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Conformational selection or induced fit? 50 years of debate resolved

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Abstract

Exactly 50 years ago, biochemists raised the question of the mechanism of the conformational change that mediates "allosteric" interactions between regulatory sites and biologically active sites in regulatory/receptor proteins. Do the different conformations involved already exist spontaneously in the absence of the regulatory ligands (Monod-Wyman-Changeux), such that the complementary protein conformation would be selected to mediate signal transduction, or do particular ligands induce the receptor to adopt the conformation best suited to them (Koshland-Nemethy-Filmer—induced fit)? This is not just a central question for biophysics, it also has enormous importance for drug design. Recent advances in techniques have allowed detailed experimental and theoretical comparisons with the formal models of both scenarios. Also, it has been shown that mutated receptors can adopt constitutively active confirmations in the absence of ligand. There have also been demonstrations that the atomic resolution structures of the same protein are essentially the same whether ligand is bound or not. These and other advances in past decades have produced a situation where the vast majority of the data using different categories of regulatory proteins (including regulatory enzymes, ligand-gated ion channels, G protein-coupled receptors, and nuclear receptors) support the conformational selection scheme of signal transduction.

Introduction

A central issue for the cybernetics of living organisms, and consequently for drug design, is how do regulatory ligands (e.g., metabolites, neurotransmitters, and hormones) control biochemical reactions by binding to specific regulatory sites on specialized proteins or receptors? The hypothesis that a conformational change in the protein mediates such "allosteric" interactions between regulatory sites and biologically active sites was proposed exactly 50 years ago [1]. The mechanism that controls this conformational change was a key issue then and remains a central question of protein biophysics, with enormous importance for the design of drugs. Do the differing conformations involved already exist spontaneously in the absence of the regulatory ligands, so that the complementary conformation for a ligand would be selected to mediate signal transduction, or do particular ligands induce the receptor to adopt their adequate conformation (induced fit)? Clearly, if the former is the case, it may be possible to design drugs to stabilize a particular conformation, from a possible spectrum of states, possessing the desired biological activity.

This fundamental question has a long history, dating back to the controversy between Lamarck and Darwin about the evolution of species. An early example of this controversy took place in the early 20th century, when many new antigens were discovered, and most immunologists had difficulty accepting the notion that antibodies against all the many possible antigens preexist in an organism. The so-called instructive theory of antibody formation, advanced notably by Linus Pauling, postulated that the antigen plays a matrix role from which antibody molecules acquired a specific configuration [2]. Alternative mechanisms were not

established until over 40 years later when Tonegawa demonstrated that a wide variety of antibodies against potential antigens are in fact continuously synthesized in the organism through somatic gene recombination [3]. The antigen does not perform an instructive role, but participates in the selection of a complementary antibody. Generally, in biological sciences, the instructive model has been (and is still) commonly used to describe the relationships between living organisms and the outside world. However, convincing experimental evidence rarely, if ever, shows that the environment leaves a direct inheritable "imprint" on the organism.

For proteins other than antibodies, Koshland's 1959 induced-fit theory of enzyme action [4] did not, at the time it was proposed, concern the regulation of enzyme activity by a metabolic signal, only the specificity of enzyme action. His view was that a "steric fit" was essential for the reaction and that such a "fit occurred only *after* a change in shape of the enzyme molecule had been induced by the substrate." Such an "instructive" mechanism was also initially suggested in 1963 to account for the conformational change mediating signal transduction in bacterial regulatory enzymes [5].

It was within this background that in 1965 a paradigmatic shift from instruction to selection occurred—from "the pressure of facts"—with the Monod-Wyman-Changeux proposal (extended in 1966 by Rubin and Changeux) of a model postulating a small number of discrete conformational states *independent* of ligand structure and occupancy [6,7]. The Monod-Wyman-Changeux model provided a substantial economy of means and explained simply many kinetic properties of native enzymes, in particular cooperative ligand binding and its regulation. Specifically, it posed two unifying concepts: (a) regulatory proteins have a quaternary structure with identical subunits symmetrically organized into finite assemblies (oligomers); and (b) oligomers undergo reversible transitions between a small number of discrete conformations, which primarily affect the quaternary organization, preserve its symmetry, and are accessible in the absence of ligand. In this model, ligands selectively stabilize the state to which they preferentially bind, and thereby trigger signal transduction. In its initial formulation, the Monod-Wyman-Changeux model dealt with protein oligomers to account for the positive cooperative phenomena (and oligomeric structure) encountered with most regulatory proteins. A more general formulation was suggested in 1967 by Changeux et al. [8] based upon the conformational transition of a single protomer modulated or not by the interaction with other protomers in a membrane lattice. This model thus lays the ground for a general thermodynamic mechanism of "conformational selection" subsequently elaborated by Burgen and others [9-13].

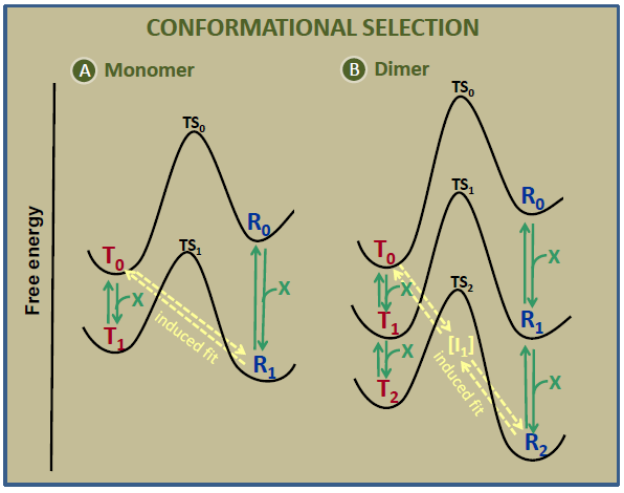
The Monod-Wyman-Changeux and Changeux et al. (1967) models contrast with the Koshland-Nemethy-Filmer sequential, induced-fit mechanism, which posits that proteins can adopt multiple conformational states, including stable "intermediate" (or mixed states), with tertiary changes *complementary* to ligand structures and caused by ligand binding. This model involves "a progressive change" of conformation and it is assumed that a subunit adopts the required conformation only when the ligand is bound to it [14]. We will discuss several recent advances that have made it possible in recent years to distinguish between these two models. One is to use new techniques to measure whether the structure and kinetics of proteins behave as predicted by one model or the other and can be quantitatively fitted by one model and not by the other.

