

The role of dynamic conformational ensembles in biomolecular recognition

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Molecular recognition is central to all biological processes. For the past 50 years, Koshland's 'induced fit' hypothesis has been the textbook explanation for molecular recognition events. However, recent experimental evidence supports an alternative mechanism. 'Conformational selection' postulates that all protein conformations pre-exist, and the ligand selects the most favored conformation. Following binding the ensemble undergoes a population shift, redistributing the conformational states. Both conformational selection and induced fit appear to play roles. Following binding by a primary conformational selection event, optimization of side chain and backbone interactions is likely to proceed by an induced fit mechanism. Conformational selection has been observed for protein-ligand, protein-protein, protein-DNA, protein-RNA and RNA-ligand interactions. These data support a new molecular recognition paradigm for processes as diverse as signaling, catalysis, gene regulation and protein aggregation in disease, which has the potential to significantly impact our views and strategies in drug design, biomolecular engineering and molecular evolution.

Highly specific and tightly regulated interactions between biological macromolecules are at the basis of all processes in living organisms. An understanding of the fundamental mechanisms of molecular recognition is therefore central to understanding biology at the molecular level. The two textbook mechanistic explanations for molecular recognition are Fischer's 'lock-and-key' model¹ and Koshland's 'induced fit' hypothesis². In the 'lock-and-key' model, the conformations of the free and ligand-bound protein are essentially the same, whereas 'induced fit' posits that conformational differences between these two states are the result of the binding interaction driving the protein toward a new conformation that is more complementary to its binding partner. In their simplest forms, both the lock-and-key and the induced fit models treat the protein as if it exists in a single, stable conformation under given experimental conditions. However, proteins

are inherently dynamic and sample a vast ensemble of conformations. Thus, thermally accessible conformational substates other than the 'native' (that is, lowest energy) conformation may play important roles in molecular recognition³ (see Fig. 1). The alternative model of conformational selection takes into account this conformational heterogeneity and argues that weakly populated, higher energy conformations are responsible for recognizing and binding to partners with subsequent population shift toward these conformers⁴⁻⁶. Invoked by kinetic data to explain antigen recognition and antibody specificity⁷⁻¹⁰ and generalized to apply to all binding events, the conformational selection and population shift model^{4-6,11} challenges the assumption that conformational differences between free and ligand-bound protein automatically implicate an induced fit-type mechanism of molecular recognition. Given the impact that the 50-year-old induced fit hypothesis has had on chemistry and biology and the central role of molecular recognition in all biological processes, an alternative to this decades-old theory based on fundamental physical principles deserves careful consideration.

The conformational selection model derives from the energy landscape theory of protein structure and dynamics^{3,5,11,12}. The energy landscape theory in biology is most familiar in terms of the 'protein folding funnel'^{3,5,13}, but it also has major consequences in terms of binding interactions and protein function^{4,5,14}. A protein free energy landscape consists of different conformations or 'substates' in dynamic equilibrium. The populations of the substates follow statistical thermodynamic distributions, and the heights of the energy barriers separating the substates define the timescale of conformational exchange. If the free energy barriers are low relative to the Boltzmann energy ($k_B T$), thermal fluctuations can lead to significant population of more than one conformational state in solution. A ligand may interact with the lowest energy conformation, or with one of a number of higher energy conformational substates that are populated in solution^{4,5,11}. In all cases, the binding interaction does not 'induce' a conformational change; it merely leads to a population shift—that is, a redistribution of the relative populations of conformational substates that already pre-exist in solution. Within this context, the lock-and-key model is a limiting case of conformational selection when the interaction partner selectively binds to the lowest energy conformation.

Structural differences between the 'beginning' (free) and 'end' (bound) thermodynamic states, as observed in X-ray and NMR structures, do not by themselves reveal the process of molecular recognition. A structural demonstration of induced fit would require data sampled over the entire course of the binding interaction and would need to show how interaction with the binding partner directly leads to specific structural changes in the protein⁸. Structural

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evidence for conformational selection would entail the characterization of alternative conformations in the free state that resemble the final ligand-bound conformation⁸, and this would need to be complemented by studies demonstrating that the ligand interacts with a small population of a 'bound' substate (throughout this review we will use the term 'bound' to refer to a conformational substate of the unliganded protein that has a conformation resembling that of the ligand-bound state). In the past, structural characterization of conformations other than the one most highly populated in solution or in the crystal has been difficult. Fortunately, single molecule, NMR and other spectroscopic techniques are now beginning to shed

more light on the true conformational diversity of proteins in solution^{15–19}. These methods have revealed the presence of conformational substates that resemble the bound state and that form part of the conformational ensemble populated by the unliganded protein; the existence of such 'bound' substates had previously been inferred only through computational protein dynamics^{20–22}. Intriguingly, these results come from a host of molecular recognition events including protein-ligand, protein-protein, protein-DNA and RNA-ligand interactions. In this Perspective, we discuss recent structural support for the conformational selection model and the consequences of a free energy landscape picture of protein structure and function in terms of drug design, biomolecular engineering and molecular evolution.

