Biophysical Chemistry 186 (2014) 46–54

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Review

Single molecule insights on conformational selection and induced fit mechanism

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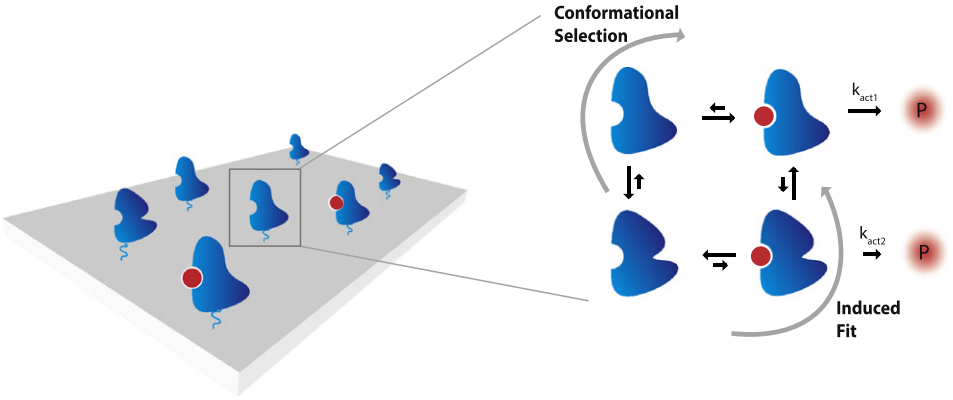
HIGHLIGHTS

- Single molecule studies decipher the mechanism of biomolecular recognition.
- Conformational selection and ligand recognition of proteins and enzymes.
- Allosteric regulation of monomeric enzymes operates via conformational selection.
- Single molecule insights for nano-medicine and drug design.
- Single molecule insights for de novo protein design with tailor made functionalities.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 October 2013
Received in revised form 1 November 2013
Accepted 1 November 2013
Available online 13 November 2013

Keywords:
Conformational selection
Induced fit
Single enzyme
Allosteric regulation
In silico drug design
De novo protein synthesis

ABSTRACT

Biomolecular interactions regulate a plethora of vital cellular processes, including signal transduction, metabolism, catalysis and gene regulation. Regulation is encoded in the molecular properties of the constituent proteins; distinct conformations correspond to different functional outcomes. To describe the molecular basis of this behavior, two main mechanisms have been advanced: 'induced fit' and 'conformational selection'. Our understanding of these models relies primarily on NMR, computational studies and kinetic measurements. These techniques report the average behavior of a large ensemble of unsynchronized molecules, often masking intrinsic dynamic behavior of proteins and biologically significant transient intermediates. Single molecule measurements are emerging as a powerful tool for characterizing protein function. They offer the direct observation and quantification of the activity, abundance and lifetime of multiple states and transient intermediates in the energy landscape, that are typically averaged out in non-synchronized ensemble measurements. Here we survey new insights from single molecule studies that advance our understanding of the molecular mechanisms underlying biomolecular recognition.

Contents

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0301-4622/$ – see front matter
http://dx.doi.org/10.1016/j.bpc.2013.11.003

1. Intro

Proteins are dynamic entities, sampling a plethora of interconverting conformations via structural fluctuations on a broad range of time scales from nanoseconds to minutes [1–3]. This fine-tuned network of motions and its remodeling by regulatory inputs drives biomolecular recognition and thus protein function and regulation. Two opposing mechanisms describing the conformational sampling underlying biomolecular recognition have been proposed: 'induced fit' and 'conformational selection' [4,5]. Induced fit posits that ligand binding remodels the protein landscape, inducing a new conformational state [6,7]. Conformational selection on the other hand proposes that this 'ligand-bound' conformation exists even before the ligand has bound, albeit as a weakly populated state [5,7]. The ligand recognizes and selectively binds this state, shifting the conformational equilibrium to make it the predominant conformation in the ensemble [8–10]. Deciphering the prevailing mechanism requires observations of weakly populated conformational states and conformational heterogeneities within ensembles of proteins.

Our understanding of these mechanisms, until recently, relied primarily on the combined results of spectrometric studies like nuclear magnetic resonance (NMR) and computational studies. NMR studies offer a wealth of information of protein conformational motions [2,3,11–13] but despite great advances [13,14] the observation of transiently formed conformational states [15,16] often remains masked due to averaging over a large ensemble of unsynchronized molecules. Computational studies on the other hand provide unprecedented insights in protein dynamics [17–23] but are limited to submillisecond time scales. The advent of single molecule techniques added new insights in the complex tapestry of protein function, the heterogeneity that is a fundamental feature of protein behavior and regulation. Despite providing limited structural information single molecule studies offered the direct observation of weakly populated states, conformational heterogeneities, transient intermediates as well as the existence of long lived stable conformational states within an ensemble of proteins with significantly different activity [24–26]. Last but not least single molecule studies offer simultaneous observation of both the conformational states of an individual protein and the ligand binding capturing and thus which of the state(s) the ligand recognizes and interacts with. Single molecule measurements thus emerge as an instrumental tool, complementing computational and spectrometric studies in attaining a comprehensive description of protein conformational and functional dynamics and in deciphering the mechanism underlying biomolecular recognition.

