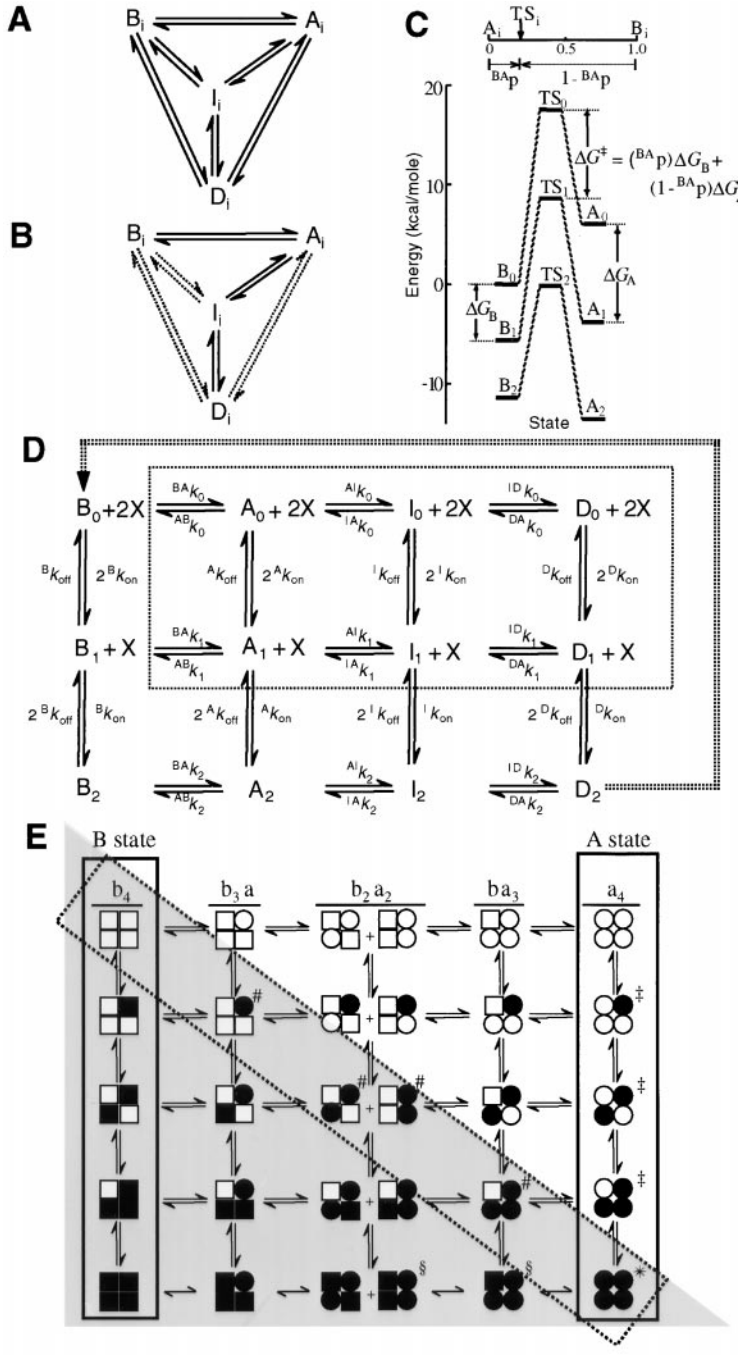

Figure 4. Interconversion Pathways and Models

(A) Tetrahedral model for interactions between four conformational states, B, A, I, and D. The abbreviations "T" and "R" used in the original MWC model were replaced by "B" and "A," respectively, for ligand-gated channels (Edelstein et al., 1996), in order to avoid confusions arising from "R" being traditionally used to designate the low-affinity "resting" state of receptors but the high-affinity state of allosteric enzymes and hemoglobin.

(B) Linear model resulting from kinetic pathway selection on the basis of the hierarchy of reaction rates (Edelstein et al., 1996); the kinetic pathways that can be ignored are indicated by the dotted lines.