Box 1: Kinetics and thermodynamics of 'induced fit' versus 'conformational selection'

Simple induced fit and conformational selection processes can be viewed in terms of a thermodynamic cycle (Fig. 1). The key difference between these two extreme mechanisms is that in the induced-fit model the bound protein conformation forms only after interaction with a binding partner, whereas in the conformational selection model, it pre-exists in the ensemble of conformations sampled by the free protein in the absence of ligand. The kinetic rate constants describing the thermodynamic cycle can dictate which molecular recognition pathway will dominate. According to this model, if the concentration of the higher energy 'bound' conformation ($[P_2]$) is larger than the concentration of the induced-fit intermediate ($[P_1L]$), conformational selection will be the preferred kinetic pathway⁹⁹. This means that for induced fit to be operative, there must be some initial favorable interaction between the ligand and the protein (K_1) before conformational change, and/or the ligand concentration needs to be sufficiently high. Of course, depending on the rate constants and protein/ligand concentrations, both recognition mechanisms may be feasible in a given system. In conformational selection, the rate of formation of the final bound complex (P_2L) depends linearly on the concentration of the higher energy 'bound' conformation ($[P_2]$) and nonlinearly on the total concentration of the protein ($[P_1+P_2]$)⁸. Unfortunately, if the population of the higher energy conformation (P_2) is very low (5%), it may be difficult to kinetically distinguish induced fit versus conformational selection processes. It should be noted that the thermodynamic box as presented is an oversimplification of the binding process, considering that many substates within the conformation ensemble could have some affinity for the ligand.

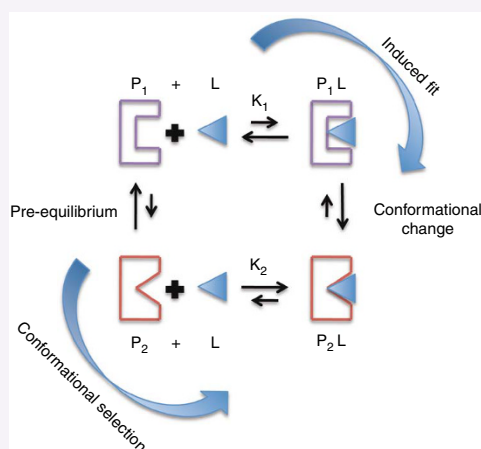


Figure 1 Thermodynamic cycle for molecular recognition processes involving induced fit or conformational selection. In conformational selection, the binding competent conformation (red, P_2) is pre-existing in solution before the addition of ligand (L). The kinetic and thermodynamic rate constants can determine whether conformational selection or induced fit is more likely^{8,99}.

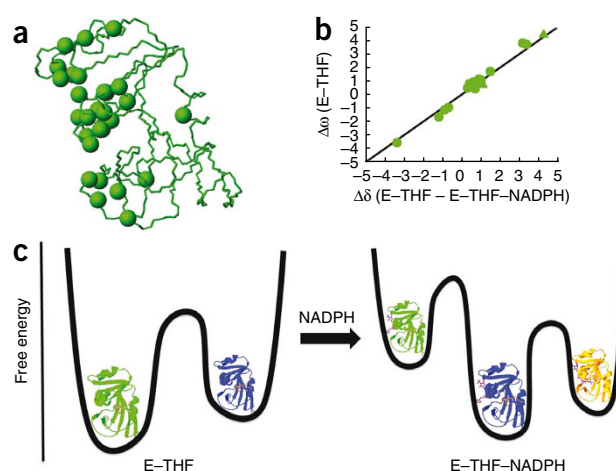
Conformational selection in protein-ligand interactions

The energy landscape theory was first used to describe the dynamic behavior of myoglobin as observed by the elegant flash photolysis experiments of Frauenfelder *et al.*³. Experiments that measured the kinetics of light-activated dissociation of oxygen from heme in myoglobin at physiological temperatures suggested a simple one-step process. However, photodissociation experiments below 200 K demonstrated non-exponential kinetics²³ and were rationalized by suggesting that myoglobin did not have a single structure under these conditions, but instead was composed of many conformational substates, each with slightly different rebinding rates³. Time-resolved X-ray diffraction²⁴ and subnanosecond spectroscopic techniques²⁵ have provided detailed insights into the complex free energy landscape of myoglobin and other heme-containing proteins. These studies lend strong support to the notion that a protein exists not as a single structure, but rather as a statistical ensemble of conformations that can give rise to complex protein kinetics.