Thermodynamics and kinetics of ligand-dependent protein conformational changes

The appropriate equations for the Monod-Wyman-Changeux and Koshland-Nemethy-Filmer models have been derived and the effect of individual parameters on the shapes and positions of ligand binding and conformational states curves established in forms convenient for testing using experimental data. A critical, yet simple, difference between the Monod-Wyman-Changeux and Koshland-Nemethy-Filmer models is that the binding and conformational state functions would most often differ in the Monod-Wyman-Changeux model while being systematically superimposed in the Koshland-Nemethy-Filmer model. Theoretical approaches combining features of both models have also been proposed [15].

A simple scheme (Figure 1) illustrates the two models; one for a monomeric protein and one for a minimal dimeric protein with identical sites. According to the Monod-Wyman-Changeux model, two conformational states (denoted T_0 and R_0) are in spontaneous equilibrium, with T_0 favored in the absence of ligand (X), as set by the isomerization constant $L_0 = [T_0]/[R_0] > 1$. Since the R state has a higher affinity for X than that of the T state, the population of protein molecules will swing in favor of R as ligand is bound. The path through the states depends on the ligand concentration. For relatively high concentrations of X, the left column will predominate (leading to the reaction pathway for dimers of $T_0 \rightarrow T_1 \rightarrow T_2 \rightarrow R_2$), since binding to T_0 is more rapid than repopulating R_0. However, at low concentrations of X, formation of R_0 may be sufficiently rapid for binding to utilize mainly

Figure 1. Conformational selection for hypothetical monomeric and dimeric proteins



(A) Monomer. (**B**) Dimer. *T* and *R* states are presented on a vertical free energy scale and include the transition state (TS) kinetic barriers for their interconversion, estimated according to linear free energy principles for the dimer [17]. Subscripts correspond to the number of ligand molecules (X) bound. The *T*–*R* transitions follow the Monod-Wyman-Changeux principles for the dimer [6] or those of Changeux et al. [8] for the monomer. The pathway for the induced-fit mechanism [14] is presented by the dashed arrows in yellow and includes an intermediate (I) state for the dimer.

the states of the right column (leading to the reaction pathway for dimers of $R_0 \rightarrow R_1 \rightarrow R_2$). A similar argument was recently presented to distinguish induced fit ($T_0 \rightarrow T_1 \rightarrow R_1$) and conformational selection ($T_0 \rightarrow R_0 \rightarrow R_1$) for a monomeric protein [11,12], but an orthodox induced-fit model would not include R_0 so their model is identical to the Monod-Wyman-Changeux model. Moreover, since the analysis was restricted to monomers (omitting the essential assumption on the quaternary organization of the protein), a key difference related to intermediate states could not be considered. As shown in Figure 1 for the hypothetical dimeric protein, only the induced-fit type mechanism in the Koshland-Nemethy-Filmer model leads to a mixed intermediate state, designated as I_1.

Another distinguishing feature of the Monod-Wyman-Changeux model is the kinetics of the allosteric transitions. In addition to the left (*T* state) and right (*R* state) columns of Figure 1, a central column is shown to represent the transition states for the conformational transitions. The logic of the Monod-Wyman-Changeux model implies equally spaced energy steps for binding of ligand to each state, as illustrated for the hypothetical dimer. It thus also implies equally spaced energies for each transition state (TS), characterized by a TS-positional parameter that fixes the spacing as *T*-like, *R*-like, or some intermediate energy [16]. The consequence of the systematic TS spacing is that for conformational states with increasing numbers (i) of ligand molecules bound, the $T \rightarrow R$ rates increase by a constant factor (${}^{TR}k_{i-1}/{}^{TR}k_i < 1$) and the $R \rightarrow T$ rates decrease by a constant factor (${}^{RT}k_{i-1}/{}^{RT}k_i > 1$), such that the product of the first ratio and the reciprocal of the second ratio equals c, the ratio of ligand binding dissociation constants for the *T* and *R* states [17]:

$$c = ({}^{TR}k_{i-1}\,{}^{RT}k_i)/({}^{TR}k_i\,{}^{RT}k_{i-1}).$$

Attention to this principle can aid in avoiding postulating improbable reaction mechanisms, as recently illustrated for pentameric ligand-gated channels [16].

Methods to investigate the relationships between ligand binding and protein conformation

X-ray crystallography is commonly used to determine the structure of large proteins at sufficient resolution to provide coordinates for its atoms. Since proteins must be crystallized, particular conformations of the protein in solution may be selected, in addition to the constraints imposed on protein conformation by the crystal structure. Independent techniques are thus needed to probe the distinction between conformational selection and induced fit; in the case of ligand binding by hemoglobin, these include fast spectroscopic techniques such as laser flash photolysis of the carbon monoxide adduct of hemoglobin and time-resolved resonance Raman, which is sensitive to structural relaxation close to the hemes, as well as time-resolved ultraviolet resonance Raman, time-resolved circular dichroism, and time-resolved magnetic circular dichroism [18]. In addition, time-resolved wide-angle X-ray scattering (TR-WAXS) has been used to accurately probe structural changes of hemoglobin in solution with nanosecond time resolution, as discussed below [19]. Finally, *in silico* molecular dynamics techniques allow detailed time and space resolution into representative behavior for carefully selected systems. Probing the relationship between molecular structure, movement, and function in molecular dynamics simulations represent an interface between laboratory experiments and theory that can be understood as a "virtual experiment", and are especially useful to distinguish conformational selection from induced fit.

Experimental evidence for selected examples

Hemoglobin

Studies on the cooperative binding of oxygen by hemoglobin in many respects paved the way to the understanding of the role of conformational changes in protein regulation [20]. The first models naturally relied on an induced-fit–sequential mechanism with the

pioneering work of Adair relating the sigmoid oxygen binding curve with the tetrameric nature of the hemoglobin molecule [21] and Pauling's introduction of the concept of heme–heme interactions, with progressively enhanced energy of oxygen binding, including three distinct intermediate states with one, two, and three O_2 bound [22].