(C) Linear free energy relations permitting interconversion rates for the B and A states to be fixed by a transition state parameter, ^{BA}p. The B and A states each bind ligand with a characteristic intrinsic affinity that leads to the energy ladders for molecules with 0, 1, or 2 ligands bound (indicated by the subscript), with the step sizes indicated by ΔG_B and ΔG_A, respectively. The two rate constants of interconversion of the fully liganded forms, $B_2 \rightarrow A_2$ and $A_2 \rightarrow B_2$, fix the height of the transition state barrier, TS_2. The position, ^{BA}p, on a hypothetical scale from 0 to 1 (shown at the top of the panel), characterizes the transition state such that the more the transition state resembles A, the closer ^{BA}p is to 0. The rates for the unliganded and singly liganded forms are then determined by the heights of the transition state barriers TS_1 and TS_0, which are at steps above TS_2 fixed by $\Delta G^{\ddagger}$ in terms of ^{BA}p, ΔG_B, and ΔG_A according to the relationship indicated (Edelstein and Changeux, 1998). When applied to the detailed kinetic studies on muscle receptors, a value of $^{BA}p = 0.2$ was obtained (Edelstein et al., 1996), indicating that with each ligand binding step the increase in the B → A rate is considerably larger (~250-fold) than the decrease in the A → B rate (~4-fold).

(D) The ligand binding and interconversion reactions for the linear model based on the MWC-type principles. For the purely sequential model, the states within the dashed rectangle would not be considered. In addition, the "cyclic pathway" invoked to explain recovery from a strong, desensitizing agonist pulse without passage via the open state (Katz and Thesleff, 1957; Franke et al., 1993) is presented by the dashed double line.

(E) A schematic description of the conformational states for a protein with four subunits with two tertiary states (open squares and open circles), and with ligand binding or voltage activation indicated by the closed symbols. The two-state model (Monod et al., 1965) corresponds to the vertical columns at the left and right extremes; the sequential model (Koshland et al., 1966) corresponds to the descending diagonal. Other states within and below the diagonal (in the shaded triangle) correspond to a model for voltage-gated *Shaker* channels (Zagotta et al., 1994). In addition, specific states (see The Intrusion of "Intermediate States" in Nonnicotinic Channels) are indicated as follows: #proposed subconductance states for drk1 channels (Chapman et al., 1997), §proposed subconductance states for *Shaker* channels (Zheng and Sigworth, 1997), *probable full conductance states for all models, and ‡possible alternative subconductance states in the context of the MWC model.

B state in one study (Sine et al., 1995b) to identical affinities for the two sites in another study (Wang et al., 1997a). Simulations based on the allosteric model show that the same single channel data can be represented by mechanistic schemes with equivalent or with nonequivalent binding affinities at the two sites, but much different single binding properties are predicted in the two cases. Independent measurements of single binding events would thus provide critical data capable of distinguishing between the two modes (Edelstein et al., 1997b). In addition, the formalism incorporating nonequivalent sites readily accommodates the differences

in affinity for competitive antagonists that lead to Hill coefficients apparently lower than 1. Such values arise in fact from the heterogeneity of nonequivalent binding sites and should be distinguished from the so-called "negative cooperativity" that implies destabilizing interactions between identical sites (Koshland et al., 1966).

Fifth, simulations of recovery after a strong, desensitizing pulse indicate that the kinetic pathway for return to B_0 can pass sufficiently rapidly through A_0 (Edelstein et al., 1996) that the "silent" channels observed during recovery may be accounted for without postulating a separate recovery pathway (see Figure 4D) that provides the basis of the "cyclic" mechanism (Katz and Thesleff, 1957; Franke et al., 1993).

The novel kinetic formulation of the allosteric model adequately fits most of the known dynamic properties of the nicotinic receptor, and it gives rise to a number of original predictions related to the notion that not all binding events lead to channel opening and not all channel opening events are the result of ligand binding. As a result, gating of the ion channel cannot be viewed solely as a ligand-triggered process but as reflecting an intrinsic structural transition of the receptor molecule, which may even occur in the absence of ligand. Moreover, at low agonist concentrations, desensitized states can be stabilized under conditions of negligible channel opening. Overall, these concepts bring new insights into the still rather enigmatic molecular mechanisms of channel opening and desensitization.

The Intrusion of "Intermediate States" in Nonnicotinic Channels

At variance with the MWC model, the KNF or sequential model (Koshland et al., 1966) does not impose any restrictions on the conformational states accessible to the regulatory oligomer in relation to its quaternary organization. Each subunit can change its tertiary structure upon ligand binding, thereby affecting through "induced fit" the chemical reactivity of the neighboring subunits.