In this review we will firstly introduce shortly the prevailing hypothesis underlying biomolecular recognition and the limitation of current averaging techniques. We will then focus on the insights acquired by recent single molecule data in deciphering the controversy between conformational selection or induced fit as the mechanism underlying enzymatic function and regulation. Comparison of the two models has been extensively discussed earlier [5–7,10,20,27,28] and will not be discussed further here. The pivotal role of NMR [2,3,11–13] and computational studies [17–23] on the mechanism underlying biomolecular recognition has been extensively reviewed elsewhere but also by GM Clore and JA McCammon in this special issue.

2. Current models underlying biomolecular recognition and state of the art

Tightly regulated biomolecular recognition is central for controlling plethora of vital cellular processes from signaling to metabolism and gene regulation as it encompasses protein-ligand and substrate, protein–protein interactions and allostery. Comprehensive description of biomolecular recognition is critical for understanding these processes and the design of novel therapeutics for controlling them. The two prevailing hypothesis underlying allostery for oligomeric proteins proposed in the 1960s, are the MWC (Monod Wyman Changeux) model [29] and the KNF (Koshland Nemethy Filmer) model [30,31] (Fig. 1). Both models describe the allosteric effect as a binding event at one partner of the oligomeric protein causing a conformational change affecting the activity of the rest of the partners. The MWC model proposes signaling proteins to exist as oligomers that preexist in equilibrium between two conformational states. Ligand interactions would shift the conformational equilibrium leading to the allosteric activation [32,33]. The KNF model on the other hand proposes ligand-binding interactions to drive the protein towards a new conformation that is complementary to the ligand [30,31].

The modern view of molecular recognition has evolved to the "conformational selection" and the "induced fit" mechanisms extending to account for monomeric proteins and to encompass their inherent dynamics (Fig. 1C). While the term conformational selection was first used in the 1980s [34,35] it is only the last decade it has gained significant ground as the prevailing mechanism underlying biomolecular recognition [5,7,36,37] by the insightful contributions of R. Nussinov, also discussed in this special issue. Conformational selection (or different incarnations of it: population selection, population-shift, selected fit, and stabilization of conformational ensembles) has been initially rationalized to explain protein ligand recognition [36,38,39] and folding of disordered structures [37]. Later studies extended CS to explain dynamics along the reaction coordinate for a plethora of monomeric non regulated metabolic [40–44], or signaling [27], enzymes and the dynamics and mechanisms of coupled binding and folding reactions [36,45]. Recent pivotal single molecule and NMR studies showed monomeric allosterically regulated enzymes to operate via mechanism similar to conformational selection [46–48]. Although we emphasized the importance of conformational selection mechanisms, it is well understood that conformational selection and induced fit constitute the two extreme ends of the molecular recognition mechanism. In fact often both conformational selection and induced fit or induced fit alone play important roles in molecular recognition [5,7,49]. Deciphering the prevalence of any of the two mechanisms to date is often based on the quantification of the kinetic rate constants describing the thermodynamic cycle for varying ligand concentrations (see Fig. 1C) [28,49]. The critical role of the kinetics rates [39] in sorting out the prevailing mechanism is described by E Di Cera in this special issue.

The key advancement in the field of protein conformational dynamics and biomolecular recognition has come from the provision of a new

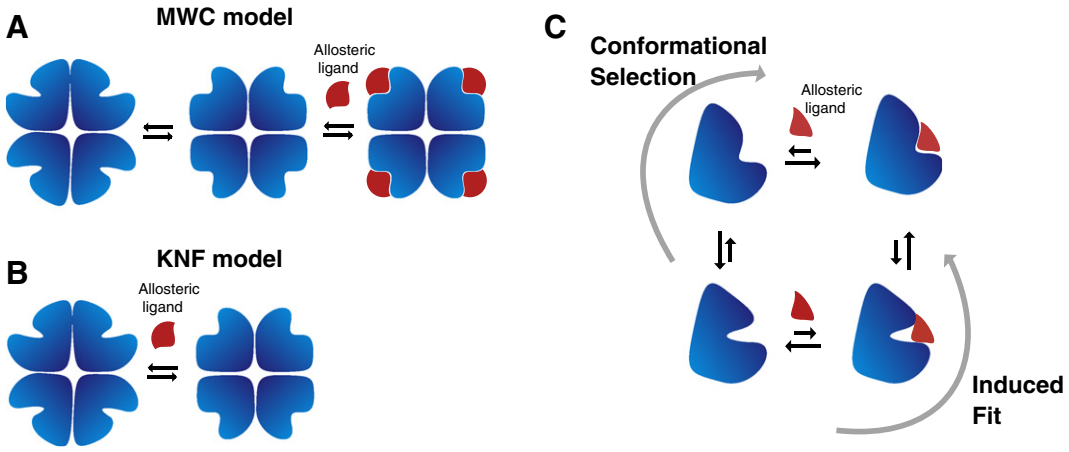

Fig. 1. Schematic simplification of MWC, KNF conformational selection and induced models for biomolecular recognition. MWC and KNF models describe the allosteric effect as a binding of the ligand on one site to cause a conformational change on the other site of the oligomer. A. The MWC model proposes allosteric proteins to exist as oligomers with at least one axis of symmetry. These oligomers interconvert between two (at least) major conformational states. Ligand interactions are proposed to shift the conformational equilibrium by selectively binding and stabilizing one of the conformational states. B. KNF model proposes ligands to bind to one protein binding site sequentially inducing a new conformational state to the rest of the monomers. C. Schematic description of the thermodynamic cycle for molecular recognition processes involving induced fit or conformational selection. These models have evolved from the MWC and the KNF model respectfully and are extended to account for the conformational dynamics of monomeric proteins and biomolecular recognition in general. The key difference between the two extreme models is proposed to lie on the kinetic and thermodynamic constant and mechanistic sequence of events. In the induced-fit model the bound protein conformation forms only after interaction with a binding partner whereas in the conformational selection model, the protein pre-exists in an ensemble of conformations that ligand binding redistributes.

