Allosteric Mechanisms of Signal Transduction

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Forty years ago, a simple model of allosteric mechanisms (indirect interactions between distinct sites), used initially to explain feedback-inhibited enzymes, was presented by Monod, Wyman, and Changeux. We review the MWC theory and its applications for the understanding of signal transduction in biology, and also identify remaining issues that deserve theoretical and experimental substantiation.

The elaboration of the allosteric theory spanned the years 1961 to 1967 and developed in two principal steps. The first issue involved the mechanisms by which a regulatory ligand (such as an enzyme feedback inhibitor) controls the state of activity of a biologically active site, such as an enzyme catalytic site, despite being structurally different from the active-site substrate. Regulatory effectors and substrates were proposed to behave as two distinct categories of ligands, which bind to their target protein at topographically "distinct sites" (Fig. 1A) (*1*) that mutually influence each other through a reversible conformational change. The proposal relied on the induced-fit theory of Koshland (*2*), which initially was developed not to explain the regulation of enzyme activity by a metabolic signal but to account for the specificity of enzyme action. This concept of indirect or "allosteric" interactions between stereospecifically distinct sites (*3*) differed from the classical explanations of enzyme inhibition through steric hindrance at a common binding site. The second issue was raised by the analysis of the complex patterns of kinetics encountered with bacterial regulatory enzymes, particularly L-threonine deaminase and aspartate transcarbamylase. Both of these enzymes showed intertwined cooperative (homotropic) interactions between identical ligands (i.e., oxygen and hemoglobin), as well as signaling (heterotropic) interactions between different ligands (i.e., between a regulatory molecule and a substrate).

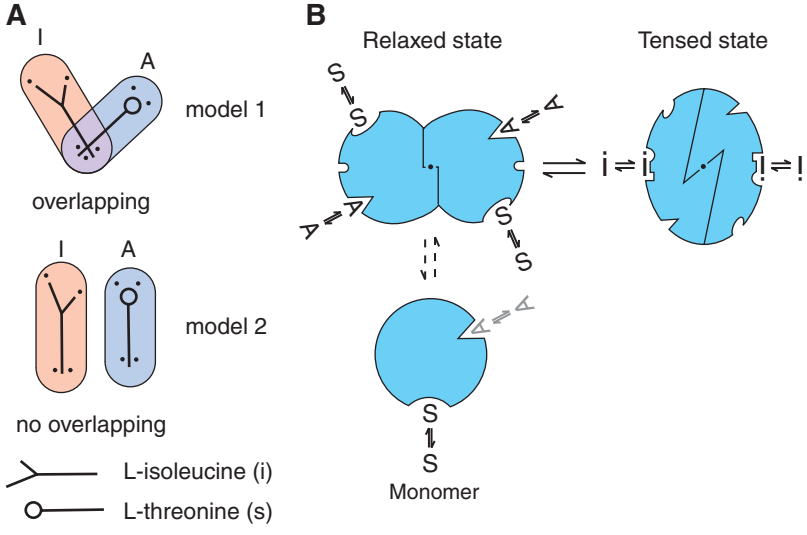

Fig. 1. Initial models of allosteric sites and allosteric transitions. (**A**) Nonoverlapping regulatory and catalytic sites for (s) substrate, L-threonine and (i) inhibitor, L-isoleucine (*1*). (**B**) Conformational transitions preserving the symmetry of the quaternary structure (*33*). Three classes of molecules are shown that bind to the enzyme (as indicated by solid double arrows): A (activator), I (inhibitor), and S (substrate). Transition between two states, R (relaxed) and T (tensed), is depicted horizontally, as well as a hypothetical monomeric state below, designated (with possible binding of A) as indicated by the gray double arrows.