The elucidation of the X-ray structures of oxy- and deoxyhemoglobin by Perutz and his colleagues (1960–1964) as a symmerical $\alpha_2\beta_2$ tetramer almost coincided with the proposal of the Monod-Wyman-Changeux model. In 1970, Perutz tried to apply the Monod-Wyman-Changeux conformational selection scheme to hemoglobin (see [23]) and Edelstein's quantitative model soon followed [24]. Perutz interpreted the hemoglobin data in terms of an equilibrium between a tense structure (*T*) with low oxygen affinity (the predominant conformation at low oxygen pressure) constrained by salt bridges between the C-termini of the four subunits, and a relaxed structure (*R*) with high oxygen affinity (the predominant conformation at high oxygen pressure) lacking the salt bridges. The equilibrium was suggested to be governed primarily by the position of the iron atoms relative to the porphyrin plane: out-of-plane in five-coordinated, high-spin deoxyhemoglobin, and in-plane in six-coordinated, low-spin oxyhemoglobin, with the tension exercised by the salt bridges in the *T*-structure transmitted to the heme-linked histidines to restrain the movement of the iron atoms into the porphyrin plane that is necessary for oxygen binding. The variations of the oxygen equilibrium curve of hemoglobin with pH, ionic strength, and allosteric effectors could be described by a simplified Monod-Wyman-Changeux model, but according to Perutz, the Bohr effect and the linearity of proton release with early oxygen uptake would require a sequential rupture of hydrogen bonds in the *T*-structure. Locally "induced" changes would then follow oxygen binding. In the case of fish hemoglobins, the O_2 binding curves at low pH appear drastically "anti-cooperative" (Hill coefficient, $n \ll 1$) and such negative cooperativity has been often reported in the biochemical literature as supporting the Koshland-Nemethy-Filmer model. Yet, heterogeneity between the two types of chains can readily lead to such behavior [25], which becomes extreme at acidic pH for certain fish hemoglobins, reconciling the data with the Monod-Wyman-Changeux scheme [26].

Detailed kinetic measurements using fast spectroscopy placed the rate of the conformational transition in the range of tens of μs and were fully consistent with the Monod-Wyman-Changeux model [27]. Recently, TR-WAXS revealed that the main structural change takes place much faster, in the 2-μs range, after photolysis of CO-hemoglobin [19]. An early transition signal already fully developed at 300 ns, corresponding to a tertiary relaxation to be followed by a main quaternary relaxation likely involving the αβ dimers relative rotation and translation occurring in a concerted way at about 2 μs. The authors assigned this effect to the *R* to *T* quaternary transition, insisting on the fact that the partially ligated states present under their conditions do not modify the pattern. The 20-μs step observed with time-resolved optical spectroscopy would then correspond to a smaller and localized structural change.

In silico calculations based on the method of conjugate peak refinement are consistent with such a two-step quaternary transition [28]. In the *R* to *T* direction the full quaternary transition separates into two distinct phases: an early large quaternary change (characterized by a 3° rotation of each α-subunit relative to the $\beta_1\beta_2$-dimer), with a lower energy barrier postulated to correspond to the 2-μs step, and a smaller late quaternary change (characterized by a 6° rotation of the $\alpha_1\beta_1$- and the $\alpha_2\beta_2$-dimers) with a higher energy barrier postulated to correspond to the 20-μs step. Although the *T*–*R* transition was initially reported to involve very little change at the tertiary level [29], the "quaternary constraint" (as described in Monod-Wyman-Changeux), must naturally be driven by underlying tertiary changes. Indeed, evidence for this concept has been presented by using silica gels to trap unstable intermediates of hemoglobin [30], indicating that a fraction of liganded hemoglobin subunits blocked by the gel in a quaternary *T* state display *R*-state kinetic properties.

Overall, the current understanding of the binding of oxygen to hemoglobin adds details to the Monod-Wyman-Changeux model by incorporating tertiary transitions and explicitly representing coupling between tertiary and quaternary levels. Overall, the conformational selection scheme largely accounts for the paradigmatic example of the cooperative binding of oxygen to hemoglobin, possibly with some small elements of induced fit limited to the immediate vicinity of the ligand-binding sites.

Aspartate transcarbamylase

Together with L-threonine deaminase [1], aspartate transcarbamoylase had a significant place in the early studies on allosteric proteins [31,32]. *Escherichia coli* aspartate transcarbamoylase catalyzes the initial step in pyrimidine nucleotide biosynthesis: the reaction of carbamoyl phosphate with L-aspartate (Asp) to form N-carbamoyl-L-aspartate and inorganic phosphate. The enzyme is composed of two types of subunits: the two larger catalytic subunits are each composed of three

identical polypeptide chains (M_r=34,000), while the three smaller regulatory subunits are each composed of two identical polypeptide chains (M_r=17,000). Each of the six active sites is located at the interface between two adjacent catalytic chains. The enzyme displays homotropic cooperativity for the substrate Asp and is heterotropically regulated by the effectors ATP, CTP and UTP in the presence of CTP.

Early tests of the Monod-Wyman-Changeux model with aspartate transcarbamoylase involved the comparison, under equilibrium conditions, between the equilibrium binding of the substrate analog succinate and the corresponding changes in the enzyme conformation monitored by following p-mercury benzoate reactivity and the sedimentation coefficient. The conformational alterations were found to approach completion at succinate concentrations, which only partially saturate the specific binding sites, excluding the precise superimposition between ligand binding and conformational change expected from the Koshland-Nemethy-Filmer model but quantitatively accommodated by the Monod-Wyman-Changeux scheme [33,34].

The structures of the low-activity *T* state (in the absence of substrates) and high-activity *R* state (in the presence of substrates or substrate analogs such as N-phosphonacetyl-L-aspartate) have been determined by X-ray crystallography [35,36]. A comparison of the *T* and *R* structures reveals that during the $T \rightarrow R$ transition, the two catalytic trimers increase their separation along the 3-fold axis by about 11 Å and rotate about 5° around the same axis, while the regulatory dimers rotate about 15° around their respective 2-fold axes [35].

Using highly deuterated, $^{1}H,^{13}C$-methyl-labeled aspartate transcarbamoylase in concert with methyl-transverse relaxation optimized spectroscopy (TROSY) nuclear magnetic resonance, the shift from the *T* to the *R* state of the enzyme can be quantitatively monitored, allowing the equilibrium constant (L_0) between *R* and *T* forms of the enzyme to be measured quantitatively [37]. The titration data establish unequivocally that both carbamoyl phosphate and its non-hydrolyzable analogue phosphonoacetamide, as well as the bisubstrate analogue phosphonoacetyl-L-aspartate, shift the equilibrium toward *R* and thus bind preferentially to this state. The presence of significant populations of both *R* and *T* conformers in the phosphonoacetamide-saturated enzyme allows a straightforward determination of the shift of the *R*–*T* equilibrium toward *R* upon addition of ATP and conversely, the complete disappearance of the *R* conformer from spectra with CTP. Such changes are in agreement with the Monod-Wyman-Changeux model for both homo- and heterotropic effects, where binding of substrate analogs and ATP to the *R* state is favored and binding of CTP to the *T* state is preferred.