conceptual framework, the funneled energy landscape [1,2,8,50,51] that defines the abundance, relative probabilities and the energy barriers of the plethora of interconverting conformational states sampled by the protein. Regulatory cofactors are proposed to recognize weakly populated states and shift the conformational equilibrium towards them [1,2,8,36,37] or alter protein dynamics [52,53]. Despite great strides especially in NMR techniques [13,14] however, it remains challenging to capture rarely sampled conformational states because they are often masked due to ensemble averaging. Single molecule studies on the other hand are uniquely suited for the direct observation and quantification of both rarely sampled states and intrasample heterogeneities. In fact one paramount finding of single molecule studies was the direct observation that enzymes within a population exist in stable states that exhibit different average activity, referred to as static heterogeneities [24–26,54,55]. These states were anticipated to originate from rare transitions between trapped native states in the energy landscape [26,54,56,57]. In addition to being crucial for a mechanism based on conformational selection the existence of such states would have profound implication for processes catalyzed by single, or low copy numbers, of enzymes such as gene expression [58]. Recent studies illustrated cell phenotype switching to occur due to a single molecule event [59–61]. Heterogeneities in transcription factor binding to the single gene may contribute to the heterogeneities in messenger RNA and thus protein production, consequently resulting in cells having the same genes but different phenotypes [60] and varying persistence to antibiotics [62,63]. Single molecule measurements have also revealed previously masked conformational dynamics, the existence of heterogeneities in enzymatic function [24,46,55,64,65] as well as transient intermediates and stochastic events [58,66] that underlie protein fidelity [15,16] (see Fig. 2A). Combined with their unique capability to measure kinetics when the spontaneous nature of the fluctuations impedes, the often perturbing, synchronization renders single molecule studies an indispensable tool in deciphering the mechanisms underlying function and regulation of proteins.

3. Single molecule approaches to deciphering biomolecular recognition

The two principal approaches employed to study biomolecular recognition at the single molecule level are single molecule fluoresence resonance energy transfer (smFRET) and single molecule functional studies. FRET studies serve as a "spectroscopic ruler" reporting distance information between donor and acceptor fluorophores placed within a certain proximity [67–70]. smFRET has offered the direct observation, the extent and the lifetime characterization of conformational motions associated with the catalysis step and substrate mediated interconversion between states with different dynamics that are often masked in non-synchronized measurements [71] (see Fig. 2A). Single molecule functional studies [24,46,55,64,65] on the other hand allowed the direct observation of activity heterogeneities both in an individual enzyme and in between enzymes of a population. They thus may offer the direct quantification of the existence lifetime and inherent activities of functional states and their dependence on substrate and regulatory inputs [46] that are averaged out in conventional kinetics (Fig. 2B). Below we will discuss the insights gained by single molecule studies on deciphering the mechanism underlying ligand and substrate recognition, as well as allosteric regulation of biomolecules.

3.1. Mechanism of enzyme substrate recognition

The conformational selection and population shift model were initially established for the folding and ligand binding of proteins [4,36,37]. The same mechanism was later successfully employed to account for substrate mediated conformational redistributions along the reaction coordinate of monomeric enzymes [27,40–44,72]. Single molecule studies play an instrumental role in this transition deciphering disputed mechanism and augmenting the findings of NMR and computational studies.

A striking example where single molecule kinetics deciphered the mechanism underlying catalysis is the lysozyme. Within each turnover cycle lysozyme undergoes a hinge conformation motion which was anticipated to be substrate induced [73]. To investigate the mechanism underlying substrate recognition and activity regulation Choi et al. tethered individual lysozymes on carbon nanotube field-effect transistors. During substrate processing, lysozyme undergoes an 8 Å, hinge-like mechanical motion generating changes in the electrostatic potential that can be converted into dynamically changing electron fluxes. The single molecule readout of lysozyme revealed the existence of an equilibrium distribution of 3 functional states; a fast fluctuating non-productive state, a slow fluctuating processing state, and a closed inactive state [74,75]. These measurements furthermore allowed the quantification of their activities, lifetimes, equilibrium distribution and their