To deal with these issues, Monod, Wyman, and Changeux (*4*) proposed two unifying concepts in their 1965 "MWC" model. The first proposes that regulatory proteins have a quaternary structure (the spatial arrangements and interactions of individual polypeptide chains that together make a complex protein) with identical subunits regularly organized into finite assemblies, or "oligomers," with symmetry properties (defined in terms of axes about which one polypeptide chain with its precise three-dimensional structure can be rotated to superimpose on another chain within the same quaternary structure) (Fig. 1B). The second postulates that, to account for the observed linkage between homotropic and heterotropic interactions, the signaling oligomers undergo reversible transitions between discrete conformations, which primarily affect the quaternary organization, preserve its symmetry, and are accessible in the absence of ligand (Fig. 1B). In other words, the cooperative structural changes intrinsic to the protein molecule determine the observed cooperative binding properties.

Such spontaneous "conformational switches," whose states are selectively stabilized by the ligands to which they preferentially bind, contrast with the sequential, induced-fit mechanism (*5*) initially suggested for hemoglobin. In an induced-fit mechanism, the very binding of the ligand to its site causes a subsequent change of conformation that would be "adapted" to the particular structure of the ligand. The initial versions of the MWC theory (*3*, *4*)—which relied on the then available structural data of Perutz for hemoglobin—dealt with regulatory enzymes, but a plausible application to hormone nuclear receptors and gene repressors was suggested. An extension of the theory to membrane receptors, in particular to neurotransmitters, was later proposed (*6*, *7*). This introduced the concepts of vectorial organization and rotational symmetry related to receptor integration into the biological membrane, along with the possible cooperative interactions in large-scale two-dimensional lattices. Detailed applications to hemoglobin were promptly pursued (*8*) and remain a subject of interest (*9*, *10*). The issue then became an experimental one: Which conformational mechanism faithfully represents the empirical observations collected with regulatory enzymes, hemoglobin, transcription factors, and

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membrane receptors and is the general mechanism of signal transduction?

Distinct Protein Domains for Signaling Ligands and the Issue of Symmetry

Crystallography and electron microscopy studies of signaling molecules—including, in addition to hemoglobin, regulatory enzymes (threonine deaminase, aspartate transcarbamylase, phosphorylase B, phosphofructokinase, L-lactate dehydrogenase), membrane receptors (acetylcholine receptor, glutamate receptor, bovine rhodopsin), and nuclear receptors (lactose repressor, estrogen, or retinoic acid receptors)—have abundantly shown that distant residues participate in the recognition of the regulatory ligand and in the biologically active site [see (*11*, *12*)]. The average distance is 30 to 40 Å. Moreover, distinct protein domains may form that show striking autonomy. In many signaling proteins, domains can be separated by biochemical or genetic methods. Such domains preserve ligand-binding capacity in solution, as found with aspartate transcarbamylase, nuclear receptors, tyrosine kinases, and the nicotinic receptors. In many of these systems, chimeric molecules joining domains with different specificities may even be functional—as shown, for instance, with the nicotinic receptor [e.g., (*13*)].

Crystallographic studies show that, in agreement with the MWC theory, most signaling proteins are made up of a finite number of identical subunits regularly organized around symmetry axes that allow their three-dimensional structure to be exactly described by defined rotations (*11*, *14*) (Fig. 2). Single rotational axes are observed in membrane receptors (Fig. 3) and nuclear receptors (*15*), and there is increasing evidence that dimerization is a requisite for function in G protein–coupled receptors (GPCRs) (*16*).

Exceptions to perfect symmetry do exist, in particular for membrane receptors in which one or several subunits can be substituted by homologous but distinct subunits coded by different genes, yielding a considerable diversification of oligomer composition [see (*12*)]. In some instances, the genesis of a functional receptor even requires association of different subunits with a defined stoichiometry, as in the case of γ-aminobutyric acid type B ($GABA_B$) receptors.