Although time-resolved Laue diffraction can provide a wealth of information about conformational heterogeneity, application of the method is restricted to proteins that can be photoactivated. This limits the structural information that can be obtained about the conformational ensembles of other proteins. Classical X-ray crystallography usually reveals only a single protein conformation under a given set of conditions. However, different protein conformations are sometimes observed when the same protein complex is crystallized in other space groups under different conditions²⁶. Alternative conformations for distinct protein molecules in the asymmetric unit of the same crystal may also be observed^{27,28}. In one noteworthy case, the monoclonal antibody SPE7 was crystallized in two different conformations in the absence of antigen. Structural analyses of the free and antigen-bound conformations suggest that each conformation is responsible for binding particular antigens²⁹. Pre-steady state kinetics further support pre-binding conformational isomerism and conformational selection in the function of SPE7 (ref. 29). Similar kinetic results support conformational selection in other antibody-antigen interactions^{7,9,10,30} and in other types of protein interactions^{31–33} (see Box 1 for a kinetic and thermodynamic comparison of conformational selection versus induced fit).

NMR is a powerful technique for studying conformational heterogeneity and the free energy landscape of macromolecules³⁴. NMR provides atomic resolution insights into both protein structure and dynamics over a large range of timescales (ps–s) and, unlike time-resolved techniques that perturb chemical and structural equilibria, directly monitors dynamics under steady state conditions. Importantly, NMR methods developed over the past decade have allowed the structural characterization of weakly populated states (as little as 1% population) in the conformational ensemble that may play a role in the molecular recognition process³⁴. In particular, relaxation dispersion techniques provide kinetic and thermodynamic

Figure 2 Conformational selection in protein-ligand interactions observed by NMR R_2 relaxation dispersion experiments. (a) Locations of conformational exchange are indicated as spheres on the structure of DHFR (Protein Data Bank (PDB) code 1RX5). (b) A linear correlation between $\Delta\omega$ (chemical shift difference between ground state and higher energy conformations) from R_2 relaxation dispersion experiments of the product binary complex of DHFR (enzyme bound with tetrahydrofolate (E–THF)) and $\Delta\delta$ from ground-state chemical shift differences between product binary and ternary (enzyme bound with tetrahydrofolate and NADPH cofactor (E–THF–NADPH)) complexes indicates that the higher energy conformation of the product binary complex is structurally similar to the ground state of the product ternary complex (that is, chemical shifts of the higher energy conformation of the product binary complex are similar to the chemical shifts of the ground-state conformation of the product ternary complex) (data taken from ref. 41). (c) The binding of the NADPH cofactor changes the free energy landscape of the enzyme. Structurally similar conformations are colored alike.



information about exchange between two or more conformational substates on the μ s–ms timescale and also provide information about chemical shifts in the higher energy state that can be used to characterize its structure (see Fig. 2). Advances toward directly incorporating chemical shifts in NMR structure refinement³⁵ and newer R_2 relaxation dispersion experiments that report on internuclear vector orientations in higher energy substates^{36,37} provide structural constraints that may allow for direct characterization of higher energy protein conformations³⁸.