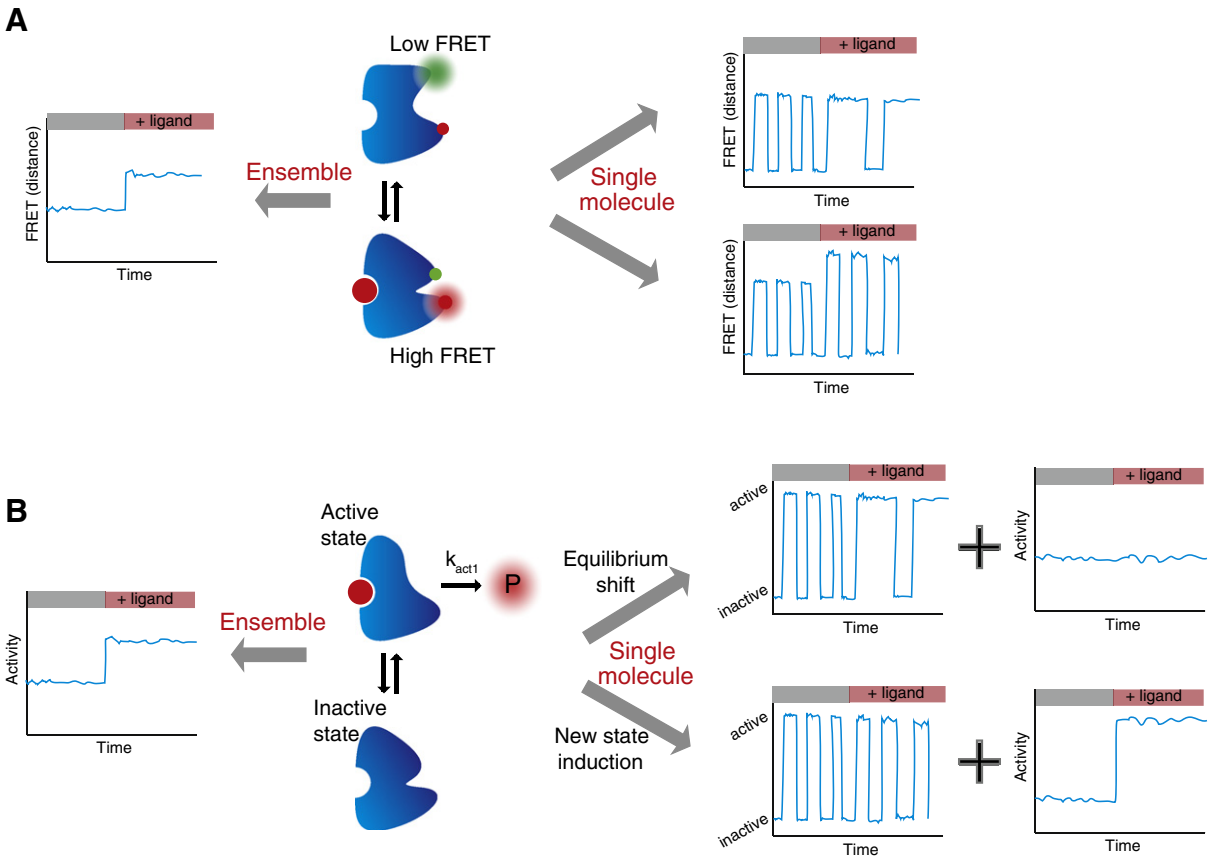

Fig. 2. Simplified representation highlighting few of the dynamic characteristics underlying biomolecular recognition that are masked in ensemble measurements but are conveniently revealed by single molecule measurements A. FRET serves as a spectrometric ruler reporting distance information. Ensemble measurements will report the ligand dependent average change in distance for a large ensemble of biomolecules, that may easily be interpreted as induced fit (left). smFRET offers the direct observation of the life times of each state and can deconvolute whether ligand interactions shift a preexisting conformational equilibrium by e.g. stabilizing a state, or e.g. induce a new conformational state or any variation of thermodynamic and kinetic constants that determine the mechanism. B. Bulk measurements in solution will report the ligand dependent average change in activity for an allosteric regulated enzyme having an active and inactive state. Single molecule kinetic studies may record both the lifetime of each functional state and its intrinsic activity. They may thus deconvolute whether the average increase in activity primarily originates from shift in the equilibrium and increased time spent in active state without changing the activity of the active state (top right), or via the induction of a new activity state (bottom right). Note that multiple permutations of the simplified scenarios depicted in A and B may occur and delineated by single molecule studies.

dependence on regulatory inputs. Changes in the type of substrate, or pH, were found to primarily redistribute the time spent in these functional states (up to ~6-fold) but only moderately alter the "processing rate" of lysozyme [74]. These studies give strong support to the notion that lysozyme operates primarily via a mechanism based on selection rather than induced fit.

Another critical finding of these studies was the direct observation that lysozyme samples a small number of functional states, only one of which is catalytically active, contrasting the majority of single molecule kinetics reporting a wide distribution of activity states [24,55,64,65,76,77]. The single active state may possibly originate from optimized active site organization and dynamics of the conformational ensemble toward what is critical for barrier crossing and catalysis [5,10,40,78,79]. Evolution might have encoded in the structure and energy landscape of lysozyme the optimized pathway for catalysis. Substrate binding appears to remodel the landscape and stabilize the "processing state" redistributing the ensemble towards that state without changing the transition energies for catalysis to occur.

A seminal advantage of single molecule techniques is the possibility of simultaneous observations of both the conformational substates of an individual protein and the ligand, allowing one in turn to capture which of the proteins states are recognized by the ligand. Such observations are critical for delineating the mechanism of biomolecular recognition but remain masked in conventional unsynchronized measurements. The group of Kim et al. used the combined readout of two and three color SmFRET to decipher the highly disputed mechanism [5,80,81] underlying the molecular recognition of the maltose binding protein (MBP) [82]. Labeling the protein with a FRET pair (Cy3, Cy5) allowed the observation of the existence of a conformational equilibrium between an open and a closed state for an individual MBP. Using a fluorescent labeled ligand (Cy7) permitted for the first time to capture in real time which state(s) the ligand recognizes and selectively interacts with. Their findings revealed 80% of the ligand binding and dissociation events to take place in the open protein state. The authors proposed MBP recognition of maltose to operate via an extended induced fit model, in which the protein exists in a conformational equilibrium where the ligand selective binding induces a structural transition to the ligand bound closed form. Their findings signify the importance of conformation equilibrium that ligand selectively recognizes and binds inducing a conformational adjustment and highlight the importance of using single molecule readout to delineate biomolecular recognition mechanisms.