Ligand-Binding Sites at Subunit Interfaces

In the original MWC theory, two unsuspected features of oligomeric proteins were missed. First, the binding sites for regulatory ligands, substrates, or pharmacological agents are, in many cases, located at subunit interfaces, with different interfaces accommodating different categories of stereospecific ligands. Second, physiological ligands (as well as synthetic drugs) may bind within an axial cavity of the molecule, along a symmetry axis. Crystallographic structure determination of several regulatory enzymes—aspartate transcarbamylase, phosphorylase B, phosphofructokinase, and bacterial L-lactate de-

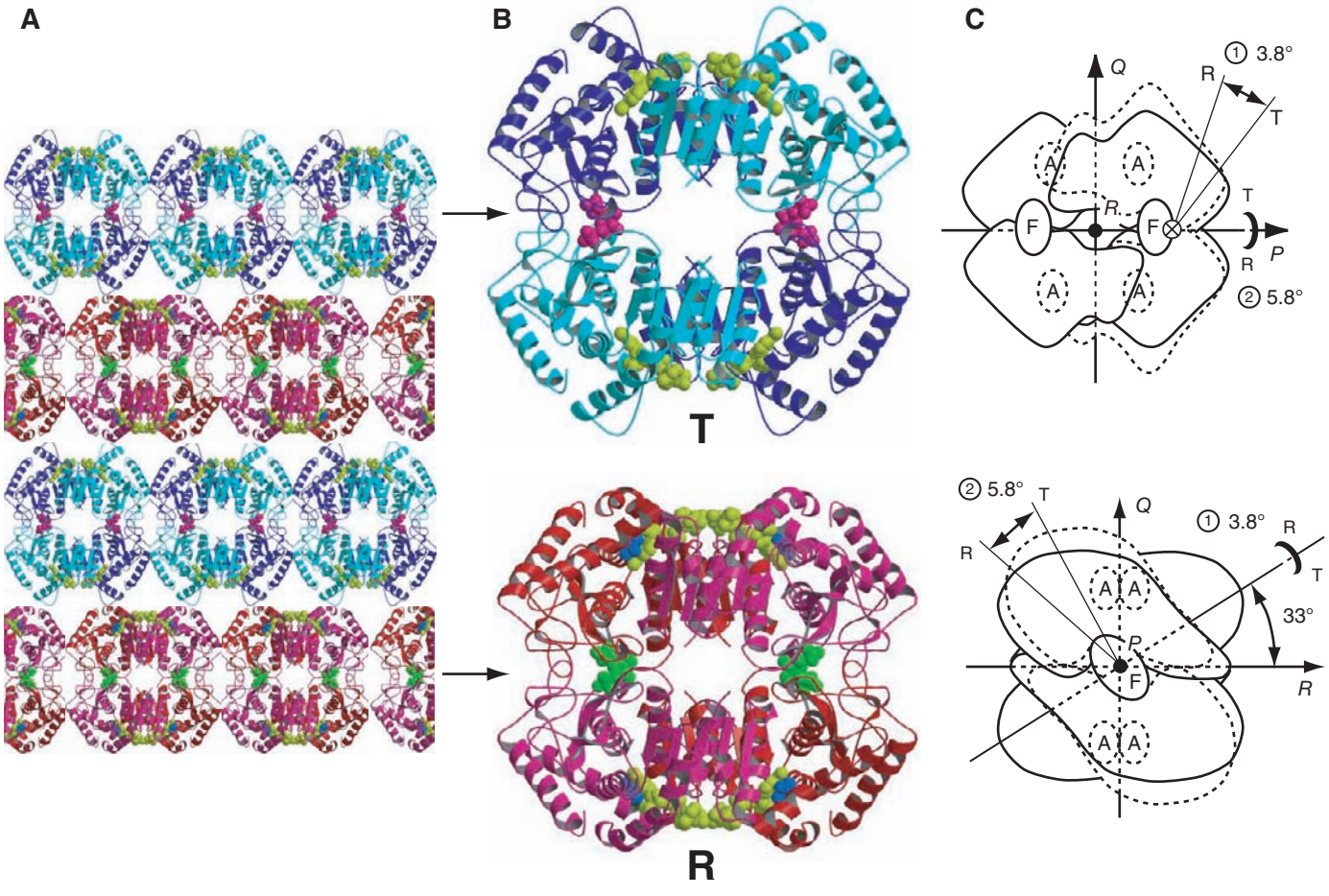

Fig. 2. Structural demonstration of the MWC model: The T and R states coexist in the crystals of bacterial L-lactate dehydrogenase (*14*). (**A**) Planar view of crystal. (**B**) T and R states enlarged showing bound ligands [coenzyme: NADH (reduced form of nicotinamide adenine dinucleotide), light green; regulatory signal: fructose 1,6-bisphosphate, fuchsia in T, green in R; substrate analog: oxalate, blue] at topographically distinct sites and conservation of symmetry of the quaternary structure with little change in tertiary organization of the subunits. (**C**) Two views showing the rotations corresponding to the T-R transition with respect to the three orthogonal axes denoted by *P*, *Q*, and *R* (viewed looking down the *R* axis in the upper schema and down the *P* axis in the lower schema). A and F refer to the analog, oxalate, and to fructose-1,6-bisphosphate, respectively.