Conformational selection processes are implicated in a number of enzymes, including RNase A³⁹, adenylate kinase^{27,40} and dihydrofolate reductase^{41,42}. The results with adenylate kinase are especially significant since this enzyme has been used as a textbook example for induced fit. NMR analyses of mesophilic (*Escherichia coli*) and thermophilic (*Aquifex aeolicus*) adenylate kinases are consistent with two-state conformational exchange between the open and closed states observed in X-ray crystal structures of free and ligand-bound adenylate kinase, respectively^{27,40}. Chemical shift values from R_2 relaxation dispersion experiments ($\Delta\omega$) obtained during catalytic turnover show a strong linear correlation between the chemical shift differences ($\Delta\delta$) between the open and closed conformational states. Moreover, there is a strong correlation between the lid opening rates (44 s^{-1} and 286 s^{-1} for *E. coli* and *A. aeolicus*, respectively) measured by R_2 relaxation dispersion and catalytic turnover ($k_{\text{cat}} = 30\text{ s}^{-1}$ and 263 s^{-1} for *E. coli* and *A. aeolicus*, respectively)⁴⁰. These results suggest that both adenylate kinases fluctuate between open and closed conformations during catalysis, and that product release depends on the closed-to-open conformational transition. Conformational exchange between open and closed conformations is not solely the function of catalytic turnover considering that similar motions are also observed in the free enzyme²⁷. In the crystal structure of the free *A. aeolicus* enzyme, there are three molecules in the asymmetric unit, each with a slightly different conformation²⁷. These conformations lie along the trajectory between fully open and fully closed conformations²⁷. Moreover, R_2 relaxation dispersion NMR spectroscopy, single-molecule FRET and paramagnetic NMR relaxation experiments are all consistent with the fluctuation of the enzyme into a ‘bound’ conformation in the absence of substrate^{27,43}.

In the case of dihydrofolate reductase (DHFR), the dynamics of five different complexes, representing all the intermediates formed in the catalytic cycle, were measured using NMR relaxation dispersion techniques^{41,42}. The conformational dynamics of DHFR are ligand dependent, in marked contrast to the ligand-independent fluctuations observed in other proteins^{27,44}. The binary complexes, with cofactor

or product bound, both fluctuate into conformations resembling the ternary complex (that is, bound with both cofactor and substrate or product), as suggested by the linear correlation between the dynamic chemical shift differences between lowest and higher energy conformations for the binary complexes ($\Delta\omega_{\text{binary}}$) and the ground-state chemical shift differences between the binary and ternary complexes ($\Delta\delta$ (binary–ternary))⁴¹ (Fig. 2). Single-molecule experiments also support the fluctuation of DHFR into other conformations in the presence of substrate or cofactor that may assist binding of the second ligand⁴⁵. The dynamics observed in complexes of DHFR appear to be functionally relevant in that each complex fluctuates into a conformation resembling the next and/or previous step in the catalytic cycle⁴¹. Thus, population re-distributions, mediated by the interactions between enzyme and ligands, play roles in substrate binding, product release and catalytic turnover. This suggests that the free energy landscape of *E. coli* DHFR is dynamic¹¹, in the sense that ligand binding (or release) can alter the nature of the thermally accessible substates in the conformational ensemble and the kinetic and thermodynamic parameters governing the conformational equilibria^{41,46–48}.

Conformational selection in protein interactions

Studies of enzyme–substrate and enzyme–product complexes demonstrate that proteins can fluctuate into conformations that resemble those of the bound state, even in the absence of ligand. These results and others^{49–54} provide structural support for conformational selection in molecular recognition between proteins and small molecules. Similar experimental support exists for conformational selection mechanisms in protein–protein^{55–60} and protein–nucleic acid interactions^{61–63}.

NMR provides a number of observables, other than relaxation dispersion, that are used to gain insights into the nature of the conformational ensemble. An especially powerful approach is to use NMR observables as restraints in molecular dynamic simulations, or alternatively, NMR observables can be used to bias populations of pre-generated conformations representing potential substates within the conformational ensemble (for more comprehensive discussion of the methodology, see reviews^{64–66} and references therein). In one approach, NMR order parameters and nuclear Overhauser effects (NOEs) were used to characterize the conformational ensembles of ubiquitin⁶⁷ and calmodulin⁵⁵. NOEs are commonly used as restraints in NMR structure determination, while Lipari–Szabo order parameters (S^2)⁶⁸ give an indication of the amplitude of protein motion on a ps–ns timescale. Intriguingly, the calculated conformational ensemble of Ca^{2+} -calmodulin includes structures that are very similar