An especially interesting enzyme is adenylate kinase since it has been used as a textbook example of the induced fit mechanism [83]. The enzyme has two "substrate lids" that within the catalytic cycle undergo a transition from the open to the close conformations. Labeling of the substrate lids with FRET pairs by the groups of D Kern and H Yang allowed the first direct observation of lid opening and closing events, and quantified their lifetimes and extent of motion both in the presence and absence of a substrate [84,85]. Adenylate kinase was found to preexist in equilibrium between the open and closed states. This equilibrium was redistributed in the presence of a substrate pointing towards a mechanism based on conformation selection and confirming the NMR and simulation studies [84]. These findings

furthermore showed that earlier ensemble FRET measurements reporting substrate induced reduction in the average distance [71] originated from averaging this equilibrium shift, highlighting the importance of single molecule measurements (see also Fig. 2A).

Increasing evidence by single molecule studies further support the redistribution of conformational equilibrium as the mechanism underlying biomolecular recognitions of multiple proteins. The group of Kapanidis et al. using smFRET identified the existence of an intermediate state and that conformational redistribution towards that state underlies fidelity decisions in DNA polymerase [16] confirming earlier findings [15]. Similarly smFRET measurements revealed the existence of a substrate dependent conformational equilibrium for dihydropholate reductase [86] suggesting a convolution of conformational selection and induced fit mechanisms. Studies on the Leucine transporter [70] and the DNA isomerising protein Z Binding protein [87] showed a ligand dependent conformational redistribution signifying the importance of conformational selection mechanism to drive substrate recognition of monomeric enzymes.

3.2. Allosteric regulation of monomeric enzymes

While the mechanism underlying allosteric regulation of mulimeric proteins are in general well understood and proposed to operate via a mechanism based on conformational selection [32,82], our understanding of the mechanism underlying allosteric regulation of monomeric enzyme remains in its infancy [33]. It is in general accepted that the binding of an allosteric effector can result in the redistribution of protein conformational ensembles along the regulatory coordinate [8–10,88,89]. Regulated enzymes however in addition to the regulatory coordinate have a reaction coordinate and a transition state to overcome for product to be formed [78,79,90]. Current understanding on monomeric enzyme regulation primarily relies on ensemble measurements that correlate redistributions of conformational equilibrium to changes in the average macroscopic activity of an enzyme. These studies intuitively assume that each of the distinct major conformational states has a well-defined activity. Ensemble kinetics, however, cannot directly measure the inherent activity of each conformational state because they report the time-average macroscopic activity of an ensemble of enzymes (see Fig. 2B). Thus, the only way to decipher whether regulatory cofactors (a) redistribute a preexisting conformational equilibrium without changing the inherent activity of each major conformational state, (b) induce a new conformational state with its corresponding inherent activity, or (c) operate via a convolution of both mechanisms a and b, is single molecule measurements.

To study the mechanism underlying the regulation of monomeric enzymes we recently examined for the first time the regulation of enzymatic catalysis at the single molecule level [46]. To this we used lipases, the workhorse enzymes catalyzing the enantioselective hydrolysis of esters in solution [91–94]. A representative model lipase is the monomeric metabolic lipase from *Thermomyces lanuginosus* that has an active site covered by an oligopeptide, the lid. Lid dynamics of TLL are not taking place along the reaction coordinate, as recently shown for other enzymes [40,84]; in the open state, the enzyme's active site is substrate accessible, and multiple turnovers may occur [89]. To monitor the lipase activity we employed the prefluorescent substrate of carboxyfluorescein diacetate, which upon hydrolysis generates the highly fluorescent product carboxy-fluorescein. Each enzymatic turnover produces a burst of florescence before the product diffuses away from the confocal volume allowing the observation of the stochastic individual catalytic events [65,77]. The continuous replenishment of fluorescent product molecules circumvent the limited observation window, due to photobleaching, of most single molecule assays and enables observations over multiple time scales (10^{-3}–10^{2} s or ~10^{3}–10^{4} turnovers per enzyme) that are critical to reliably interpret enzymatic function. Measurements and statistical analysis of stochastic turnovers revealed the existence and quantified the equilibrium distribution and interconversion rates, of two major functional states: a highly active and a practically inactive one, that correlated to the enzyme's active and inactive conformational states. To dissect the mechanism underlying allosteric regulation we tethered the lipase on surface immobilized liposomes [95–98] and titrated its proximity to its allosteric effector, the lipid membranes. We found that regulatory interactions primarily redistribute the probability of TLL to reside on the active states, but does not introduce a new functional state with altered activity (Fig. 3). We thus introduced "functional selection" instead of conformational selection arguing in addition that for activity regulation, function is a more important observable compared to structure. These studies provide new insights compared to earlier ensemble studies proposing that enzymes with lid must operate in an induced fit mechanism [99] and point towards a mechanism based on selection in agreement with NMR studies [47,48]. They furthermore extend beyond the reaction coordinate allowing the direct measurement of changes in a free energy landscape upon allosteric regulation of enzymatic activity. Besides calculating energy barriers along the reaction coordinate [40,84], they allowed the quantification of energy barriers along the regulatory coordinate [40,70,89]. Regulatory interactions were found to equally stabilize the highly active functional state and the reaction transition energy indicating TLL to operate via a mechanism akin to "conformational selection".