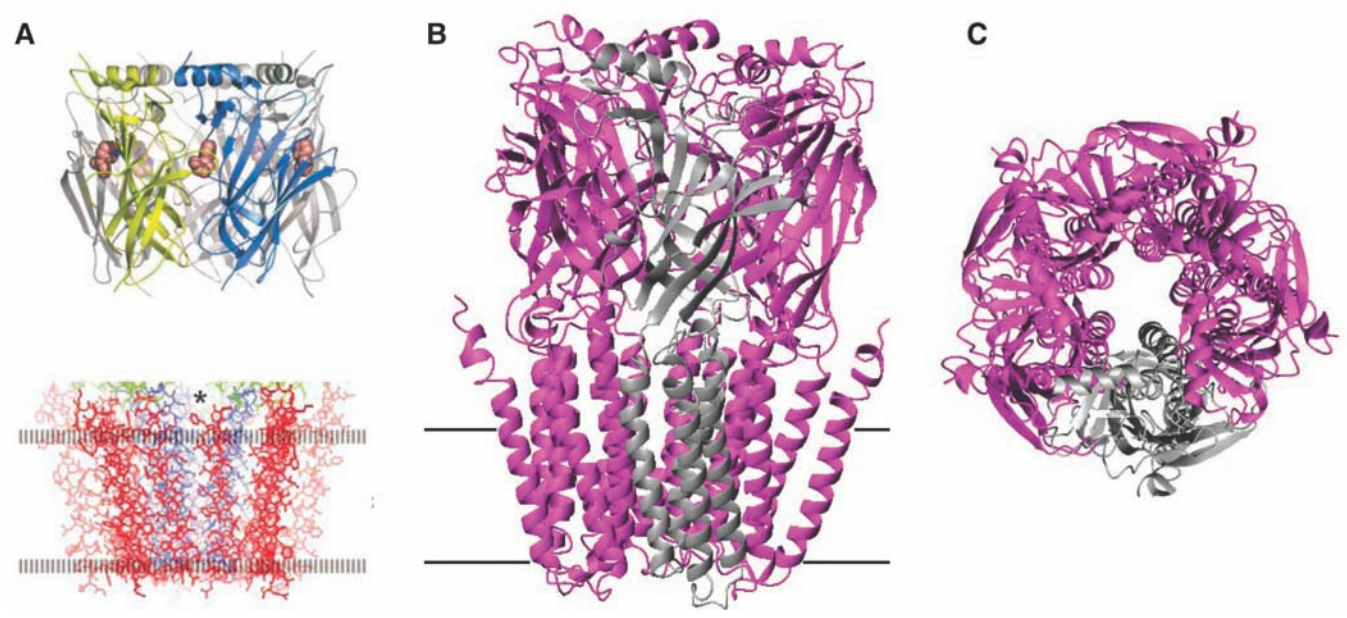

Fig. 3. The acetylcholine nicotinic receptor, a typical allosteric membrane protein. (**A**) Top: X-ray structure of the soluble molluscan acetylcholine binding protein (*34*). Bottom: High-resolution electron microscopy structure of the Torpedo nicotinic receptor transmembrane region (*35*). (**B** and **C**) A three-dimensional computer model of the pentameric α7 acetylcholine nicotinic receptor derived from the structural data in (A) (*36*), in side view (B) and top view (C). The vectorial organization of the oligomeric molecule shows the five-fold rotational axis of symmetry, the nicotine binding site at the boundaries between subunits, and the topographically distinct ion channel.

hydrogenase, among others (*11*, *14*)—revealed the location of the regulatory sites at protein interfaces distinct from the catalytic site. In membrane receptors, binding sites for signaling molecules also occur at subunit interfaces. In muscle nicotinic receptor, for instance, the acetylcholine binding sites are located at subunit boundaries and display structural differences and distinct ligand-binding properties, with no evidence for ligand binding at the three other possible interfaces. In $GABA_A$ receptors, synthetic pharmacological agents—the benzodiazepines—bind at such ''free'' interfaces (*12*), much like anti–sickle cell anemia drugs bind to hemoglobin (*11*). Yet in some GPCRs such as rhodopsin, the ligand-binding domain is located not at a subunit interface but rather within the transmembrane heptahelical domain. In others, such as the metabotropic receptors for glutamate and $GABA_B$ and the ionotropic receptor for glutamate, the neurotransmitter binding pocket lies between distinct ''lobes'' from the same subunit, but the ligand-binding cores assemble as dimers (*15*) within which pairs of sites strongly interact.