Nicotinic receptors, in agreement with the two-state MWC model, exhibit a single discrete conductance state that generally does not vary with the nature of the agonist, nor with its concentration (but see Revah et al., 1991; Camacho et al., 1993; Kuryatov et al., 1997; Zhang and Karlin, 1998). The very high resolution of the electrophysiological techniques, however, reveals that other ligand-gated channels may exhibit, in addition, multiple subconductance states that contrast with the simple predictions of the MWC scheme. Among such receptors, two categories of phenomena are observed.

In the first category, typified by glycine receptors, multiple conductance states are prevalent (Bormann et al., 1993), but their distribution does not depend on agonist concentration (Twyman and Macdonald, 1991). The simplest interpretation of such deviations is that discrete fluctuations occur in the steric organization of a few amino acid residues within the open channel state, independent of binding site occupancy and subunit interactions. Only a limited number of side chain conformations (rotamers) are indeed accessible to residues belonging to α helices (Dunbrack and Karplus, 1994), some combinations of which may be "frozen" in the channel-lining pore during the transition to the open state. In other words, these fluctuations would arise from local, sequence-dependent variations in conformation within the channel for certain receptor subunits (such as glycine receptor α1), without basically departing from the all-or-none openings predicted by the MWC model and mediated by changes in the quaternary organization of the molecule.

A more serious challenge to the fully concerted scheme is the occurrence of subconductance states that have been found to vary with the nature of the agonist, as typified by AMPA-type glutamate receptors expressed in HEK cells (Swanson et al., 1997b) or studied in vivo (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987; Jonas and Sakmann, 1992; Wyllie et al., 1993), including changes accompanying the induction of LTP (Benke et al., 1998). Subconductance states have also been reported for GluR6/GluR3 chimeric receptors by following the changes upon slow dissociation of the competitive antagonist 6-nitro-7-sulphamoyl-benzo(F)quinoxalinedion and were interpreted as corresponding to partially liganded receptors (Rosenmund et al., 1998) (despite the fact that occupancy of two binding sites was found to be necessary to reach the first subconductance state). Ruiz and Karpen (1997) also reported a progression of subconductance states related to ligand binding for retinal rod cGMP-gated channels, although such states were not reported in the studies of similar channels by Liu et al. (1998). Such agonist-dependent subconductance states to some extent resemble the patterns reported for drk1 voltage-gated receptors (Chapman et al., 1997). They may possibly reflect common channel gating mechanisms, distinct from those found within the nAChR family, with an increased flexibility (Figure 4E) in the relationship between the ligand binding site and the ion channel involving a specialized pore device (MacKinnon, 1995; Zagotta and Siegelbaum, 1996).

Figure 4E represents all possible conformational states accessible in theory to a tetrameric oligomer with subunits possessing two tertiary conformational states (squares versus circles), each subject to ligand binding or voltage-activated transitions (from open to closed). Among these various possibilities, an especially interesting one is that the subconductances states arise from local changes in subunits that reside within a common allosteric quaternary state, for instance the A state (distributed in the vertical column at the right in Figure 4E). These substates, which have so far been neglected in current models for K^+ channels, would then be analogous to the tertiary-level contributions to the H^+ Bohr effect in hemoglobin (Edelstein, 1975). Indeed, the equivalent of a pH effect on current–voltage relationships has recently been produced for *Shaker* channels by introducing a histidine into the segment S4 responsible for voltage sensitivity (Starace et al., 1997). Each agonist molecule bound to the homologous tetrameric receptor (as each individual Bohr proton for hemoglobin) would then produce local conformational changes that modulate activity without causing a major transition to another quaternary conformational state. An extensive data set has recently been interpreted in terms of a model involving three sequential and two concerted transitions (Schoppa and Sigworth, 1998), but models based on a cascade of concerted transitions equally fit the data.

Moreover, it should be noted that spontaneous open states have been reported for cGMP and NMDA receptors (Picones and Korenbrot, 1995; Tibbs et al., 1997; Turecek et al., 1997), but, as already noted, such states are *not* allowed in the strict observance of the KNF-type model. Analysis of the spontaneous openings and their possible subconductance states may thus offer useful clues for distinctions between the different models.

