ACCOUNTS
of chemical research

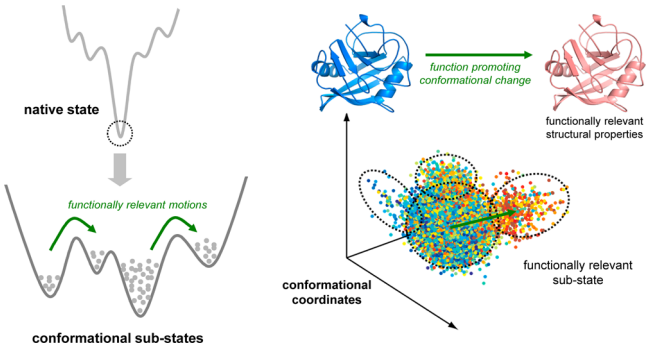
Protein Conformational Populations and Functionally Relevant Substates

ARVIND RAMANATHAN,[†] ANDREJ SAVOL,[‡,§] VIRGINIA BURGER,[‡,§] CHAKRA S. CHENNUBHOTLA,[*,§] AND PRATUL K. AGARWAL[*,⊥,∥]

[†]*Computational Science and Engineering Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States,* [‡]*Joint Carnegie Mellon University—University of Pittsburgh Ph.D Program in Computational Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, United States,* [§]*Department of Computational and Systems Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, United States,* [⊥]*Annavitas Biosciences, 2519 Caspian Drive, Knoxville, Tennessee 37932, United States, and* [∥]*Computational Biology Institute, and Computer Science and Mathematics Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States*

RECEIVED ON APRIL 9, 2013

CONSPECTUS



Functioning proteins do not remain fixed in a unique structure, but instead they sample a range of conformations facilitated by motions within the protein. Even in the native state, a protein exists as a collection of interconverting conformations driven by thermodynamic fluctuations. Motions on the fast time scale allow a protein to sample conformations in the nearby area of its conformational landscape, while motions on slower time scales give it access to conformations in distal areas of the landscape.

Emerging evidence indicates that protein landscapes contain conformational substates with dynamic and structural features that support the designated function of the protein. Nuclear magnetic resonance (NMR) experiments provide information about conformational ensembles of proteins. X-ray crystallography allows researchers to identify the most populated states along the landscape, and computational simulations give atom-level information about the conformational substates of different proteins. This ability to characterize and obtain quantitative information about the conformational substates and the populations of proteins within them is allowing researchers to better understand the relationship between protein structure and dynamics and the mechanisms of protein function.

In this Account, we discuss recent developments and challenges in the characterization of functionally relevant conformational populations and substates of proteins. In some enzymes, the sampling of functionally relevant conformational substates is connected to promoting the overall mechanism of catalysis. For example, the conformational landscape of the enzyme dihydrofolate reductase has multiple substates, which facilitate the binding and the release of the cofactor and substrate and catalyze the hydride transfer. For the enzyme cyclophilin A, computational simulations reveal that the long time scale conformational fluctuations enable the enzyme to access conformational substates that allow it to attain the transition state, therefore promoting the reaction mechanism.

In the long term, this emerging view of proteins with conformational substates has broad implications for improving our understanding of enzymes, enzyme engineering, and better drug design. Researchers have already used photoactivation to modulate protein conformations as a strategy to develop a hypercatalytic enzyme. In addition, the alteration of the conformational substates through binding of ligands at locations other than the active site provides the basis for the design of new medicines through allosteric modulation.

10.1021/ar400084s

Introduction

For more than a century, proteins have been viewed as rigid molecules;[1] an idea made familiar by the ball-and-stick models and a single structure drawn on paper. However, proteins are dynamically active molecules that constantly undergo a range of internal motions or conformational fluctuations.[2] Experimental techniques including X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) have been widely used to obtain information about protein structures, providing insights into their mechanisms of functioning. The information obtained from these and other techniques reveals that functioning proteins (in their native states) do not have a fixed structure but exist in slightly different but related conformations. Given the success of structural information in explaining many aspects of protein function, the observed deviations in protein conformation have been largely ignored. More recently, however, it has been proposed that protein function may involve multiple conformations; so the observed internal motions may not be inconsequential or random thermodynamical fluctuations but may be involved closely in many aspects, including protein folding, interaction with the surrounding environment, and its designated function.[3,4]