The 11-Å expansion of the enzyme observed during the $T \rightarrow R$ transition has also been monitored by small-angle X-ray scattering (SAXS) [38,39]. Although the *T* conformation was practically identical in solution and in the crystal, differences were observed in the *R* conformation [40]. For instance, the distance between the two catalytic trimers was found to be 2.8 Å larger than in the crystal structure. A salt link was observed only in the low-activity, low-affinity *T*-state quaternary structure, between Lys-143 of the regulatory chain and Asp-236 of the catalytic chain. Its disruption by an Asp-236-Ala mutation yielded an interesting enzyme. SAXS reveals a spontaneous and reversible equilibrium between the *R* and *T* states in solution, and in the absence of ligand, critical evidence in favor of the conformational selection mechanism [41].

The quaternary change observed during the $T \rightarrow R$ transition has been monitored by SAXS at a time resolution as short as 5 ms [39]. After mixing with substrates or substrate analogs at 5°C it appeared to be a single phase under some conditions and biphasic under other conditions. Most strikingly, after addition of N-phosphonacetyl-L-aspartate to the enzyme, the transition rate was more than one order of magnitude slower than with the natural substrates. The results on the homotropic interactions are consistent with a concerted transition between structural and functional states of different affinity and activity for aspartate. Addition of ATP along with the substrates increased the rate of the transition from the *T* to the *R* state and also decreased the duration of the *R*-state steady-state phase. Addition of CTP or the combination of CTP/UTP to the substrates significantly decreased the rate of the $T \rightarrow R$ transition and caused a shift in the enzyme population towards the *T* state. These results suggest a destabilization of the *T* state by ATP and a destabilization of the *R* state by CTP and CTP/UTP, consistent with the *T*- and *R*-state crystallographic structures. The data are adequately fit by a simplified equation derived for the two-state Monod-Wyman-Changeux model. With aspartate transcarbamoylase as with hemoglobin, convergent data from different groups are thus consistent, at the present stage of investigation, with the conformational selection scheme.

Nicotinic acetylcholine receptor

We can attribute the muscle target for nicotine and curare to the original definition in 1905 by Langley [42] of a receptor for a neurotransmitter (acetylcholine) and the characterization of its ionic response as the opening of a cation channel by electrophysiological recording by

Gopfert and Schaefer and by Eccles in the 1930s, long before the molecule was suggested to be an allosteric membrane protein [43,44] and identified from fish electric organ [45,46]. Convergent structural and biochemical data [47] show that nicotinic acetylcholine receptors are integral membrane proteins with a molecular mass of ~290 kDa, forming a cylinder of ~8 nm in diameter and ~16 nm in length comprising five identical or homologous subunits symmetrically arranged around a central ionic channel. Each subunit consists of a large N-terminal extracellular domain, a transmembrane (TM) domain comprising four segments (TM1–TM4), and a variable cytoplasmic domain. There are 2–5 acetylcholine-binding sites within the extracellular domain, located at the boundary between subunits, and topographically distinct from the functionally linked cationic ion channel, located on the axis of symmetry of the transmembrane domain and bordered by the TM2 helices. The TM1 and TM3 segments and especially TM4 contact lipid molecules in the plasma membrane. The crystal structures at high resolution (ca. 3Å) of recently identified bacterial homologues of nicotinic acetylcholine receptors reveal a striking conservation of secondary and tertiary motifs that include the extracellular domain β-sandwich and the four transmembrane α-helices [48,49]. Together they delineate a core structure that is conserved in the superfamily from prokaryotes to eukaryotes, demonstrating that the interactions between acetylcholine sites and ion channels occur between topographically distinct sites and are thus typically allosteric [50]. The issue of conformational selection versus induced fit is raised by the mechanism of ion-channel opening by acetylcholine since, as expected, the early models that were proposed [51] were based on the sequential scheme, the open-channel state always being an agonist-bound state. The following arguments support the view that the conformational selection model more adequately fits the available data.

(a) Patch-clamp recordings of nicotinic acetylcholine receptors at the neuromuscular junction reveal, in the presence of acetylcholine, discrete stochastic current jumps between a low and a maximum unitary current corresponding to closed and open states of a single ion channel [51] (even if single "openings" of the ion channel are actually interrupted by brief closed periods and occasional channels found of smaller amplitude than the usual [full] open-channel current). The rise times are within a μsec and the duration of the steady current in the msec time scale. The durations of the full openings are found to vary with the nature and binding affinity of the agonist, but not the maximum unitary current or intrinsic conductance, indicating the invariance of the all-or-none ionic gating response with the structure of the ligand.

(b) Spontaneous channel openings can be recorded in the absence of acetylcholine [52], and the antagonist α-bungarotoxin causes the frequency of openings to decay with time, results that are inconsistent with a mechanism by which the ligand "induces" the opening of the channel.

(c) Mutations were discovered initially in the channel domain α7-nicotinic acetylcholine receptor [53], but also scattered throughout the receptor. These mutations enhance the response of the receptor to acetylcholine and promote spontaneous channel openings (some of them naturally occurring and causing human disease such as autosomal dominant nocturnal frontal lobe epilepsy and congenital myasthenia). Systematic analyses of these mutations show that they alter the unliganded equilibrium between discrete channel states (open and closed) but not the energy from ligand binding. They are accounted for by the Monod-Wyman-Changeux model [54], providing strong evidence for the conformational selection scheme for acetylcholine receptor gating by acetylcholine.