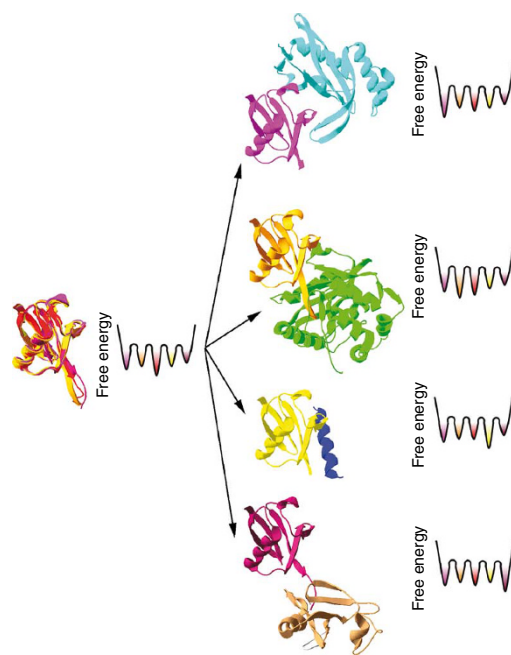
Figure 3 A schematic illustration of molecular recognition processes involving ubiquitin. The NMR-derived conformational ensemble of ubiquitin indicates that all 'bound' conformations exist in the absence of protein binding partners⁶⁶ (left). Although the conformational ensemble encompasses all 46 of the known crystal structures of ubiquitin, only 5 are shown here for clarity (PDB 1F9J, 1S1Q, 1XD3, 2D36 and 2G45). The free energy landscapes are hypothetical considering that the relative population of each conformation in the ensemble and the energy barriers separating the conformations are not known.

to the conformation of calmodulin bound to myosin light chain kinase⁵⁵. Calmodulin consists of N-terminal and C-terminal domains separated by an interdomain linker. The NMR-derived conformational ensemble of Ca^{2+} -calmodulin suggests that the unliganded C-terminal domain adopts a 'bound' conformation much more frequently than does the unliganded N-terminal domain. Based on these results and other biophysical evidence⁵⁵, the authors postulate a molecular recognition process in which myosin light chain kinase first interacts with the C-terminal domain, followed by population shifts within the conformational ensemble such that the 'bound' conformations of the N-terminal domain become more populated⁵⁵. Although a multistep process, the mechanism of binding involves shifting of populations within an ensemble of pre-existing conformations^{4–6,8,11} rather than 'induction' of a new conformation not observed in the free protein. Recent single-molecule atomic force microscopy experiments further support a population shift mechanism for myosin light chain kinase binding to Ca^{2+} -calmodulin⁵⁷.

One shortcoming of the classical Lipari-Szabo NMR order parameters is that they only report on ps–ns timescale protein dynamics and therefore do not give information about dynamic processes on slower timescales that might also be relevant for molecular recognition. Newer approaches utilize structural data from residual dipolar couplings (RDCs)⁶⁵ and/or paramagnetic relaxation enhancement (PRE)⁶⁶ to characterize conformational ensembles on slower timescales. For example, RDCs that report on internuclear vector orientations can be used both in NMR structure determination and to identify protein motions on the ps–ms timescale^{65,69}. Thus, RDC-derived order parameters that are smaller in magnitude than the Lipari-Szabo order parameters suggest additional motions on the ns–ms timescale that are not captured by the classical order parameters. By measuring RDCs resulting from partial alignment in a large number of media, the conformational ensemble of ubiquitin was calculated up to the microsecond timescale⁶⁰. The most striking feature of the conformational ensemble is the presence of 'bound' conformations in the free state of ubiquitin (Fig. 3). In fact, the backbone conformations in all 46 of the known X-ray crystal structures of ubiquitin in complex with various partner proteins are represented within the conformational ensemble, despite the absence of any crystallographic information in ensemble refinement⁶⁰. This gives strong evidence that 'bound' conformations of ubiquitin are present in the free protein, in the absence of protein interaction partners, and that conformational selection-type processes are important for molecular recognition in ubiquitin. However, subsequent conformational changes may be induced, especially in the side chains, after the initial binding interaction⁶⁰. Kinetic studies implicate conformational selection in Alzheimer's A β amyloidosis⁷⁰, which can also be considered a protein-protein recognition event⁷¹.

Conformational selection in RNA and DNA interactions

The principles of energy landscape theory can also be applied to protein–nucleic acid recognition and to the structure and dynamics of RNA and DNA^{63,72}. For example, the specificity of *lac* repressor