A second paramount finding of these single molecule results was the existence of two, rather than a distribution of, functional states that correlated with the major conformational states of TLL. Current studies on enzymatic activity regulation, based primarily on structural evidence and ensemble kinetics, intuitively assume each of the enzymatic major conformational states to have a well-defined activity. This is indeed true for the majority of ion channels [100] where the open conformation has a well-defined ion flux rate. To date however the vast majority of single molecule activity measurements on non regulated enzymes provide data and interpretation thereof supporting the existence of a wide distribution of functional states [24,46,55,64,65]. Interestingly, even though the enzymatic behavior of most single enzymes could be interpreted on the basis of a simple, often two state, model [24,46,101–103], the authors employed more complex multistate models to explain enzymatic behavior. The putative absence of discrete functional states raises concerns on the intuitive, albeit non-validated, assumption of a one to one correspondence between conformational and functional states, which is critical for enzymatic allosteric regulation to operate via a selection mechanism. The direct observation of discrete functional states does not any longer preclude selection as a regulatory mechanism, and furthermore supports the simplification of two state models employed by ensemble techniques such as NMR and theoretical calculations [79,101] to describe enzymatic behavior.

What is the origin and implications of a low number of discrete functional states [46,57,74,75] observed for multiple lipases, reductase and lysozyme? The energetically favored structures of a given protein, the amplitudes and frequencies of sampling them are encoded in its sequence and are subject to evolutionary pressures [104]. Evolution may thus have optimized the tight control of enzymatic function for proficient regulation by discarding unneeded functional states, maintaining few or two of them with large activity differences. The existence of a few functional states does not avert the dynamic nature of proteins. In fact regulated proteins often oscillate between active and inactive conformational states and most ion channels exist in equilibrium between an active and inactive conformational and functional states [100]. The high activity state(s) of enzymes might stem from optimized conformational coordinate and dynamics toward what is important for barrier crossing and catalysis [5,10,40,78,79] while the practically inactive states would stem from any improper active site organization or dynamics relevant for catalysis. This phenotype may indeed minimize conformational sampling in the enzyme's landscape and form the basis of an efficient intramolecular binary switch [105] critical for the proficient regulation of metabolic and signaling output [106]. Both the activity ratio and the equilibrium distribution between these functional