An original position for a site binding a signaling molecule, which was not mentioned in the MWC theory but appears perfectly consistent with it, is the axial cavity of the protein molecule. Initially demonstrated for 2,3-diphosphoglycerate with hemoglobin, it served as a structural model for the action of synthetic or natural channel blockers that target a large number of ion channels, including the nicotinic receptor and the Na^+ and K^+ channels. These blockers were instrumental in the first identification of a channel lumen at the intersection of the five subunits of the nicotinic receptor and the demonstration of its interactions with the acetylcholine binding site 20 to 40 Å away (*12*). The strategic location of binding sites for signaling molecules at protein subunit (or lobe) interfaces adequately fits with the concept of the MWC theory that the conformational transitions primarily concern a reorganization of their quaternary structure.

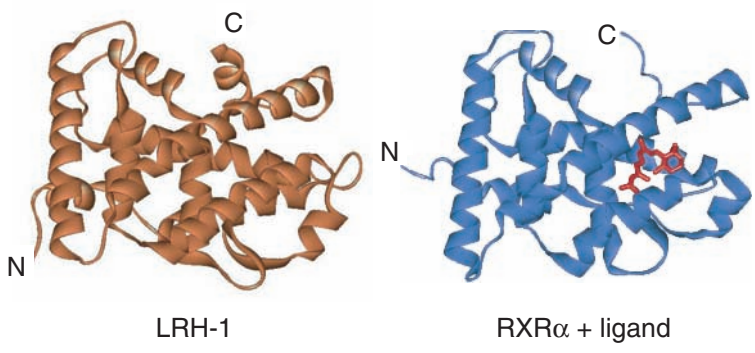

Fig. 4. Structure of the ligand-binding domain (LBD) of LRH-1, a constitutive orphan nuclear receptor (*20*) on the left (PDB 1PK5) and the standard RXRα LBD bound to 9-*cis*-retinoic acid (PDB 1FBY) on the right. The LRH-1 molecule is in an active conformation, as observed by the similarity with the structure of RXRα, but has no ligand in its binding pocket, in contrast to the presence of 9-*cis*-retinoic acid for RXRα.