In the familiar representation, one can imagine the potential energy landscape of a protein to be formed of *hills* and *valleys* of varying heights and depths, populated by conformations (see Figure 1).[5] Not just during the process of folding, even near the native state, a protein's landscape contains several energetic or conformational wells separated by energy barriers. Within each well, the collection of different conformations (or conformational population) shares significant similarity in terms of their structures, internal energies, and other features including dynamical motions. This subpopulation of protein conformations within each of these valleys represents a *substate*. Protein conformational fluctuations driven by thermodynamical motions enable the protein to sample a range of conformations and substates.[6] Internal protein motions span time scales ranging over more than 12 orders of magnitude, from femtoseconds (10^{-15} seconds) to milliseconds and higher ($>10^{-3}$ seconds).[7] Motions at fast time scales enable sampling of the conformations within the substates, while conformational transitions at long time scales enable the protein to transition from one substate to another.

Emerging evidence has tied the kinetics of interconversion between conformational states with the speed of protein folding.[8] In the case of folded and functioning proteins, the rate of interconversion between functionally important substates has been tied to the overall rate of the protein function.[9] The ability to accurately and quantitatively characterize various substates and populations within these substates is widely being sought because it could provide insights into designated function of proteins such as enzyme catalyzed reactions. For example, characterizing the conformational population and substates in the vicinity of transition state area has provided new insights into the enzyme mechanisms as well as detailed understanding of interplay between protein dynamics and enzyme kinetics.[5,10,11] Additionally, it has been discussed that binding of allosteric ligands can alter the conformational substates and the kinetics of interconversion between functional substates, thereby controlling target activity.[12,13] Unfortunately, significant challenges are encountered in identifying and obtaining quantitative information about conformational substates as well as relating the properties of these states to the biophysical mechanism of protein function.

In this Account, we discuss the recent developments that are being used to obtain information about conformational populations and functionally relevant substates of proteins, and how the information obtained provides new insights into functioning of enzyme systems. Some discussions about challenges and potential ways to overcome them are also discussed.

Experimental Techniques Provide Clues about Conformational Diversity

Several experimental techniques have shed light on internal dynamics of proteins and other biomolecules. Unfortunately, the narrow (but improving) windows of resolution for individual instruments and techniques have limited the information that can be collected at the wide range of time scales involved. X-ray crystallography has provided detailed conformational information for the protein in free or ligand-bound states and information about stable and intermediate states along the reaction pathway.[14] NMR experiments have been able to obtain information regarding ensembles of protein structures either in functioning or nonfunctioning states, in the absence or presence of ligands,[15,16] providing insights into the range of natural motions exhibited by different proteins.[9,15] Other experimental techniques, including but not limited to fluorescence resonance energy transfer (FRET),[17] hydrogen–deuterium exchange,[18] and neutron scattering,[19,20] have provided information about the fluctuations that occur within the protein structure as well as some insights into the ensemble, or the population of conformations. Collected information indicates that the