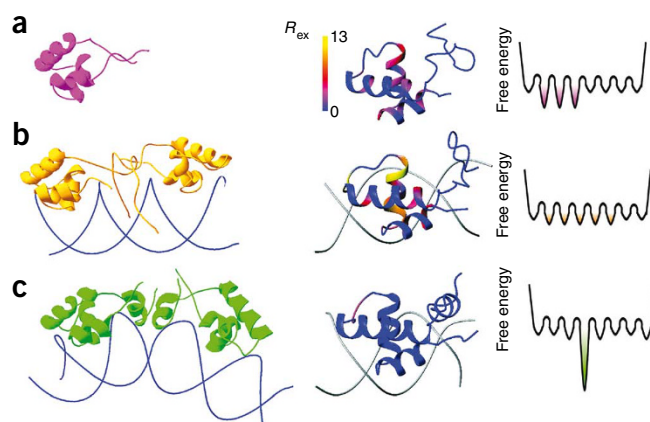
headpiece binding to DNA can be explained by describing differences in the protein free energy landscape when bound to either cognate or noncognate DNA^{61,72}. NMR studies indicate that there are both structural and dynamic differences between noncognate and cognate DNA–*lac* repressor complexes⁶¹ (Fig. 4). Protein motions on the μs – ms timescale that are observed in the DNA-free *lac* repressor headpiece are significantly increased when it binds to nonspecific DNA, especially for residues at the protein–DNA interface⁶¹. However, upon binding the cognate DNA sequence, the μs – ms timescale motions observed by NMR are effectively quenched⁶¹. Many of the residues that display changes in μs – ms timescale dynamics adopt alternative conformations in the NMR-derived structural ensemble of the non-specific complex⁶¹. Together, these results suggest that the *lac* repressor headpiece samples many conformations in the DNA-free form and when bound to nonspecific DNA, but that a single protein conformation is selected when the protein binds to its specific DNA recognition sequence^{61,72}. The molecular recognition mechanism described for the *lac* repressor would allow the protein to bind initially to non-specific DNA and allow for a fast one-dimensional diffusional search for the cognate recognition site^{73,74}, while also providing a mechanism for tight binding with the target sequence.

The structural and dynamic diversity of RNA rivals that of proteins⁶³. Many RNA molecules also exist as conformational ensembles containing interconverting substates, and binding interactions can be rationalized based on population shifts and conformational selection processes⁶³. For example, the RDC-derived dynamics of the transactivation response (TAR) RNA from the human immunodeficiency virus type-1 demonstrate that the RNA samples 'bound' conformations even in the absence of ligand⁷⁵. The motions of the two TAR RNA helices appear to be highly correlated, and they trace out a dynamic trajectory that encompasses the conformations observed in nearly all of the ligand-bound structures⁷⁵. Thus, conformational selection appears to be universal in molecular recognition processes involving biomolecules.

Conformational selection in protein regulation

Conformational selection is closely related to the Monod–Wyman–Changeux (MWC) theory of allostery⁷⁶. The MWC theory also

Figure 4 DNA recognition by the *lac* repressor headpiece. (a–c) Differences in structure (left) and dynamics (middle) between *lac* repressor headpiece in the free state (PDB code 1LQC) (a), *lac* repressor headpiece bound to noncognate DNA (PDB 1OSL) (b) and *lac* repressor headpiece bound to cognate DNA (PDB 1L1M) (c). The middle column shows R_{ex} , the contribution to the NMR R_2 transverse rate constant from μ s–ms time scale conformational fluctuations, mapped onto the structures of the *lac* repressor protein (adapted from ref. 72 with permission from the American Chemical Society). The free energy landscape (shown schematically on the right) is rough, with many interconverting substates in the complex of *lac* repressor headpiece with noncognate DNA, but a single dominant conformation is formed in the complex with cognate DNA.



envisions the presence of two or more protein conformations in solution, each with different binding or functional characteristics. For example, the enzyme aspartate transcarbamoylase is suggested to exist in two conformations: a fully active R state and a less active T state⁷⁷. Although the crystal structures of the R and T states of aspartate transcarbamoylase are well characterized, only recently has it been possible to detect both conformations in solution by NMR and to directly monitor the population redistributions upon the addition of allosteric ligands^{78,79}. Results from the NMR studies are fully consistent with the MWC model.

Population redistributions of active and inactive protein conformations can also explain other regulatory processes⁸⁰. Phosphorylation of the bacterial signaling protein NtrC leads to a large conformational change⁸¹. NMR studies demonstrate that unphosphorylated NtrC accesses a conformation similar to that of phosphorylated NtrC, which suggests that phosphorylation results in a population redistribution between active and inactive conformations that pre-exist in solution⁸¹. Likewise, NMR relaxation dispersion experiments demonstrate that the photoswitch LOV2-J α fluctuates between ‘dark’ inactive and ‘light’ active conformations⁸². Shining light on the protein results in a population redistribution that favors the active conformational substate. Thus, changes in protein populations represent a general mechanism for protein function that interconnects molecular recognition and protein regulation.