The Allosteric Transition and "Constitutive" Receptors

A critical statement of the MWC theory was that, in essence, the conformational transition that links the multiple sites present on the allosteric oligomer and mediates signal transduction involves states that are populated in the absence of ligand and may spontaneously interconvert with each other. Moreover, it was postulated that these conformations are present in small numbers and differ in the strength of the interactions between subunits, but preserve the symmetry of the subunit assemblies. Crystallographic studies of hemoglobin, phosphofructokinase, bacterial L-lactate dehydrogenase, aspartate transcarbamylase, and other regulatory enzymes revealed that, as predicted, the transitions between a low-activity, low-affinity T state and a high-activity, high-affinity R state can be resolved at the subunit assembly level into rotations about symmetry axes (*11*). However, minor changes of the tertiary structure of individual subunits can be expected to take place. In other words, the quaternary organization am-

plifies the tertiary changes and gives rise to the cooperative interactions (*11*, *14*, *17*). In the case of membrane receptors, crystallographic analyses of the ligand-binding domain of ionotropic (*18*) and metabotropic (*19*) glutamate receptors have identified a resting conformation—in which the above-mentioned lobes are mostly "open" and which is stabilized by competitive antagonists—as well as a "closed" glutamate or agonist-bound conformation. The tertiary folding of the two lobes does not differ between the two situations (*18*, *19*). Similarly, the homo- or heterodimeric ligand-binding domains of several nuclear receptors display different conformations in the agonist-bound versus antagonist-bound states, with a few characteristic changes in helix structure within a largely common protein fold structure [see (*20*)]. Crystallographic studies elucidated the structural changes of multisubunit potassium channels between the closed and open states (*21*). In agreement with the MWC theory, a hydrophobic gate located in the axis of symmetry frees ion movement through the concerted symmetrical motions of the transmembrane helices.

A key statement of the MWC theory is that the conformational R-T equilibrium is an intrinsic property of the allosteric oligomers accessible in the absence of ligand, with the ligand stabilizing the conformation to which it binds with higher affinity. This concept was initially demonstrated by biochemical methods with regulatory enzymes such as aspartate transcarbamylase (*22*), but it took a long time to be accepted by the physiology and pharmacology communities. Two sets of evidence changed the frame of thought: First, a broad spectrum of receptors are found spontaneously (or constitutively) active in vivo in the absence of ligand, as observed initially for ionotropic receptors (*23*). Several synthetic pharmacological agents referred to as inverse agonists have been identified that stabilize—through binding to allosteric sites—the receptor in its inactive or resting conformation (*24*). Second, mutations have been identified in various receptor systems that cause a constitutive activation, or gain of function, in the absence of ligand. These include regulatory enzymes, ligand-gated ion channels such as the acetylcholine receptor (*12*), many GPCRs (*25*), and nuclear receptors (*20*). In several of these systems, these mutations alter the pharmacological response so that antagonists may become agonists. Such mutations occurring spontaneously in human populations can cause diseases such as congenital myasthenia, frontal lobe nocturnal epilepsy, familial male precocious puberty, and retinitis pigmentosa. Moreover, in the case of GPCRs and receptor tyrosine kinases, many of these mutations can be oncogenic.

Structural studies of a constitutively active orphan nuclear receptor (LRH-1) have brought a striking confirmation of the MWC theory (*20*). At 2.4 Å resolution, the receptor in the absence of hormone adopts an active conformation with a large but empty hydrophobic pocket (Fig. 4). Adding bulky side chains in this pocket results in full or greater activity, which indicates that although the receptor could accommodate potential ligands, they are not a prerequisite for constitutive activity.