This discussion reveals the difficulty in interpreting high-resolution electrophysiological data in terms of molecular models that rely upon stereochemical information for membrane proteins. The various attempts to model the conformational transitions of these molecules emphasize the distinction between major discrete quaternary transitions, which would be common to both pentameric and tetrameric receptors (e.g., B and A states), and local reorganizations at the subunit level more directly linked to ligand (or voltage) gating (which apparently would be more probable in tetrameric receptors). How fractional ligand binding may exert "partial" effects on the quaternary organization is unclear at this stage. Ultimately, structural studies at atomic resolution should provide access to an objective description of these subconductance states and to the distinction between those corresponding to a local flexibility within a common quaternary "open" state and those representing more complex patterns of mixed conformational states.

Extremely Pleiotropic Mutations and Pathology

A specific prediction of the occurrence of allosteric interactions is that mutations occurring at pivotal locations in the pathway between the ligand binding site and the biologically active site may dramatically affect their properties or their relationships. In hemoglobin, for example, the mutation *Kansas* (βN102T) confers a low oxygen affinity and low Hill coefficient because the R state is destabilized, whereas the mutation *Chesapeake* (αR92L) displays a high oxygen affinity and a low Hill coefficient because the T conformation is destabilized (Edelstein, 1971). "Gain-of-function" mutations may apply to the conversion of allosteric inhibition to activation in regulatory enzymes, such as threonine deaminase (Sanchez and Changeux, 1966) or phosphofructokinase (Lau and Fersht, 1987).

In the case of membrane receptors, novel insights are provided by site-directed mutations in the homopentameric α7 neuronal nicotinic receptor that produce dramatic pleiotropic alterations of functional properties. For example, point mutations were introduced at one of three sites of the M2 segment (Thr-244, Leu-247, or Val-251—the first two of which are homologs of chlorpromazine-labeled amino acids [Revah et al., 1990] in *Torpedo*). Each mutation plausibly located on successive turns of the α helix generates a receptor that, when expressed in oocytes, displays an apparent affinity for acetylcholine (ACh) up to 200-fold higher than for wild type, no longer desensitizes, and possesses high and low conductance states (Revah et al., 1991; Devillers-Thiéry et al., 1992). Moreover, a competitive antagonist of the wild-type receptor, dihydro-β-erythroidine (DHβE), becomes a full agonist (with 10-fold higher apparent affinity than ACh) for L247T receptors but only a partial agonist for T244Q and V251T receptors. These gain-of-function mutations, as well as others responsible for congenital myasthenic syndromes (Sine et al., 1995b; Edelstein et al., 1997a; Léna and Changeux, 1997; Engel et al., 1998) occur primarily in M2 (see Figure 6A) but also in other domains such as loop B of the agonist binding site (Corringer et al., 1998). Their mechanistic interpretation poses a serious challenge to the KNF-type sequential models for channel activation. On the other hand, the MWC-type model provides simple and plausible explanations on the basis of changes in the equilibrium between conformations (L phenotype, α7 V251T and T244Q), in addition to (or as an alternative to) changes in the ligand binding constant (K phenotype, α7 Y92F and W148F) or ion channel conductance (γ phenotype, α7 L247T) of individual conformational states (Galzi et al., 1996b).

One of the most striking mutant phenotypes observed for receptors implicated in congenital myasthenic syndrome is produced by the M2 mutation εT264P (Ohno et al., 1995). In single channel recordings, the profiles of open channel dwell times display three peaks, instead of the single peak for wild-type receptors. The standard sequential-type model (Figure 4D), which does not include non- or mono-liganded open states, fails to accommodate these data. On the other hand, they are adequately represented by the allosteric scheme in terms of a decrease of L (from 9×10^8 to 100) that facilitates the B → A transition. As a consequence, significant channel opening is predicted for receptors with no ligand or one molecule of ligand bound (Edelstein et al., 1997a), and the three peaks for the mutant receptors are readily interpreted as reflecting non-, mono-, and biliganded molecules (with reasonable agreement between theory and experiment; see Figure 5). These simulations also illustrate that the distinct single binding events would outnumber the single ionic events for the wild type, while the reverse would be true for the myasthenic mutant. Abundant spontaneously open channels that nicotinic antagonists selectively close are observed in the double mutant α7 L247T–V251T (Bertrand et al., 1997). Pleiotropic phenotypes also occur after homologous mutations in the 5-HT_3 (Yakel et al., 1993) and $GABA_A$ (Chang et al., 1996; Pan et al., 1997) receptors. Moreover, in the case of the glycine receptor α1 subunit, mutations at the extracellular extremity of M2 at position R271 (Shiang et al., 1993; Langosh et al., 1994; Rajendra et al., 1994) are responsible for startle disease (for additional startle mutations, see Shiang et al., 1995) and might also affect L, but with the mutation increasing its value above the wild-type level (Galzi et al., 1996b).