(d) *In silico* normal-mode analysis application to a model of α7-nicotinic acetylcholine receptor, based on the then-available structural data, shows that the lower frequency mode corresponds to a global quaternary twist motion of the protein [55], resulting from a tilt of each subunit that causes anticlockwise motion in the upper part of the nicotinic acetylcholine receptor pentamer. This motion occurs concomitantly with a bending of the subunits at their extracellular domain–transmembrane domain interface, a pore dilatation over its entire length and a structural reorganization of the acetylcholine-binding site. The twist mode accounts for key features of receptor channel opening and closing by agonists and antagonists, and for key features of the above mentioned mutations in the nicotinic acetylcholine receptors [55,56]. The atomic mechanism of channel opening can be seen using the X-ray structures of the bacterial nicotinic acetylcholine receptor homologs stabilized in closed versus open conformation. Despite low sequence identity (18%), their common core structure undergoes a quaternary twist, similar to that described above, that contributes to at least 29% of the closed to open transition, and each subunit undergoes tertiary deformations. These deformations involve a substantial rearrangement of the subunit interfaces and a downward motion of the β1–β2 loop. This is apparently coupled to a tilt of the TM2 and TM3 segments and generates a wide opening in the upper part of the pore (from 2 to 12 Å diameter) [48]. The recently determined structure of the homopentameric glutamate-gated ionic channel-α from *Caenorhabditis* stabilized in an open conformation

[57] further reveals an unanticipated superimposition with the previously identified *Gloeobacter* receptor structure [48,49], thus supporting the striking invariance of a channel-gating transition stabilized by chemically unrelated ligands of proteins that are very distant evolutionarily.

(e) One advantage of the electrophysiological recordings of ligand-gated ion channels is that they reach similar time ranges as TR-WAXS with hemoglobin, giving one hope that they may improve the resolution of the intrinsic conformational change that mediates signal transduction in the presence (or absence) of ligand and answer some outstanding questions. How does the cooperative transition proceed in an oligomeric protein? Are there "intermediate" or "flip" states involved? One-microsecond molecular dynamics simulation of the *Gloeobacter* receptor reveals a channel closure initiated by local but large fluctuations in the pore that take place at the top of the M2 helix, followed by a global tertiary relaxation resulting in a quaternary twist of the whole molecule [58]. Future investigations should determine whether or not the simulation matches the structural data within the appropriate time scale. In addition, fast and slow desensitization processes have been identified in muscular and neuronal nicotinic acetylcholine receptors within a broad range of time scales that imply different local conformations within both transmembrane domains and extracellular domains [47,59]. Additional conformational states still need to be added to the Monod-Wyman-Changeux scheme for these data to fit [17,60], within the conformational selection scheme.

(f) Last but not least, abundant structural studies have identified the three-dimensional structure of a broad diversity of nicotinic ligands bound to the acetylcholine binding site of the acetylcholine receptor or an acetylcholine-binding protein homolog from mollusk [61]. Other investigations focused on the stabilization of the pyridine in nicotine, where the unprotonated nitrogen is a hydrogen-bond acceptor for stabilizing this ring [62]. It seems that a network of H_2O molecules is stabilized and connects to solvent in the vestibule. In turn, polar side chains in the receptor and acetylcholine-binding protein are involved. Little if any "induced-fit" reorganization is detected at this level of resolution within the site with ligand binding, with the notable exception of a C-loop motion that primarily maps the size of the bound ligand. Acetylcholine-binding protein possesses a rigid organization and lacks the complement of conformational changes associated with acetylcholine receptor activation. On the other hand, rigid body docking procedures with the quaternary twist model of acetylcholine receptor conformational transition [55] reveal a complex between the antagonist α-cobratoxin and muscle nicotinic acetylcholine receptor that matches the basal "resting" state conformation of the receptor but not its active open-channel conformation, in agreement with the experimental data suggesting a "closing" of the binding site during gating (twist mode) [63]. In other words, *in silico* simulation and empirical data are consistent with a conformational selection model. Further studies are, however, needed for a deeper understanding of the (probably multiple) allosteric transitions of the nicotinic acetylcholine receptor to account for the activation and desensitization processes together with the relevant design of pharmacologically efficient drugs.

G protein-coupled receptors

G protein-coupled receptors (GPCRs), the largest class of cell-surface receptors, are activated by a diverse range of ligands, including hormones, neurotransmitters, ions, odorants, and photons of light. Once activated, they couple to a wide range of signaling molecules and effector systems. GPCRs are encoded by about 800 genes in humans and are the target of many therapeutic agents that are currently in use. X-ray crystallography structures of GPCRs are characterized by seven-trans-membrane helices and an eighth helix that lies approximately parallel to the intracellular membrane, as well as substantial variations in functionally divergent regions—especially on the extracellular side of the receptor, which is responsible for ligand interactions. Binding of small extracellular ligands modulates the capacity of GPCRs to catalyze GDP/GTP exchange in heterotrimeric guanine-nucleotide-binding proteins (G proteins $G\alpha\beta\gamma$) at the distant site facing the cell interior. Signal transduction mediated by GPCRs is thus, by definition, allosteric. A rich literature exists on GPCR-mediated signal transduction and only a few features relevant to the allosteric transition mechanism shall be mentioned here:

(a) GPCRs can adopt multiple oligomeric states

The relationship between the formation of GPCR homo- or hetero-dimers and signal transduction is still debated, and may vary with the GPCR class (A, B, or C) [64-69]. Rhodopsin and the β2-adrenergic receptor, which belong to class A GPCRs, signal efficiently through G proteins when reconstituted into lipid nanodiscs containing only a single receptor molecule, and thus, these class A GPCRs may function without the need for oligomerization. Numerous studies, however, have revealed more complex activation mechanisms due to the ability of diverse GPCRs to adopt several conformations, each linked to specific signaling cascades (see section below: (d) *Different ligands may activate different pathways in*

β-adrenergic receptors) and to form dimers or even larger oligomeric complexes offering new signaling possibilities. For instance, for the class A serotonin receptor $5HT_4$, activation of one protomer in a dimer is sufficient for G-protein activation, but coupling efficiency increases by 100% when both protomers are activated [70]. For the class C metabotropic glutamate (mGlu) and $GABA_B$ receptors, dimerization is mandatory for receptor activation: the mGlu receptor forms homodimers stabilized by an inter-subunit disulphide bridge and the $GABA_B$ receptor is an obligatory heterodimer ($GABA_{B1}$+$GABA_{B2}$). Furthermore, time-resolved fluorescence resonance energy transfer experiments show that $GABA_B$ heterodimers can form stable tetramers (that are present in the brain) with a decrease of G protein-coupling efficiency [71]. Recently, in one among many examples, a model system of a GPCR heteromer consisting of μ and δ opioid receptors was established, showing that μ receptor ligands are capable of allosterically enhancing δ receptor radioligand binding and *vice versa*, suggesting an allosteric receptor model [72]. Abundant investigations are currently in progress to evaluate the functional significance of these diverse allosteric interactions as an uncharted landscape for drug development [66], within the conformational selection scheme.