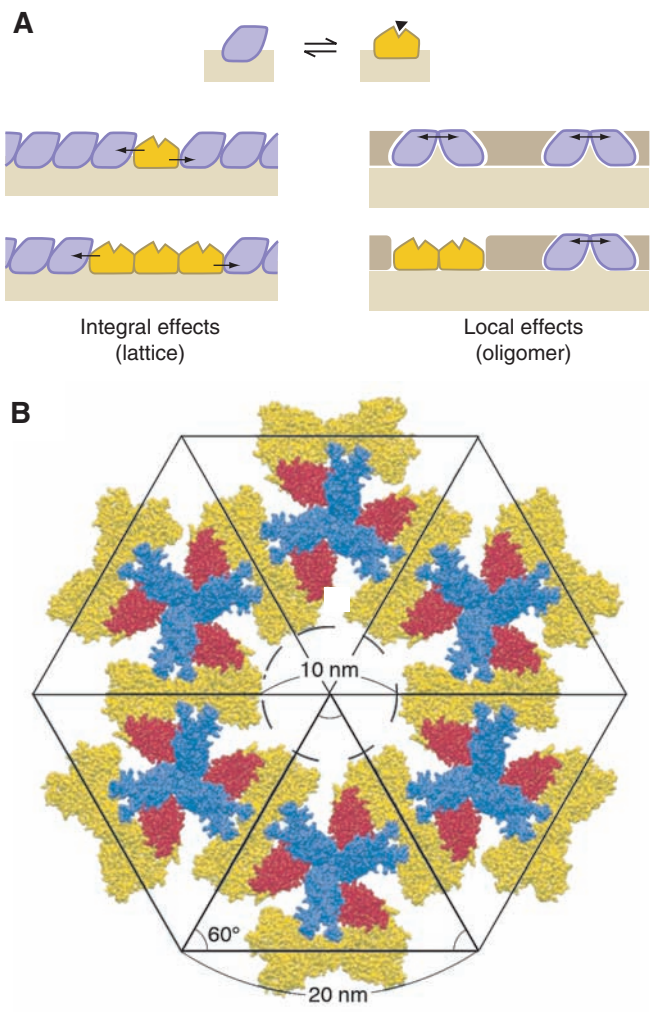

Fig. 5. Allosteric membrane lattice. (**A**) Extension of the allosteric theory to a membrane lattice (*6*). (**B**) The extended cooperative lattice of *E. coli* chemotactic receptors as visualized looking onto the plasma membrane; receptors are in blue, and the linking proteins CheW and CheA are in red and yellow, respectively (*37*).

Cascade of Multiple Transitions in Membrane Receptors and the Question of "Intermediate" States

The original MWC theory hypothesized that the allosteric oligomers exist under a minimum of two discrete (R, T) conformational states with conserved symmetry. Such minimal representation may suffice to account for the kinetics of regulatory enzymes (*11*, *14*) and the all-or-none gating of ion channels in the picosecond to millisecond time range (*12*). However, in most ligand-gated ion channels—and in some GPCRs—the observed kinetics recorded upon prolonged exposure to the signaling ligand involve multiple transitions occurring on a time scale that is much slower (10 ms to several minutes). A cascade of transitions involving high-affinity, slowly interconverting, inactive states must be postulated to account for the physiologically reversible process of desensitization (*12*).

High-resolution electron microscopy studies with the heteromeric muscle nicotinic receptor suggest that exposure to the neurotransmitter causes a nonsymmetrical quaternary reorganization of the molecule, with α subunits more tangentially inclined than other subunits with respect to the axis of symmetry of the molecule (*26*). Yet the structures of the active and desensitized states at atomic resolution remain to be determined. On the other hand, both active and desensitized states have been shown to occur in the absence of ligand. The slow conformational transitions to desensitized states have been proposed to play a critical role in the short-term regulation of synaptic efficacies (*12*).