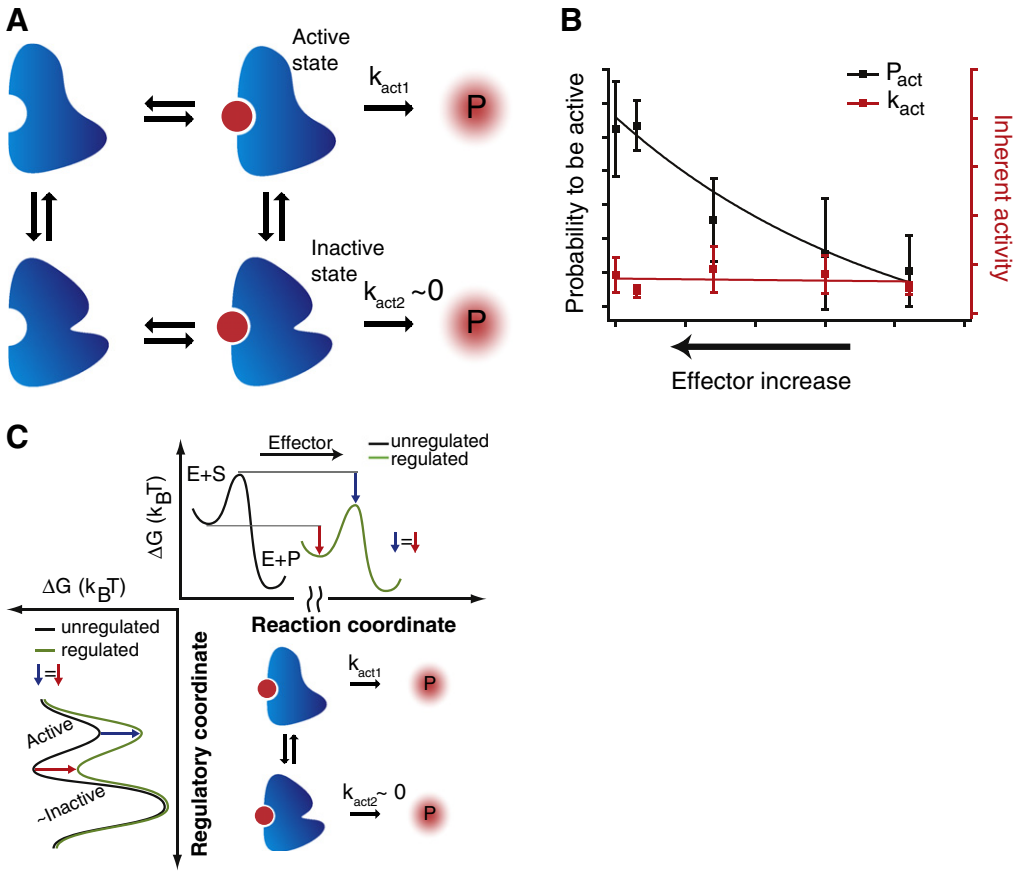

Fig. 3. Allosteric regulation of monomeric enzymes. A. Allosterically regulated enzymes have a regulatory coordinate and reaction a coordinate. Allosteric regulation of enzymes may operate via changes in one or both of a) the equilibrium between the two states, and b) the inherent activity of the state. B. First single molecule measurements on allosterically regulated lipase enzyme deconvolve the parameters underlying allosteric activity regulation. The lipase preexists in equilibrium between an active and inactive functional state. Controlling its access to the effector primarily shifts the equilibrium increasing the probability to reside in the active functional state, but does not induce a new functional state. C. Quantifications of free energy landscape remodeling for both the regulatory coordinate (vertical axis) and the reaction coordinate (horizontal axis) of the lipase upon regulation as revealed by single molecule studies. Regulatory interactions equally stabilize the highly active functional state and the transition energy of the reaction. Allosteric regulation operates via a mechanism based on functional selection. Adapted with permission from [46]. Copyright 2013 American Chemical Society.

states may differ between enzymes with enzymes optimized to work close to the diffusion limit to have minimized conformational sampling. Regulatory interactions appear to shift the population distribution without altering the transition energy barriers reactants have to pass along the reaction coordinate and thus the inherent activity of each state.

3.3. Allosteric regulation of signaling proteins

A tightly controlled regulation of signaling proteins is critical for multiple cellular processes and single molecule investigation on signaling proteins is slowly beginning to emerge. An especially significant class of proteins is the transmembrane G-protein-coupled receptors (GPCRs) that are responsible for the majority of transmembrane signal transduction in response to hormones and neurotransmitters and constitute the largest drug targets. Despite great strides in characterizing signaling receptors the path to comprehensively understanding the full spectrum of conformational motions and their dependence on regulatory inputs remains challenging [33,107,108] and mechanism reminiscent of both conformational selection [109] and induced fit [110] have been proposed. The first single molecule characterization of β_2AR was recently achieved by the use of antibrownian electrokinetic trapping methods (ABEL trap) [111]. This methodology allowed the trapping of β_2AR proteins solubilised in detergents for a few hundreds of milliseconds. Employing an environmentally sensitive chromophore coupled in the transmembrane helix 6 of β_2AR that is sensitive to ligand binding, the group of W.E. Moerner identified β_2AR to sample a spectrum of conformational states. Agonist binding was found to alter the lifetimes of these states and to cause a clear shift in their equilibrium distribution, indicative of a mechanism reminiscent to conformational selection.

Despite the immense methodological advance of these studies several challenges lie ahead in understanding the fundamental details underlying the allostery of GPCRS. GPCRs are inherently dynamic and their conformational sampling is proposed to depend not only on the bound ligand but also on the associated signaling proteins, and the membrane environment [107]. Reconstituting them in surface tethered membrane systems [112], liposomes or nanodiscs, may provide the biocompatible membrane environment and extension of the observation window required for capturing the full spectra of conformational dynamics and its conditioning by ligand and signaling protein presence. The recent advances in single molecule techniques constitute them as central tools in deciphering whether additional signaling proteins operate via a mechanism based on conformational selection or induced fit.

3.4. Mechanism of RNA molecular recognition

The structural and dynamic diversity of RNA rivals that of proteins and the principles of energy landscape theory can also be applied to its structure and dynamics. RNA recognition had been widely assumed to primarily operate via induced fit mechanisms [113,114]. Careful investigations and reconsiderations driven primarily by smFRET studies however suggests that the induced fit mechanism is not the panacea describing the function of ribonucleic acids. Riboswitches is such an example where both mechanisms of conformational selection and induced fit or only induced fit were used to explain the experimental

observations [115]. To decipher the mechanism of a pseudoknot formation that drives ligand recognition of *Streptococcus pneumoniae* R6 preQ1 riboswitch, Soulièrea et al. employed smFRET measurements [116]. Using three distinctly fluorescently labeled RNA constructs allowed the authors to obtain multiple structural perspectives in smFRET and identify the role of ligand or Mg^{2+} binding on the riboswitch dynamics. Their findings revealed the riboswitch to spontaneously achieve a pseudoknot formation that is intrinsically unstable. Ligand binding stabilizes the pseudoknot formation by ~10× fold, shifting the existing equilibrium towards that state in accordance with a mechanism based on conformational selection. The authors furthermore highlighted that the precise relationship between structure, dynamics and function would be greatly aided by measurements where ligand binding and the aptamer fold could be measured simultaneously. To date this can only be achieved by multicolor smFRET studies. In agreement with these studies recent findings on ribosomal subunit L7A [118] initiation Factor 3 [119] demonstrate that the RNA samples 'bound' conformations even in the absence of ligand and equilibrium distribution is shifted in response to specific external stimuli. Though much remains to be learnt for RNA interaction, the emerging single molecule insights support the generic nature of conformational selection mechanism to underlie the function of RNA function and recognition [117,120,121]. Thus the existence of conformational and functional equilibrium encoded in the structure and dynamics of biomolecules that are redistributed in the presence of regulatory inputs, emerges as a unifying concept generically underlying biomolecular recognition of multiple types of biomolecules.