With respect to tetrameric receptors, pleiotropic changes in the physiological and pharmacological properties of tetrameric receptors also follow mutations within the neurotransmitter binding site (Mano et al., 1996; Laube et al., 1997; Krupp et al., 1998) and within the membrane domains (Sommer et al., 1990; Villarroel et al., 1998), although their interpretation in terms of the MWC model remains to be established (see Paas, 1998).

Concerning GPCRs, "constitutive" mutations at various loci distributed within the receptor molecules result in a dramatically enhanced activation of the G protein–linked biological activity in the absence of agonist (Lefkowitz et al., 1993). The MWC model applied to "monomeric" GPCRs provides a simple explanation of these

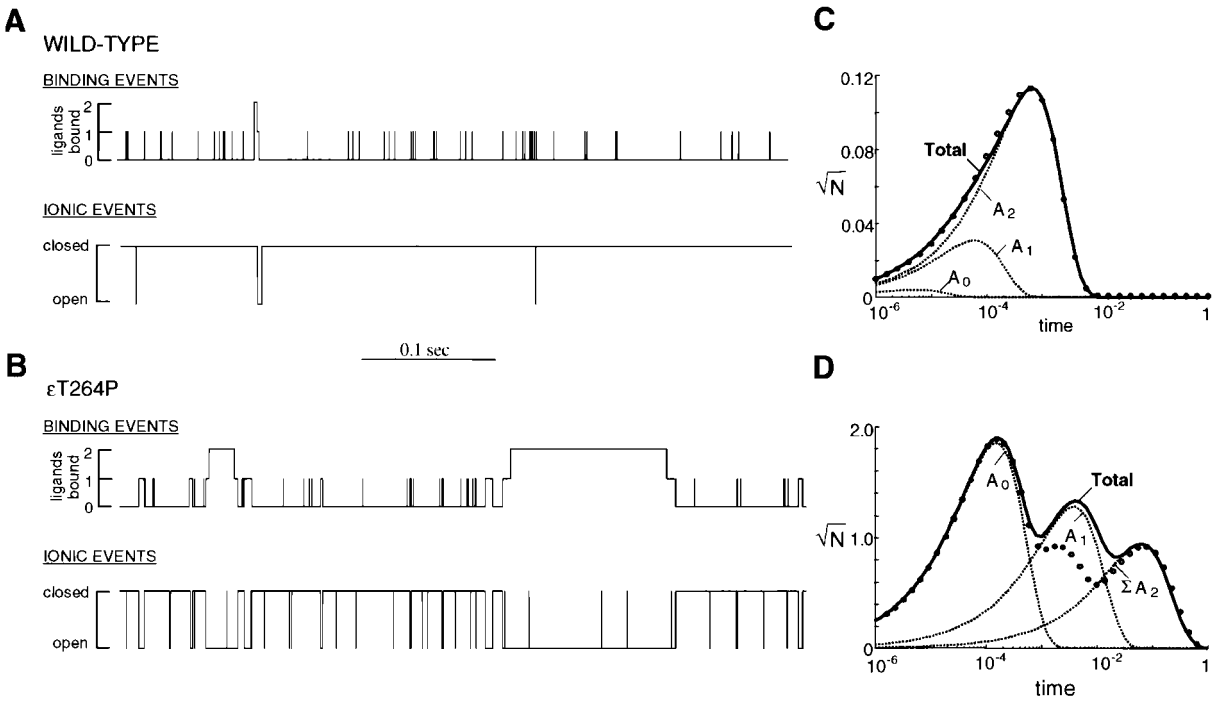

Figure 5. Stochastic Simulations and Open Channel Dwell Times for Normal and Myasthenic Mutant εT264P Receptors

(A) Simulations of binding and ionic events for human wild-type muscle receptors expressed in HEK cells using parameters (Edelstein et al., 1997a) based on experimental observations (Milone et al., 1997). The single binding simulations are based on a theory that incorporates multiple conformational transitions (Edelstein et al., 1997b).

(B) Simulations as in (A), but for εT264P mutant muscle receptors expressed in HEK cells using parameters (Edelstein et al., 1997a) based on experimental observations (Ohno et al., 1995).