A most appealing aspect of the MWC theory is its simplicity. Nonetheless, the postulated two-state "concerted" transition has been and still is a debated issue. The alternative sequential model postulates multiple conformations, each with different numbers of ligand molecules bound (*5*). From the theoretical side, more sophisticated models, which include combinations of subunit tertiary conformations, have been proposed (*10*, *12*, *27*). Experimentally, data from enzyme kinetics revealing "negative" cooperativity or from patch-clamp recordings (disclosing, for instance, subconductance states in

non-nicotinic channels) have been interpreted as evidence for intermediate states, but none of these observations are fully conclusive. In particular, for the latter examples, the strictly electrophysiological techniques used do not allow for distinctions between local fluctuations of amino acid side chains and changes in the quaternary organization (*12*).

Hemoglobin has been the paradigmatic system to investigate the mechanism of cooperative ligand binding and, in particular, to test the MWC theory (*8*, *9*). Single-crystal studies, together with a vast array of equilibrium, spectroscopic, and complex kinetic analyses (including time-resolved spectroscopy from the picosecond to the millisecond regime), have shown that the cooperative binding of oxygen is mediated by quaternary changes consistent with a two-state mechanism [(*9*, *10*), but see (*28*)]. However, synthetic allosteric inhibitors such as inositol hexaphosphate and bezafibrate also change O_2 affinity without a change of quaternary structure (*29*). Trapping of transient unstable conformations by encapsulation in silica gels revealed tertiary conformations of individual subunits that coexist within a given quaternary conformation (*10*). The data can be explained by a generalization of the MWC theory to tertiary states (*30*). Similar evidence for trapping of intermediate states within two principal quaternary states has been obtained for *Escherichia coli* aspartate transcarbamylase (*17*). X-ray crystallography of a mutant enzyme for which the allosteric equilibrium is shifted toward the T state showed that in the presence of substrate analogs that push the equilibrium toward the R state, intermediate T′ or R′ conformations could be captured with differential rotation and separation of the catalytic trimers. As in the case of hemoglobin, conditions can be found that trap intermediates within the two principal quaternary states. At this level of resolution, the MWC theory has reached its limits of applicability.

Supramolecular Allosteric Assemblies

Membrane receptors frequently cluster at specific sites in the cell, such as the postsynaptic membrane beneath a nerve ending. Such localized distribution results from their interaction with scaffolding molecules that can modulate their activity (*31*). An extended MWC theory addressed multimolecular planar arrays of identical subunits forming two-dimensional cooperative lattices (*7*). However, studies on diverse specialized membranes did not reveal the anticipated phase transitions. It seems that such scaffolds, if they allow allosteric interactions with individual receptor molecules, may prevent multimeric transitions that would interfere with the repetitive firing of the synapse in millisecond time scales. Bacterial chemotaxis receptors (*32*), however, do appear to form cooperative lattices (Fig. 5). A few molecules of attractant cause a large change in swimming bias, which is mediated by a conformational change that spreads in a large lattice of receptor trimers of dimers. Allosteric cross-talk between 10 to 100 ryanodine receptors has also been reported in heart muscle sarcoplasmic reticulum and may occur between the elementary subunits of flagella or actin filaments.

Conclusions: The Quest for Theory

After 40 years, the allosteric theory of signal transduction has been applied to signaling molecules as diverse as regulatory enzymes, nuclear receptors, and the various classes of membrane receptors. It has even been extended to ribo switches within which folded RNA domains serve as receptors for specific metabolites and to the allosteric cascade of spliceosome activation. As expected, each signaling system displays features of its own. But the concept of signal transmission mediated by discrete conformational transitions that exist before ligand binding would appear to be universal, as is the occurrence of mutations—often pathological—causing constitutive activation of the receptor in the absence of ligand. The simplicity of the theory facilitates its experimental test. However, both the theory and the available technology have reached their limits. This is an important area for future research. Another lies at a more macroscopic scale: It includes the deciphering of the networks of allosteric interactions taking place in supramolecular assemblies within the cell and between cells. The future study of allosteric proteins is more promising than ever, and we expect that theorizing will become more important as we refine our understanding of the mechanisms that allow elaborate physiological control of protein function.

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