(b) GPCR mutations can make them constitutively active without ligands

More than 40% of all wild-type GPCRs tested exhibit constitutive basal activity [73], including the β2-adrenergic, H3 histamine, ghrelin, and the thyroid-stimulating hormone receptors; see [74] for review. Recent evidence for spontaneous visual pigment activation (or dark noise) has also been presented [75]. Mutations in GPCRs cause more than 30 different human diseases, and among them about 100 are constitutive mutations that are themselves responsible for more than 10 diseases (including congenital stationary night blindness for rhodopsin or Kaposi's sarcoma for KSVH-GPCR) [74]. The $\alpha_{1\beta}$-adrenergic receptor was the first GPCR in which point mutations were shown to trigger receptor activation in the absence of agonist [76]. To extend the generality of this finding, similar mutations were performed in the β2- and α2-adrenergic receptors, which are coupled to G_s-mediated stimulation or G_i-mediated inhibition of adenylyl cyclase, respectively [77-79]. Both β2- and α2-receptor mutants exhibited increased constitutive activity leading to increased or decreased agonist-independent adenylyl cyclase activity, respectively. Since then, spontaneously occurring activating mutations have been discovered in various GPCRs, including rhodopsin, that are responsible for a number of human diseases [80]. The constitutive effects of these mutations, together with basal activity and its shift toward the resting conformation by negative allosteric modulators (awkwardly named "inverse agonists") shown by many of the receptors, was interpreted by Lefkowitz and colleagues [81] in terms of an adapted version of the Monod-Wyman-Changeux scheme. The spontaneous equilibrium between R and R^* and the relevant value of L would allow a significant level of R^* to be present in the absence of agonist. The equilibrium would be further shifted in favor of R^* as a consequence of a constitutive mutation or saturating concentrations of agonist, with the amplitude of the shift depending on the consequences of the mutation on the value of L. As discussed for nicotinic acetylcholine receptors [82,83], the occurrence of such constitutive mutations that yield active receptor molecules in the absence of agonist rules out the "induced-fit" mechanism of receptor activation.

(c) Resting and active conformations of GPCRs have common X-ray structures

In addition to the spontaneous occurrence of the activation transition, recent X-ray crystallography structures for rhodopsin, β-adrenergic, and adenosine A_{2A} GPCRs in antagonist-resting versus agonist-bound states with a variety of ligands show a similar global conformation, and this suggests that GPCRs have a common activation mechanism that is independent of the structure of the ligand bound—see references in [84,85]. Rhodopsin structures have been of immense value for understanding the activation transition. Rhodopsin's structure is stabilized by multiple hydrogen bonding networks within the seven-transmembrane core involving structurally bound water molecules and conserved motifs forming an "ionic lock" in GPCRs that constrains TM3 and TM6. The recent structures solved for rhodopsin and non-rhodopsin GPCRs in the active and resting conformations reveal a slightly increased distance between TM3 and TM6, which is sufficient to break the ionic lock that appears to be a common prerequisite for an active receptor state. Non-rhodopsin GPCR structures show, in addition to a highly variable organization of the ligand-binding domain, a larger diversity among the extracellular ends of the TM segments and conformations of the connecting loops. Yet, common features emerge from this diversity. For instance, residues in the binding pocket that interact with the ligand are, as in the case of nicotinic acetylcholine receptors, more contracted or compact in the active conformation (helices III, V, and VII), which is stabilized by the agonist whatever its nature [84,85]. This structure corresponds to an opening out of the helices on the cytoplasmic surface, particularly helices V and VI, creating a cavity into which the C-terminus of G-α protein binds. Movements on helices V to VII are

supported by a relatively stable core bundle composed of helices I to IV. Interestingly, comparison of the crystal structures of ligand-free opsin [86] and constitutively active rhodopsin that retains retinal [87] reveals a common active GPCR structure, ruling out an induced-fit activation mechanism. Further understanding of these specific agonist-stabilized conformational changes in GPCRs may help to establish a structural basis for the design of functionally selective ligands. In any case, the data are strikingly consistent with a universal conformational selection scheme.

(d) Different ligands may activate different pathways in β-adrenergic receptors

β-adrenergic receptors classically mediate responses to the endogenous ligands adrenaline and noradrenaline by coupling to $G_s\alpha$ and stimulating cyclic adenosine monophosphate (cAMP) production. Yet, drugs designed as β-adrenergic receptor agonists or antagonists can activate alternative cell signaling pathways, thereby resulting in a multitude of signaling outputs [67,88]. For example, β3-adrenergic receptor-stimulated activation of p38 mitogen-activated protein kinase (MAPK) is mediated by a cAMP–protein kinase A pathway in adipocytes, whereas in CHO-K1 cells expressing the β3-adrenergic receptor, increasing levels of cAMP cause inhibition of p38 MAPK phosphorylation [89]. Such differential capacity for pathway activation of different ligands has been referred to as "biased agonism/antagonism" or "ligand-directed signaling". These terms refer to responses where drug A has higher efficacy than drug B for one signaling pathway, but a lower efficacy than drug B for a second pathway, thus with the potential to influence pharmacological activity and clinical efficacy. The observation that ligands A and B have the capacity to elicit distinct active conformations of the same receptor might, at first glance, be interpreted in terms of an induced-fit mechanism, yet the spontaneous occurrence of multiple conformations, which in turn display altered coupling efficiency to different effectors, should equally be considered.

Lac repressor and nuclear receptors

The *lac* repressor is a well-studied bacterial protein that forms, together with the specific DNA element of its operator, a genetic switch of the *lac* operon [90,91], which has become a model of transcription regulation by nuclear receptors. More than 4,000 point mutations have been introduced and characterized and have opened many avenues, including those leading to structural investigations. Several structures of the *lac* repressor have been solved to various resolutions with different molecules bound to the effector site. The *lac*-repressor tetramer is composed of four identical 360-amino-acid chains. The N-terminal 60-amino-acid region contains a helix-turn-helix motif of the operator-binding headpiece typical for DNA-binding proteins. The core comprises residues 62–332 and is subdivided into two topologically similar regions with the ligand-binding site between them. Finally, preceded by a flexible loop, the C-terminal residues 339–360 form an α-helix, which is involved in tetramerization via the formation of a four-helix bundle with the C-terminal part of the other monomers. The complete tetramer can best be described as a dimer of dimers, with a pseudosymmetric interface. Each monomer binds to one inducer molecule with equal affinity, but dimer formation is required for DNA binding. The proposed mechanism for *lac*-repressor activation is the occurrence of two conformations with different organizations of the DNA-binding domains. The conformation stabilized by the inducer IPTG (isopropyl β-D-1-thiogalactopyranoside) shows a reduced affinity for the operator thus triggering the genetic switch. The occurrence of constitutively active mutations and the adequate fit of the data using the Monod-Wyman-Changeux model [92], again, favor the conformational selection mechanism.