Implications and future directions

The studies described above clearly indicate that proteins and RNA can access ‘bound’ conformations even in the absence of a binding partner. However, the mere fact that the ‘bound’ conformation is present in the ensemble of conformations sampled by the free protein does not in itself implicate this conformation in the recognition process. The Dbl homology (DH) domain of the oncoprotein Vav1 provides a striking example of the role of a higher energy conformational substate in biological function⁸³. NMR relaxation dispersion experiments show that the DH domain fluctuates between a ground-state conformation, in which an autoinhibitory helix binds to and blocks the active site, and a weakly populated higher energy conformation in which the autoinhibitory helix is dissociated from the catalytic surface⁸³. Mutations that alter the equilibrium distributions of the two states change the catalytic activity of the DH domain and the rate of phosphorylation of the autoinhibitory helix by the Lck kinase; the catalytic activity and the phosphorylation rate are linearly dependent on the population of the higher energy ‘helix dissociated’ conformation⁸³.

Although in the preceding sections we emphasized the importance of conformational selection mechanisms, it is likely that both conformational selection and induced fit play important roles in molecular recognition. Following initial binding through a conformational selection mechanism, it is probable that further changes to the

protein structure and underlying free energy landscape are required to optimize the intermolecular interactions; such conformational rearrangements constitute an induced fit process. The co-existence of both mechanisms is evident in interactions of the maltose-binding protein. The ligand-free protein fluctuates between a predominantly open form and a minor partially closed species⁴⁹. Maltose could potentially interact with both conformations, but further structural changes would need to take place to form the final ligand-bound, fully closed conformation⁴⁹.

The energy landscape view of protein folding and function has numerous practical consequences in areas such as drug design, protein engineering and molecular evolution. For example, small-molecule inhibitors can be designed against higher energy conformations present in the conformational ensemble to target different conformations of the binding pocket^{84–86}. Along these lines, several molecular docking procedures have previously been designed to account for protein flexibility in molecular recognition^{86–89}. In one procedure, a conformational ensemble is pre-generated using experimental information (for example, different X-ray crystal structures or NMR-derived conformational ensembles) and/or computational data (for example, molecular dynamics simulations, normal mode analyses, principal component analyses); similar conformations are clustered, and binding partners are then docked to representative target conformations⁸⁸. Further energy minimization procedures and/or molecular dynamics simulations can account for additional small-scale motions in the interaction complex. The two stages of the procedure (initial docking of the conformational ensemble, followed by refinement and/or molecular dynamics) essentially represent conformational selection and induced fit processes, respectively. However, a large hurdle in accounting for protein target flexibility in the docking procedure is the increased computation time. The experimental information presented in this review suggests that low-lying conformational substates are primarily responsible for molecular recognition; consequently, biased docking to highly populated conformational clusters (that is, the lowest energy conformational substates) may significantly diminish the computational time. Experimental techniques such as those described here can be used to further decrease the size of the conformational ensemble required for docking.

Nonetheless, applying energy landscape theory to drug design is not straightforward. Not all conformations in the ensemble are likely to be equally ‘druggable’; this reflects key differences in energetics and/or accessibility of the potential binding pocket in different members of the conformational ensemble. In such cases, enhanced exploration of the conformational space of the target through additional computational procedures may be required⁸⁶. An alternative strategy for incorporating the conformational ensemble in drug design is the search for

allosteric inhibitors (or activators) that would alter the distribution of the conformational ensemble. Though considerable progress has been made toward this goal⁹⁰, and allosteric drugs are already in the market or are currently under development⁸⁵, unfortunately, no general strategy is currently available for developing allosteric inhibitors to interact with binding pockets that exist only in a subset of substates in the conformational ensemble. A better understanding of protein dynamics and the role of conformational entropy in determining inhibitor binding affinity is urgently required. The complexity of the interactions that determine the overall binding affinity is evident from recent studies of calmodulin, which show clearly that changes in protein dynamics before and after peptide binding have a significant entropic effect on peptide binding affinity⁹¹. Thus, the notion that higher affinity binding always results in a more stable, rigid complex may not be entirely correct, and attention must also be paid to the nature of the conformational ensemble populated by the protein in the ligand-bound state.