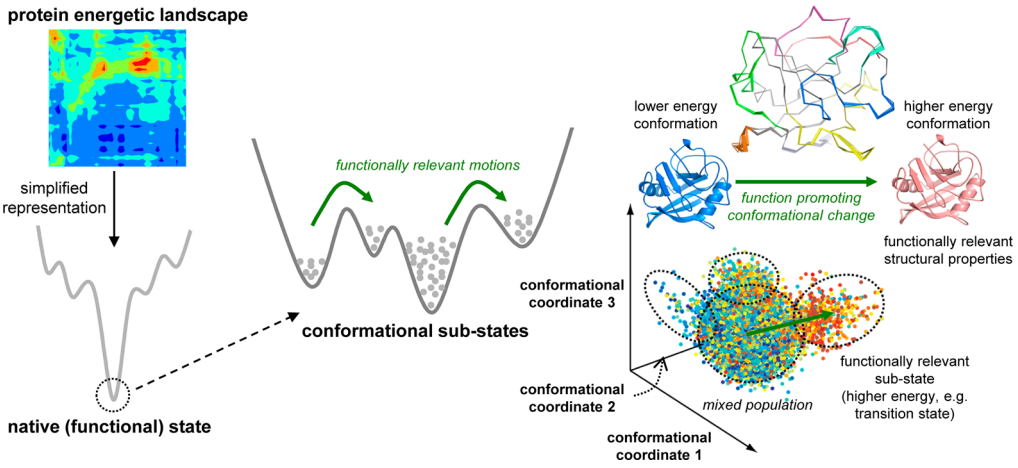


FIGURE 1. Proteins in their native (functioning) state sample conformations with considerable diversity. A group of conformations sharing similar structural and dynamical features forms a conformational substate and corresponds to a well in the energetic landscape. Internal protein motions at fast time scales allow sampling of conformations within substates, while motions at longer time scales allow interconversion between substates (green arrows). Characterizing and quantifying these diverse populations and substates has important implications for understanding designated protein functions. For example, the intrinsic motions of an enzyme allow accessing functionally relevant conformational substates. Note the conformational coordinates on the right panel correspond to global conformational fluctuations.

populations do not sample all areas of the protein landscape evenly but that they are arranged in a hierarchy of subpopulations (or substates) separated by energy barriers.[2,5,21] Depending upon the distribution of the conformations, the native state may consist of several substates with distinct features. Some of these substates contain conformations with structural and dynamical features that promote the designated function of the protein (Figure 1).

Recent advances particularly in NMR and X-ray crystallography techniques have provided insights into the conformational diversity of proteins at multiple time scales. Alber, Kern and co-workers have developed a novel approach that characterizes conformational ensembles at ambient temperatures using X-ray crystallography.[14] NMR and paramagnetic resonance enhancement techniques have provided insights into the conformational transitions and intermediate substates involved in protein folding and ligand recognition.[9,12,22] Obtaining quantitative information from the experimental techniques about the conformational substates and relating it to the mechanism of protein functions remains difficult with challenges associated with each technique. For example, X-ray crystallography is biased toward end states (and sometimes an intermediate state) with high populations but does not typically provide information about the rarely populated substates or conformation transitions between the substates. On the other hand, NMR can provide qualitative information about motions of various protein residues and identify exchange rates between the different substates as well as also identify potentially different substates. However, prior knowledge or assumptions are required regarding the number of substates to model the obtained data.

Computational Methods Enable Identification and Characterization of Substates

Computational techniques are beginning to show remarkable improvement in their ability to identify and quantitatively characterize conformational substates associated with protein function.[5,10,23,24] Coarse-grained models, even though low-resolution, provide important information about a protein's intrinsic ability to visit various conformational substates especially when used with other techniques including atomistic simulations.[25−27] More broadly, molecular dynamics (MD) simulations have been used to obtain atomic level information for conformational substates. However with computational methods, the ability to explore distant areas of the conformational landscape is always an important concern. When MD techniques are used, the landscape sampled may strongly depend on starting structure as well as time scale of simulations. Till a few years ago, typical MD simulations ranged from a few to hundreds of nanoseconds, while a protein undergoes conformational fluctuations that occur on microsecond to millisecond time scales. Recently, tremendous increase in available computing power and access to MD simulations at longer time scales through specialized computing hardware have enabled the collection of statistical information for conformational substates in various stages of a protein's life cycle, including during folding and designated biological function.[28,29]

A number of methodologies have been developed and applied to improve the sampling of the conformational landscape. These include umbrella sampling simulations, metadynamics, and accelerated MD among others. Umbrella sampling simulations allow sampling in higher energy regions which would otherwise not be sampled or only visited infrequently.[30] In a typical application of umbrella sampling, multiple MD simulations with biasing potentials applied at different values of an appropriate reaction coordinate are used to explore higher energy regions along an enzyme's reaction pathway.[31] Metadynamics works by adding a history dependent biasing potential to the visited areas to discourage the simulations from visiting these areas repeatedly, enabling sampling of newer areas of the landscape .[32] The benefit of these techniques includes obtaining quantitatively meaningful information in the higher energy regions, which would otherwise not be explored by conventional MD.[33]