The recent elucidation of the X-ray crystal structure of the eukaryotic nuclear receptor PPARγ–RXRα heterodimer [93] reveals striking analogies between the nuclear receptor superfamily [94] and the *lac* repressor. Even if the occurrence of typical allosteric modulatory sites for coactivator peptides [93] and the reciprocity of the interaction between distinct DNA- and ligand-binding domains [95] strengthen the generality of the molecular mechanisms involved, further structural information is urgently needed to understand the allosteric transitions of these important transcription regulators [96].

Ribonucleotide reductases

Ribonucleotide reductases catalyze the production of deoxynucleotides from ribonucleotides in all living species. It is an unusual regulatory protein that uses an apparently unique allosteric mechanism, with ATP and deoxynucleotides as effectors, to direct substrate specificity, ensuring that the enzyme produces the appropriate levels of the four deoxynucleotides for DNA replication [97]. In *E. coli*, the enzyme is a complex of two non-identical protein dimers, R1 and R2. R2 contains a non-heme dinuclear iron centre that, together with molecular oxygen, oxidizes a specific tyrosine residue of R2 to create a stable tyrosyl radical required for the formation of a transient thiyl radical in R1. R1 is the catalytic subunit and also contains the allosteric sites for effector binding. During each turnover of the enzyme, a proton-coupled electron transfer occurs from the tyrosine of R2 over a distance of 35 Å to the catalytically active cysteine of R1 via a complex network of specific amino-acid loops.

Crystallographic studies of the catalytic subunits from different classes of reductases in combination with the allosteric effectors: dTTP, dATP and dGTP and with and without the cognate substrates GDP, CDP, ADP delineate structural changes associated with effector binding that determine the specificity of the enzymes towards reduction of the appropriate substrate. An amino acid region referred to as "loop 2" spans the effector and substrate-binding area and is present in a different distinct conformation with each effector–substrate pair. Different conformations of the protein link the bound effector to the substrate site and create the specificity of the enzyme catalytic reaction. An unanswered but fascinating question is: To what extent does the protein spontaneously display these multiple conformations in solution as discrete entities in the absence of the cognate ligands or alternatively are they "induced" by the actual binding of their multiple ligands from a common, unique, though highly flexible form?

Conclusion

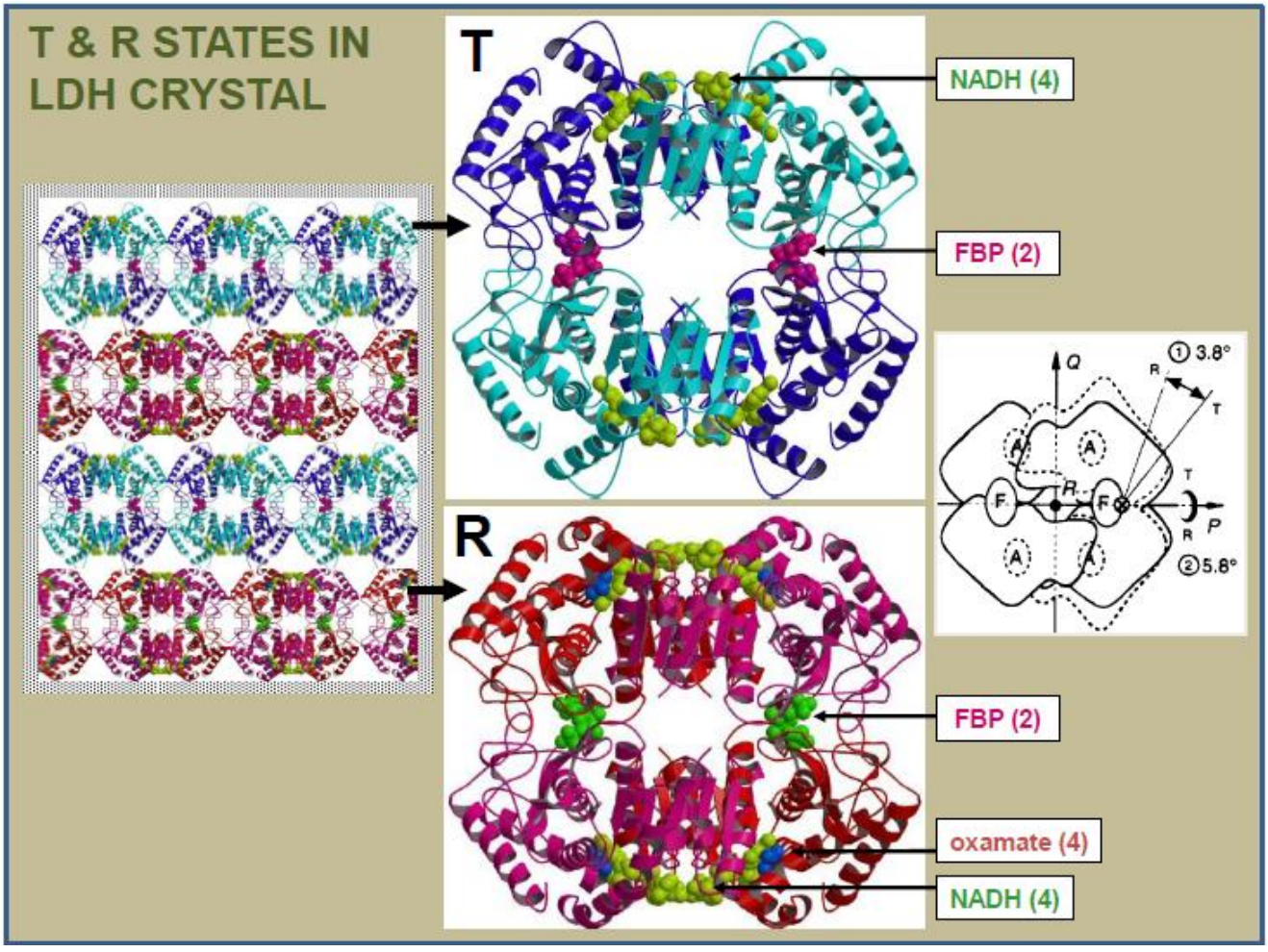
Since the static lock-and-key concept of the early 20th century for enzyme action and receptor drug responses, considerable evidence has revealed that proteins show a remarkable flexibility upon ligand binding. This view is reinforced by the discovery that, in addition to the classical "competitive drug action" and design [98,99], ligands may regulate, in an allosteric manner, the properties of far distant sites on proteins through a conformational change [46]. Relevant to a long historical controversy dating back to Lamarck and Darwin, the issue of distinctions between ligand-induced conformational change and selective stabilization of pre-existing conformational states *independent* of ligand structure and occupancy has proven to be of fundamental importance in understanding the mechanism of signal transduction from the viewpoints of both basic and clinical/pharmacological applications.