(C) Dwell time profiles predicted by the MWC-type model and experimental data points for wild-type receptors. The individual points (open circles) are obtained from the kinetic rate constants presented in the legend to Figure 8b of the article by Milone et al. (1997) to represent activation over a wide range of ACh concentrations.

(D) Dwell time profiles predicted by the MWC-type model and experimental data points for εT264P mutant receptors. The individual points (open circles) are presented for the sum of the three components of the experimentally observed open channel dwell times corresponding to the published values of Ohno et al. (1995) for 0.3 μM ACh of τ_0 = 150 μs, a_0 = 0.67; τ_1 = 1.8 ms, a_1 = 0.16; and τ_2 = 69.5 ms, a_2 = 0.17, where τ_i and a_i are the mean open time and relative amplitude, respectively, for each component. The data for wild-type and mutant receptors were simulated with values of $^{BA}L_0$ of 9×10^8 and 100, respectively (Edelstein et al., 1997a). For εT264P, compatibility with linear free energy relations yields mean open times of τ_0 = 151 μs, τ_1 = 3.8 ms, and τ_2 = 59.5 ms. Although most single channel openings are solitary events, possible contributions from multiple events (bursts) have not been included; therefore, these mean open times should be considered as upper limits, particularly for τ_2. However, a majority of A_2 channel openings involve passage to A_1 prior to closure by passage to B_1, and the contributions of these events, as well as closure by passage directly from A_2 to B_2, have been included, as indicated by ΣA_2. The simulations correspond to 10 bins for each integer interval of log t, with peak heights based on the number of events occurring in a total time t of 1 s.

gain-of-function function mutants and also accounts for the role of "inverse agonists" that reduce both the constitutive activity of such mutants and the much smaller spontaneous activity of wild-type receptors (Hebert et al., 1996). Many of these mutations provoke genetic diseases and confer oncogenic properties (Spiegel, 1996). For example, constitutive mutations of the luteinizing hormone receptor cause familial male precocious puberty (Shenker et al., 1993). These mutations are distributed throughout the primary structure, although with a prevalence in M6 (Figure 6B).

Mutations of tyrosine kinase receptors conferring high activity in the absence of ligand have also been recognized for over a decade (Downward et al., 1984) and become oncogenic via dimerization and autophosphorylation (Huang et al., 1997). Mutant erythropoietin receptors with a single point mutation in the extracellular domain form active dimers in the absence of erythropoietin (Watowich et al., 1992). In this respect, the tyrosine kinase and cytokine receptors share with the other classes of allosteric receptors the ability to undergo spontaneous transitions to an active state, in accord with the MWC model.

The wide occurrence of gain-of-function or constitutive mutations in membrane receptors as in classical regulatory enzymes also supports the concept that, from an evolutionary point of view, these regulatory molecules may derive from ancestral unregulated molecules via a selective inhibition of their biological function, possibly as a consequence of oligomerization. Regulatory ligands would then "relax" these inhibitions via quaternary changes of the molecular structure. In any case, these few examples illustrate the usefulness of the MWC model for the interpretation of rather paradoxical gain-of-function mutations whose pathological effects contrast with those of the standard null mutations.

Concluding Comments

When, about 30 years ago, the extension of the MWC model to membrane receptors was proposed, it was, as

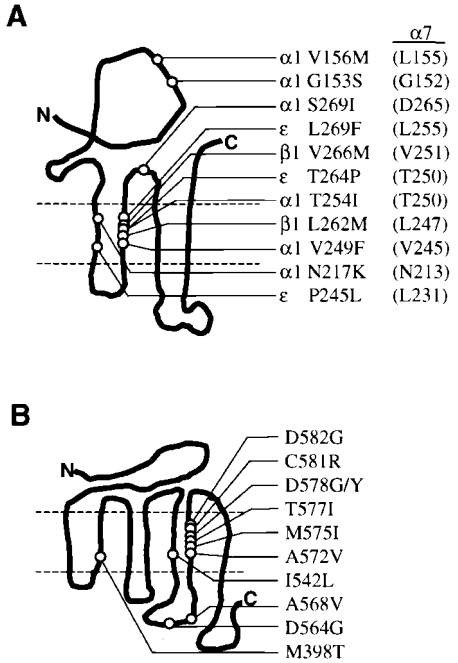

Figure 6. Gain-of-Function Mutations Associated with Pathologies of Nicotinic and G Protein–Coupled Receptors

(A) The congenital myasthenic syndrome mutations of the neuromuscular junction acetylcholine receptor with gain-of-function properties. The individual mutations are aligned with respect to the corresponding position in chick α7 nAChR; references to the original articles and the basis for their functional characterization as gain of function were published previously (Edelstein et al., 1997a).