Once the conformations sampled during MD simulations and related techniques have been collected, one of the most commonly used approaches to identify the conformational substates is to project the sampled conformations over principal component analysis modes (particularly the first few modes, which correspond to global motions). However, this approach has shown limited success due to bias toward second-order correlations of protein motions resulting in conformational populations with mixed properties.[5,11]

Recent advances in theoretical methodology, including the characterization of the degree of anharmonicity associated with protein motions, have allowed the identification of conformational substates and the quantitative estimation of populations in these substates.[5] Grubmüller and co-workers have developed full correlation analysis that includes nonlinear and higher order correlations between protein motions; this method provided insights into functionally relevant motions of T4 lysozyme and the hexapeptide neurotensin.[34] Long trajectories at microsecond time scale have been used to investigate conversion between an inactive state (stabilized by intrahelical and interhelical salt bridges) and the active state for β_2 adrenergic receptor from the G-protein-coupled receptor (GPCR) family.[35] The challenge of discovering hidden conformations has been addressed using accelerated MD for maltose binding protein.[36] Also note that the characterization of the metastable states with accurate statistical information about the conformational populations has allowed detailed understanding of the process of protein folding for a number of proteins.[8] On the other hand, computational investigations from the group of Schwartz have led to the discovery of motions at fast time scales that act as *promoting vibrations* in enzyme catalysis.[37,38] The promoting nature of these motions or vibrations corresponds to enabling the reactive trajectories to go over the activation energy barrier.

There is a widely acknowledged need to integrate multiple experimental techniques with computational models to obtain a more complete picture with quantitative information about how conformational substates and transitions between them relate to protein function.[5] As recently demonstrated for ribonuclease A the use of NMR chemical shifts in conjunction with MD simulations has allowed the characterization of two major functional substates and the conformational equilibrium between these states.[39]

Function Promoting Conformational Substates in Enzyme Catalysis

Information collected by a number of different techniques reveals that the active sites of enzymes are optimized for providing an environment that is structurally and electrostatically complementary to the transition state of the catalyzed reaction. Computational models based on active sites and neighboring residues are often able to account correctly for the energy difference between the ground state and the transition state.[40,41] However, the features of enzymes that enable reaching the transition state (higher energy conformations) have mostly remained a mystery. It has been hypothesized that the conformational fluctuations of enzyme allow sampling of higher energy conformational substates, and the rate of catalysis in certain enzyme-catalyzed reactions may be limited by the rate of conformational fluctuations that enable access to these higher energy but functionally important conformational substates.[2,5]

As a result of experimental and computational methods, new insights are coming to light regarding the roles of conformational substates in designated protein functions such as enzyme catalysis.[5,9,14] It has been shown that the conformational landscapes of several enzymes appear to have intrinsic characteristics that enable them to sample function-promoting conformation substates containing structural properties relevant for stages along the catalytic cycle. We discuss two widely investigated enzyme systems in detail.

Dihydrofolate Reductase (DHFR). Enzyme DHFR catalyzes hydride transfer in the folate mechanism and has been widely investigated for the role of protein motions in the enzyme mechanism.[3,42] Pioneering work by Wright and coworkers using NMR investigations has shown the existence of multiple conformational substates that are sampled along