Considerations of conformational heterogeneity and conformational selection could potentially aid greatly in the design of protein function and engineering of enzymes^{92,93}. Despite impressive successes, initial attempts to design artificial enzymes resulted in relatively poor catalysts, compared to naturally occurring enzymes. Similarly, catalytic antibodies raised against transition-state analogs are also inefficient catalysts, in general, compared to their natural enzyme counterparts⁹⁴. This suggests that either we do not have a firm enough understanding of the chemistry and transition-state complexes involved, or that we ignore and do not select for other critical parameters that are essential for efficient enzyme catalysis. In the case of one newly engineered enzyme, *in vitro* evolution was able to select for catalysts with activities similar to those of naturally occurring enzymes⁹². Although this result argues that a combined computational design and directed evolution approach can yield effective biocatalysts, not all mutations introduced by *in vitro* evolution can be readily explained. Current enzyme design efforts tend to consider only the chemical steps in an enzyme's catalytic cycle, overlooking essential steps such as substrate binding, product release and conformational change that could severely limit the catalytic turnover rate in a natural enzyme. Studies of enzymes such as dihydrofolate reductase⁴¹, adenylate kinase²⁷ and RNase A³⁹ suggest that conformational selection-type processes occur throughout the catalytic cycle and can govern steady state turnover⁴¹. Hence, we argue that in the future, consideration of low-lying conformational ensembles and conformational changes (that is, re-distribution of the conformer populations) in parallel with optimization of chemistry, substrate binding and product release will be essential for the design of more effective catalysts that rival naturally occurring enzymes in catalytic activity. Incorporation of such elements into the design strategy could also permit the engineering of regulatory elements that modify newly evolved enzyme activities via remote-site allosteric modulation. In turn, these studies will enhance comprehension of conformational ensembles in ligand interactions, enzyme catalysis and molecular evolution.

Conformational ensembles are also important in the evolution of molecular interactions. The 'bound' conformation observed in free enzymes such as adenylate kinase and dihydrofolate reductase can be regarded as the second most highly populated conformation in solution; other conformations even higher in energy and of much lower population are not detectable by NMR relaxation dispersion techniques. This suggests that the intrinsic fluctuations of a protein (or RNA) are highly 'tuned' and have evolved to be functionally relevant⁸⁴, and that it is the lowest energy subset of conformational states that are most relevant to biological function. In the case of

ubiquitin, the conformational ensemble contains a very large number of different conformational substates; these may have evolved to allow ubiquitin to interact with a diverse range of binding partners, or conversely, protein binding partners may have taken advantage of pre-existing conformational diversity. Both processes may be important in the evolution of molecular recognition. This is similar to a 70-year-old proposal for antigen-antibody interactions that suggested that both conformational and amino acid sequence diversity would allow antibodies to interact with the greatest variety of antigens⁹⁵. Darwinian evolution requires that a function exists to some extent before natural selection^{6,11}, thus protein conformational diversity and functional promiscuity are closely connected and are a potential means of molecular evolution^{96,97}. Conformational selection processes may also be more resilient to evolutionary forces than induced fit-type mechanisms¹¹. In the case of conformational selection, deleterious mutations may significantly alter the free energy landscape, but binding-competent conformers may still be present in the conformational ensemble. This is in contrast to an induced fit-type mechanism where an unfavorable mutation can critically disrupt the step-wise conformational changes required for competent binding¹¹. Except for a few studies of the maturation process of antibody-antigen interactions^{7,29,98}, there is very little information regarding the role of the conformational ensemble in directing molecular evolution. This situation is unfortunate, since a better understanding of the evolutionary aspects of conformational heterogeneity could have an immediate and positive impact on efforts to design and engineer *de novo* proteins with new functions.

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Corrigendum: Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors

Florian Mullershausen, Frédéric Zecri, Cihan Cetin, Andreas Billich, Danilo Guerini & Klaus Seuwen

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In the version of this article initially published, the chemical structures in Fig. 2a and in the graphical abstract were incorrect. The error has been corrected in the HTML and PDF versions of the article.

Erratum: S1P1 signaling just keeps going and going and going...

Stuart Cahalan & Hugh Rosen

Nat. Chem. Biol. 5, 377–378 (2009); published online 15 May 2009; corrected after print 13 November 2009

In the version of this article initially published, the chemical structures for AFD-R, FTY720-P and OC5 were incorrect. The error has been corrected in the HTML and PDF versions of the article.

Erratum: Targeting proteins for degradation

Erin K Schrader, Kristine G Harstad & Andreas Matouschek

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In the version of this article initially published, a positive competing financial interest statement was noted, where none exists. The error has been corrected in the HTML and PDF versions of the article.

Erratum: The role of dynamic conformational ensembles in biomolecular recognition

David D Boehr, Ruth Nussinov & Peter E Wright

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In the version of this article initially published, in Figure 3 the term “free energy” appears with the horizontal axes rather than the vertical axes of the energy diagrams. The error has been corrected in the HTML and PDF versions of the article.