4. Conclusions and future perspectives

Great advances have been made in characterizing enzyme dynamics, function as well as the introduction of protein landscape and its remodeling by regulatory inputs. To date however a full mechanistic understanding and harnessing of these principles for in silico drug design of novel pharmaceutics and the denovo design of biocatalysts with tailor made functionalities remains in its infancy. Current drug design techniques often assume a unique and invariant protein binding site and provide inhibitors that are pre-shaped and conformationally constrained to a fixed geometry of protein structure. Advances on single molecule studies NMR and computational studies revealed the existence of multiple protein conformational states associated with the recognition and the catalysis step in the free energy landscape [5,8,9,26]. Especially significant is the single molecule observations of heterogeneities in between enzymes of a population. These heterogeneities are anticipated to originate from rare transitions between long lived [25,26,56] conformations states (with life times of minutes or longer) in the free energy landscape and they are expected to have mechanistic and biological significance since evolutionary pressure is required to evolve and maintain them [104,122]. Indeed the existence of subpopulations of proteins with heterogeneous function is correlated to bacterial persistence to antibiotics [63]. They are also expected to have a dominant effect on stochastic cellular processes catalyzed by single, or low protein copy numbers of, proteins such as gene expression [58], threshold crossing for downstream cascade initiation [123] localized signaling and even phenotype switching [59,60]. Both the time dependent conformational fluctuations and the existence of long lived conformational heterogeneities are anticipated to have subtle and yet significant differences in the energetics of the binding pockets that underlie ligand recognition and thus protein function. Integrating these heterogeneities in the drug design process combined with recent great strides on identification of protein hotspots may contribute to the in silico design of small molecules able to: either target multiple protein conformational states, or; target multiple allosteric binding sites, or; select and tightly bind to one conformational state shifting the distribution of the conformational ensemble [9,124–126], all of which are critical for the development of novel therapeutic interventions.

The rational design of enzymes has tremendous practical potential for developing novel biocatalysts with tailor made functionalities but presents a daunting task and is a salient test of our understanding of protein behavior. A breakthrough in this field came recently with the design of enzymes with functions not catalyzed by naturally occurring biocatalysts [127,128]. Despite the immense methodological advance however there is still a significant gap between the activities of the artificial enzymes as compared to their naturally occurring counterparts [127]. Narrowing this gap is a formidable challenge and may require understandings that extend beyond the classical protein picture. Combining the readout of single molecule functional and structural measurements with NMR, free energy calculation and simulations offer profound insights on protein conformational ensembles, transition energy barriers and how they are remodeled by ligand/regulatory inputs. Implementation of conformational heterogeneity and conformational selection in the design process could profoundly contribute to the "de novo" design of protein function and engineering of enzymes with tailor made functionalities.

Despite the immense methodological advance the path to understanding protein function in vivo has been arduous and there is still a vast difference between the in vitro single molecular studies and the cellular level interactions. This difference lies primarily on the interactions spectra differences (interactions with multiple small molecules, protein partners and membranes, etc.) and time window differences (protein functional states are mostly found to last ms to sec whereas cellular processes may persist substantially longer). Bridging these requires powerful new methods combining single molecule readout native in vivo like conditions and parallel screening of biomolecular interactions. The development of high-density microarrays of biomimetic scaffolds, single liposomes [95–98] or nanodiscs [129,130], for single molecule studies could be a novel way to implement the simultaneous screening of biochemical properties, molecular function or protein–protein interaction. The combination of membrane heterogeneities [95,97,131,132] and protein or small molecule partner concentration may create distinct combinatorial permutations of regulatory inputs that may be screened with a single molecule readout. The massive parallel readout (10^3–10^4 liposomes per frame) of such arrays liposomes [95–98,131] could allow the direct observation, activity quantification, abundance and redistribution by regulatory inputs of multiple protein native states.

A comprehensive description of how proteins operate in real time requires connection between different timescales and complementary interdisciplinary approaches. Computational studies have the unbeatable edge in describing fully the dynamics of protein in the regime of picoseconds to microseconds but only recently attained milliseconds resolutions [133] approaching time scales that are relevant for biology. Spectrometric techniques such as NMR are especially powerful tools for studying protein conformational dynamics, catalysis and regulation in multiple time scales (picosecond to seconds) [2,3,40,53,84] but are limited to monitoring the ensemble average property of a population of biomolecules. Single molecule fluorescent techniques provide direct observation of dynamic and functional heterogeneities unattainable with any other method and occurring in the biological relevant regime of millisecond to minutes, but offer limited structural insights. Our ability to develop novel pharmaceutics, reengineer and tightly regulate protein function will rely both on progress in these fields and in integrating their readouts. A holistic understanding of the choreography of enzyme dynamics and their dependence on biomolecular regulatory interactions that underlie and tightly control cellular functions may thus begin to emerge.

Acknowledgments

This work was supported by the UNIK research initiative of the Danish Ministry of Science, Technology and Innovation through the "Center for Synthetic Biology" at the University of Copenhagen, and the Lundbeck Foundation Center of Excellence "Biomembranes in

Nanomedicine" and the UCPH Excellence Programme for Interdisciplinary Research' provided by University of Copenhagen.

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