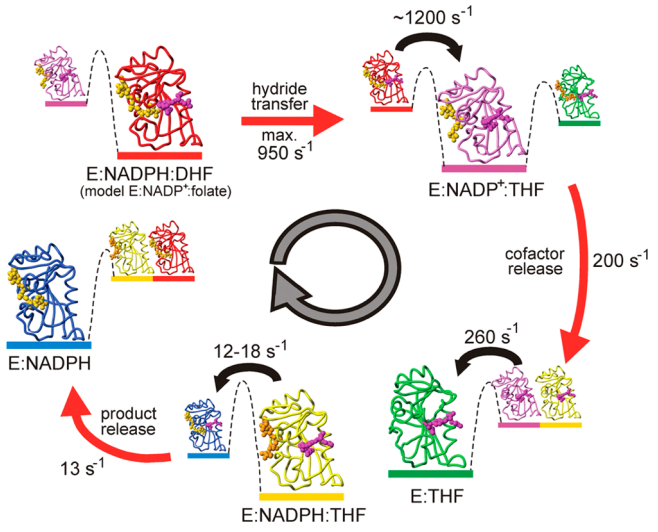


FIGURE 2. Catalytic cycle of dihydrofolate reductase (DHFR) involves protein sampling a range of conformational substates. The substates sampled are shown in different colors. Each state also samples a minor population of conformations from neighboring substates, indicated as smaller protein(s). Substate sampled by enzyme (E) enables the conversion of substrate dihydrofolate (DHF) to tetrahydrofolate (THF) by utilizing cofactor nicotinamide adenine dinucleotide phosphate (NADPH). $NADP^+$ indicates the oxidized form of the cofactor. The rate of interconversion between substates (black) and the intermediate steps during the enzyme catalytic cycles (red) are shown. From ref 9. Reprinted with permission from AAAS.

the catalytic cycle (see Figure 2).[9,15] There are at least five major substates along the reaction pathway. It has been proposed that while the protein is in each conformational substate, it also samples the neighboring states in the catalytic cycle with a minor fraction of populations in these states. The exact populations of conformations in these minor states proved difficult to quantify using NMR; however, they are estimated to be at least 1−2%, because this is the lower limit for detection by R_2 relaxation dispersion experiments. A major finding of these studies has been the relationship between the rate of conformational substate sampling and the kinetics of the various steps along the reaction pathway. In particular, the rate-limiting hydride transfer reaction occurs at the rate of 950 s^{-1}, which coincides with the rate of conformational transitions of the enzyme structure between two different conformational states.[4]

As an interesting consequence, the understanding of interconnection between enzyme dynamics and catalysis has allowed design of mutant DHFRs with lower catalytic activity. These mutants, M42W/G121V[43] and N23PP/S148A,[42] referred to as *dynamic knockouts*, specifically reduced the flexibility of the catalytically important Met20 loop in DHFR that shows multiple conformations during the catalytic cycle, and its flexibility has been implicated in the hydride transfer step. However, it has also been argued that the N23PP/S148A dynamic knockout may not affect the chemical step of hydride transfer.[44]

Cyclophilin A (CypA). The peptidyl−prolyl *cis/trans* isomerization reaction catalyzed by enzyme CypA has also been investigated for the link between internal dynamics and enzyme mechanism. NMR spin relaxation experiments by Kern and co-workers have indicated the existence of a hierarchy of protein motions with internal motions also coinciding with the time scale of the reaction, about 9000 s^{-1}.[45] Further, by ambient temperature X-ray crystallography with mutations, hidden conformations with relevance to catalytic mechanism has been unmasked.[14] On the other hand, computational techniques have allowed detailed insights to be obtained into the conformational substates associated with CypA mechanism (Figure 3). A new methodology, quasi-anharmonic analysis (QAA), in combination with reaction pathway sampling allowed organization of the conformational space into a multilevel hierarchy of conformational substates.[5] The conformations sampled along the reaction pathway allowed identification of substates associated with reactant, product, and more importantly the transition state regions. Characterization of the conformational substates also allowed the identification of conformational transitions between these substates (see the black arrows in Figure 3 A-B) that enable the sampling of the transition state region. The observed displacements in these transitions agree very well with the NMR spin relaxation data.[45] Therefore, the emerging picture indicates that the enzyme−substrate complex samples a variety of conformations between the reactant and product state. Intrinsic conformational transitions allow access to substates that contain structural and dynamical features that allow the reaction to reach the transition state.[31] It has been hypothesized that the rate of these conformational transitions is tied to the rate liming events in the enzyme mechanism.[2,5]

Apart from enzyme catalysis, emerging evidence also indicates that the conformational substates may be involved in the recognition and molecular binding pathways of protein function.[12,24] Further, the comparison of conformational states in the presence and absence of ligands bound to proteins is offering new insights for allostery.[13] Strategy of designing ligands that bind to sites away from primary binding sites but still control changes in conformational populations and therefore modulate target activity could open doors for discovering new medicines based on positive and negative allosteric modulators.[46−48]