The recent progress in various methods of structural determinations and elaborate computations based on principles of molecular dynamics have clearly established the spontaneous occurrence of discrete global fluctuations of protein conformation and the establishment of a preset conformational equilibrium between functional states of the protein [100]. For oligomeric proteins in solution or membrane-bound (hemoglobin, aspartate transcarbamoylase, and various homo- or hetero-oligomeric receptors) spontaneous changes at the quaternary level resulting in receptor activation in the absence of ligands have been well documented by both approaches. In several instances, in agreement with the Monod-Wyman-Changeux model, there is direct evidence—as illustrated in Figure 2 for bacterial lactate dehydrogenase—for fully concerted changes of quaternary structure, without intermediate states and with unambiguous conservation of symmetry [101]. For GPCRs, which have been recently found to exist mostly as homo- or heterodimers, a similar conclusion has been reached so that there is a common global activation mechanism of the basic receptor unit for different GPCRs that involves discrete movements of transmembrane helical groups. Moreover, mutations have been found in several different types of allosteric proteins, discussed above; such mutations can change the conformer equilibrium, rendering a conformer far more or far less stable and, in the extreme case, stabilizing without ligand to give constitutive activity. In other words, a fully active conformation can occur in the absence of ligand, definitively ruling out an induced-fit mechanism. These observations suggest that, in the course of evolution, some mechanisms of conformational selection have been selected that are shared by entire groups of proteins and that these occur independently of the diversity of regulatory ligands involved. Consistent with the Monod-Wyman-Changeux scheme, the quaternary organization of the proteins has been the principal target of the evolutionary changes that lead to the genesis of signal transduction mechanisms in proteins.

This commonality of mechanisms does not exclude the possibility that, at a fine-structure level, local ligand-dependent movements in the Å range might take place that would be consistent with the "induced-fit" mechanism [100], as initially suggested with the "non-regulatory" enzyme *E. coli* isocitrate dehydrogenase for enzyme activation [102]. This might also be the case, for instance, with the reordering of the C-loop of nicotinic acetylcholine receptor following nicotinic ligand binding noted above [103] or for ionotropic glutamate receptors, where changes in degree of closure of the bilobed agonist-binding site quantitatively control the open probability of discrete sub-conductance states of the ion channel [104]. Yet, it should be emphasized that at the present level of resolution, the detailed mechanisms for such local conformational changes have not been identified at the atomic level and may equally be interpreted in terms of conformational selection—within an equilibrium of multiple (more than two) alternative structures [105]—or of an "adequate" induced-fit mechanism.

Progress will likely result from the rapid advances in computational power and improved algorithms, permitting longer simulations for complex systems that occur over time frames (microseconds to milliseconds) relevant to the phenomena discussed here; in particular, this could be achieved using a special purpose machine for long molecular dynamics simulations [106] involving protein molecules and ligands that can reveal new

Figure 2. Co-crystal of T and R states of bacterial L-lactate dehydrogenase (LDH)



Left panel: Ribbon representation of the crystal showing alternating rows of molecules in the *T* and *R* states. **Middle panel:** Individual molecules in the *T* and *R* states complexed with NADH (the reduced form of nicotinamide adenine dinucleotide), 1,6-fructose-bisphosphate (FBP), or oxamate (analogue of the substrate pyruvate). Oxamate is present at a concentration that shifts the allosteric equilibrium 50% to the *R* state. **Right panel:** Schematic sketch of a *T*-state dimer (dashed outline) superimposed on the *R*-state tetramer (solid outline) showing the rotations for interconversion between the two states. Adapted from images supplied by So Iwata.

allosteric targets [107]. Other advances are likely to arise from new experimental approaches that build on the power of TR-WAXS as described for hemoglobin [19]. These approaches are anticipated to open new opportunities for pharmacology and drug design of many neurological and psychiatric diseases. Indeed, drugs will have to be targeted not to a single, fixed state of ligand binding proteins but instead, more selectively, to one of the several conformations of such proteins. However, it would be foolhardy to predict exactly what the next 50 years will produce.

In conclusion, in answer to the question: "conformational selection or induced-fit?", the response is that following 50 years of debate and experimentations, the vast majority of the data support the conformational selection (Monod-Wyman-Changeux) scheme of signal transduction.

Abbreviations

ADP, adenosine diphosphate; Asp, L-aspartate; ATP, adenosine-5′-triphosphate; cAMP, cyclic adenosine monophosphate; CDP, cytosine diphosphate; CTP, cytosine triphosphate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dTTP, deoxythymidine triphosphate; G_i, inhibitory G protein; G_s, stimulatory G protein; GABA, gamma-aminobutyric acid; GDP, guanosine diphosphate; GPCR, G protein-

coupled receptor; GTP, guanosine-5′-triphosphate; KSVH-GPCR, Kaposi's sarcoma-associated herpesvirus–G protein-coupled receptor; MAPK, mitogen-activated protein kinase; mGlu, metabotropic glutamate; PPARγ, peroxisome proliferator-activated receptor gamma; RXRα, retinoid X receptor alpha; SAXS, small-angle X-ray scattering; TM, transmembrane; TR-WAXS, time-resolved wide-angle X-ray scattering; TS, transition state; UTP, uridine-5′-triphosphate.

Competing interests

The authors declare that they have no competing interests.

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