(B) The gain-of-function mutations of the human luteinizing hormone receptor responsible for familial male precocious puberty (Shenker et al., 1993; Latronico et al., 1995; Laue et al., 1995); other references are available from Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/Omim; OMIM number 152790).

noted above, viewed by skeptics as adding one unlikely hypothesis on top of another. In the following years, structural and dynamic studies with hemoglobin and globular regulatory enzymes brought detailed experimental validation of the original scheme (Perutz, 1989; Iwata et al., 1994; Henry et al., 1997; see also Janin, 1989; Maddox, 1990). Moreover, several membrane receptors for neurotransmitters were biochemically identified (e.g., Changeux, 1981; Karlin, 1991) and several of them cloned and sequenced (e.g., Lefkowitz et al., 1988; Numa, 1989). The wealth of data derived from expression of cloned genes and electrophysiological recordings on native and recombinant proteins has nevertheless provided only a fraction of the information necessary for a critical evaluation of the MWC model (Jackson et al., 1990; Colquhoun and Sakmann, 1998; Green et al., 1998). The references to the most fully understood three-dimensional structural properties of allosteric proteins (Perutz, 1989; Iwata et al., 1994) and the modeling of protein conformational transitions, as presented here, illustrate that the mechanistic understanding of signal transduction by neurotransmitter receptors must integrate three-dimensional protein biochemistry and stereochemical analysis of conformational transitions, together with biophysical and electrophysiological recordings, within a multidisciplinary framework analogous to that which led to their initial identification (Changeux, 1981). While a number of chemical and functional "signatures" can already be identified, more definitive tests of the currently available models must await high-resolution structural information and dynamics, particularly to distinguish between fully concerted transitions in quaternary structure and more locally induced conformational changes.

Investigations at the level of the three-dimensional structure of receptors are certain to open a new era of research. Insights into their three-dimensional structure have been recently presented for the ligand binding pocket of GluR2 receptors from a crystal structure with bound kainate (Armstrong et al., 1998). The general folding pattern is similar to homology models proposed using the three-dimensional coordinates of bacterial periplasmic binding proteins as templates (Paas et al., 1996; Sutcliffe et al., 1996; Laube et al., 1997; Swanson et al., 1997a; Paas, 1998). Similarly, for the binding site of cGMP receptors, a reasonable model can be based on the known structure of the bacterial catabolite-activating protein (Weber and Steitz, 1987). Model building based on proteins that resemble receptors in the acetylcholine nicotinic family have been hampered by the weak homologies for the proteins so far structurally identified, plastocyanin and pseudoazurin (Tsigelny et al., 1997) and the biotin repressor (Gready et al., 1997). Similar limitations apply to the model for the transmembrane region based on the structure of an enterotoxin (Ortells and Lunt, 1996). On the other hand, a small region of similarity exists between ligand binding residues of nAChR and substrate binding residues of acetylcholinesterase (Figure 3D). This similarity may provide some additional clues to the structure in this region, since the three-dimensional structure of acetylcholinesterase has been established (Sussman et al., 1991). In contrast to the limited progress in structural studies on ligand-gated channels, K^+-specific channels have recently benefited from a spectacular advance (Doyle et al., 1998).

The three-dimensional organization of GPCRs has considerably benefited from high-resolution cryoelectron microscopy of bacteriorhodopsin (Henderson et al., 1990) and vertebrate rhodopsin (Unger et al., 1997). The assembly of GPCR into symmetrical oligomers remains, however, to be documented on the basis of three-dimensional structural observations. The three-dimensional structures of the kinase domains for several proteins in the family of EGF receptor protein tyrosine kinases have been resolved (Hubbard et al., 1994; Mohammadi et al., 1996, 1997), but the overall transmembrane organization of the functional dimers remains to be identified (Hubbard et al., 1998).