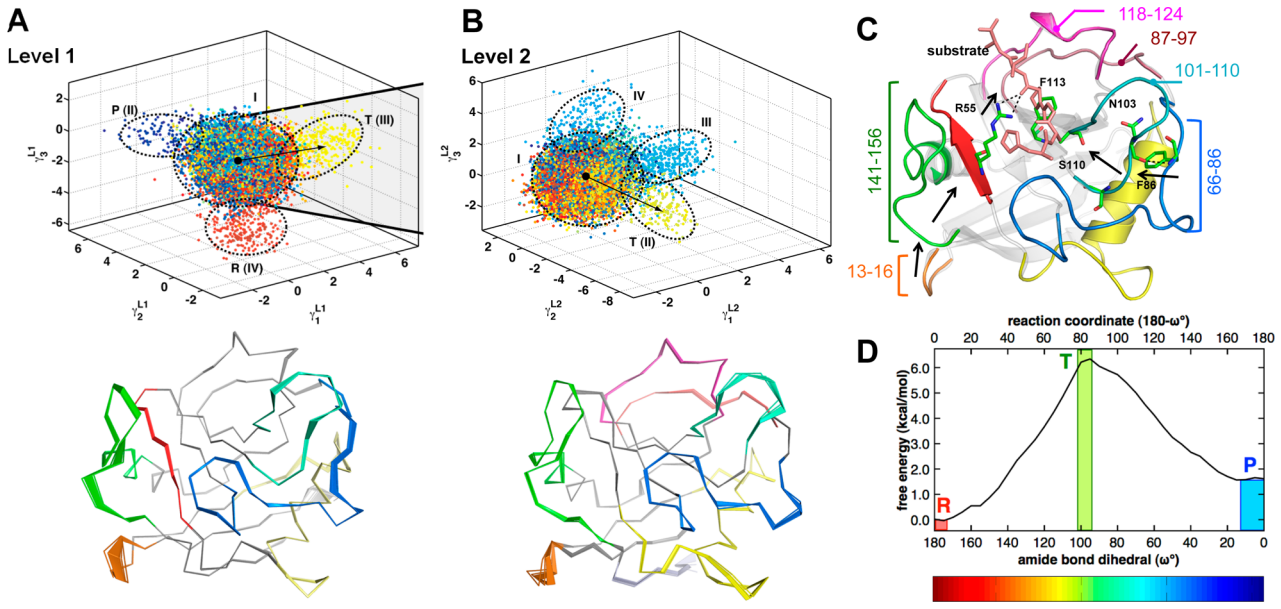


FIGURE 3. The function-promoting conformational substates associated with the *cis*/*trans* peptidyl−prolyl isomerization catalyzed by human cyclophilin A (CypA). The conformational hierarchy of CypA was revealed by a new computational methodology, quasi-anharmonic analysis (QAA), based on conformations sampled by molecular dynamics trajectories along the reaction pathway. Two levels of substates in the hierarchy are shown, (A) level 1 and (B) level 2. The axes in subplots A and B are anharmonic basis vectors (labeled as γ) identified by QAA, corresponding to global conformation fluctuations of CypA. The superscript of γ denotes the level of QAA, while subscript denotes the rank of QAA vectors (based on magnitude of the conformational change, see ref 5 for details of ranking methodology). Note that the conformational states with homogeneous properties are identified as functional substates, while the mixed populations are further decomposed into additional states at subsequent levels. Each colored dot in the top subpanels corresponds to a single conformation and is colored according to the reaction coordinate value along the reaction pathway (see below). The identified substates include reactant (R), transition state (T), and product (P). The interconversion between various substates and functionally relevant substate T is indicated by black arrows in the top part of panels A and B, while the corresponding CypA motions are shown below. (C) The identified motions correspond to a network of coupled motions extending from the flexible surface regions all the way to the active site connected by hydrogen bonds. (D) Free-energy profile for the *cis*/*trans* isomerization of the bound peptide. The color bar corresponds to reaction coordinate used for coloring conformations in panel A. Reproduced from ref 5, Ramanathan, A. et al. PLoS ONE 6 (1), e15827. doi: 10.1371/journal.pone.0015827.