While the detailed organizational plan must await structural determinations at the atomic level, many of the properties of ligand-gated channels reviewed here can be satisfactorily integrated into a mechanistic scheme based on an MWC-type model of concerted allosteric transitions, with a cascade of multiple fast and slow transitions. In ligand-gated channels, the principle of symmetry in quaternary structure is satisfied for homooligomeric forms, with pseudosymmetry among "equivalent" protomers prevailing for the heterooligomeric cases. Yet, the transmembrane polarity and the

correlative limitations to single axes of symmetry normal to the membrane restrict the symmetry properties, resulting in less quaternary constraint and lower cooperativity. The presence of ligand binding sites at protein interfaces follows the pattern observed for allosteric enzymes, but heterooligomerization introduces a large potential for specific variations of physiological significance. In addition, subtle structural differences within the ion channel may lead to the significant differences in conductance and ion specificity observed among diverse receptor forms. Finally, among the tetrameric receptors, multiple conformational states at the level of individual subunits have been invoked, although without structural demonstration, including a series of transitions for *Shaker* channels involving both movements of individual subunits as well as concerted steps (Liu et al., 1998; Schoppa and Sigworth, 1998).

Extensions of the allosteric model to GPCRs and growth factor receptor kinases illustrate some common features of these membrane protein systems. In addition, such comparisons serve to emphasize that both ligand-gated channels and GPCRs can participate in signal transduction involving either high concentration "pulse" or "leak" release of neurotransmitter. The latter may be especially important for many neuronal neurotransmitter-gated ion channels (e.g., $GABA_A$ receptors [Nusser et al., 1998] as well as α7 nAChRs [Zhang et al., 1996]) that function at extrasynaptic locations. Conversely, the GPCRs generally function in response to the arrival of agonist in the leak mode, but responses in the pulse mode may occur for metabotropic glutamate receptors at certain synapses (Nakanishi, 1994; Salt and Eaton, 1996).

With respect to functional diversity, the common principles of protein architecture and conformational dynamics reviewed here lead to a broad spectrum of properties derived from the various modes of subunit assembly and a capacity for "molecular integration"—in particular coincidence detection—of diverse signals via the multiple allosteric sites carried by individual receptor molecules with transmembrane polarity (Heidmann and Changeux, 1982). The molecular diversity produces specialized functions, as yet only poorly understood, that associate particular receptor oligomers with discrete neuronal and synaptic distributions. As these distinct roles and distributions emerge, a wealth of novel targets should be identified for future pharmacological agents designed for specific receptors or neuronal networks. As exemplified by the interpretations of gain-of-function mutations for both ligand-gated ion channels and GPCRs, the MWC model also provides a useful framework to understand human pathologies (Lefkowitz et al., 1993; Léna and Changeux, 1997).

When the interplay of both ligand-gated and GPCRs are considered (see, for example, Wang et al., 1997b), the combinatorial arrangements of receptors reach very large proportions. Many such receptor–receptor interactions (Fuxe and Agnati, 1991) underlie the fine-tuning associated with modulation of synaptic transmission through changes of receptor efficacy. The particular role of phosphorylation has been noted (see Huganir and Greengard, 1990) and may underlie aspects of synaptic memory mechanisms, possibly involving coincidence detection in systems involving NMDA receptors (Wigstrom and Gustafsson, 1985) and calcium-stimulated phosphorylation (Bliss and Collingridge, 1993; Lisman et al., 1997) or transmembrane interactions involving extracellular zinc and cytoplasmic factors (Ascher, 1998). Calcium permeability of nAChR could lead to similar phenomena, with subsequent phosphorylation events shifting the receptor population between responsive and desensitized states (Edelstein and Changeux, 1998). Combinations of these processes may generate more subtle learning rules, such as a sliding threshold mechanism (Bienenstock et al., 1982; Mayford et al., 1995; Kirkwood et al., 1996; Edelstein and Changeux, 1998). Future research directions in this area are indicated by investigations using transgenic mice that permit the evaluation of changes in the allosteric properties of receptors to be correlated with behavioral responses (Picciotto et al., 1995, 1998; Chen and Tonegawa, 1997; Maldonado et al., 1997; Milner et al., 1998). Ultimately, decoding the complex network of interactions among these various receptors may lead to an understanding of the conformational transitions associated with variations in synaptic strength, thereby constituting allosteric learning models (Heidmann and Changeux, 1982; Dehaene and Changeux, 1989, 1997). After 30 years, allosteric receptors are thriving, and future applications can be expected in physiology, cognitive sciences, and medicine.

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