Joint Experimental−Theoretical Methods Could Enable New Insights

Unfortunately, the ability to obtain quantitatively accurate and reliable information about the conformational populations and substates remains beyond the reach of a single instrument or a single theoretical approach. Therefore, there is a need for a joint experimental and computational methodology that allows the identification and characterization of conformational states. A number of investigations, including from the groups of Sali,[49] Hilvert,[50] and Roux[51] among others, have already demonstrated the benefits of utilizing joint computational−experimental strategies. Resulting models that enable quantitative predictions will be vital in teasing apart the contributions of various factors during protein function. Moreover, the exchange of already available raw experimental and computational data with a wider community can further speed up the discovery process.

Information regarding conformational sampling on different time scales is already available from different experimental techniques including X-ray crystallography. As also argued by others, reporting more than a single structure,[52] and possibly also reporting the raw data, may allow access to more information about the conformational substates and therefore offer insights into function.[14] Computational studies are increasingly becoming useful analysis tools; however, a significant emphasis on reporting of average geometrical quantities (such as distances and angles) overshadows the intrinsic property of the protein to sample structures outside the most populated regions, especially the functionally relevant conformational substates that are visited infrequently (*rare* or *hidden* conformational states). In many cases, it is the visit to these rare conformational substates that defines the kinetics of protein folding or biological function. Promoting the availability of raw data from experiments and their use by computational scientists could improve the quantitative estimates of protein conformational populations.

To summarize, viewing the protein not just as a single static structure but as an internally active molecule will

provide new insights into its function and its interaction with the environment. Areas already benefiting from this approach include, particularly, the understanding of enzyme mechanisms and drug design based on allosteric modulation.[5,9,13,46−48,53] This emerging dynamical view of proteins has wide implications for a better understanding of biophysical mechanisms of functioning protein and, more practically, for drug design as well as protein engineering. For example, the identification of reaction promoting conformational fluctuations and modulation of the conformations enabled by photoactivation has already led to the development of a hypercatalytic enzyme engineering strategy, showing ∼3000% improvement in activity over the wild-type enzyme.[54]

Acknowledgements Supercomputing time for computational studies at Oak Ridge National Laboratory's NCCS was made available through peer-reviewed INCITE (BIP003) and ALCC (BIO022) allocations.

BIOGRAPHICAL INFORMATION

Arvind Ramanathan obtained an M.S. from Stony Brook University in 2005 and a Ph.D. from Carnegie Mellon University in 2010. He is currently an associate staff scientist at Oak Ridge National Laboratory. His current research interests include the use of theoretical methods and computational simulations to study the mechanistic details of intrinsically disordered proteins and enzyme catalysis. He has published over 10 papers on these topics.

Andrej Savol received B.S. and B.A. degrees from the University of Pittsburgh in 2007. He subsequently joined the joint CMU−Pitt Ph.D. program in Computational Biology. Currently, he is a fifth year student. His research applies machine learning techniques to problems in protein biophysics, including the identification of folding pathways and inferring structural ensembles from small-angle scattering data. He has published four papers on these topics.

Virginia Burger received a BS/MS in Mathematics from the University of Vienna in 2008. Currently, she is a doctoral candidate in the Joint CMU−Pitt Ph.D. Program in Computational Biology. Her work focuses on segmentation of large biological datasets; she has published four papers on this topic.

Chakra Chennubhotla received his Ph.D. from the University of Toronto in 2004. He is an assistant professor in the Department of Computational and Systems Biology (School of Medicine) at the University of Pittsburgh. His group develops statistical inference algorithms for disease diagnosis and prognosis using data from molecular biophysical simulations and molecular diagnostic imaging. He has published over 20 papers with contributions in sparse principal component analysis, eigencuts, quasi-anharmonic analysis, and multiscale eigensolvers for analyzing biomolecular simulations data.

Pratul Agarwal received Masters from IIT-Delhi in 1997 and Ph.D. from the Pennsylvania State University in 2002. He is a Senior Staff Scientist at Oak Ridge National Laboratory. His research interests include molecular biophysics and the use of high-performance computing for scientific applications. His group develops and uses computational techniques to investigate the inter-connection between protein structure, dynamics, and function. He has published over 40 papers and serves on the editorial boards of the journals *PLoS ONE* and *Microbial Cell Factories*.

FOOTNOTES

*Corresponding authors. Chakra S. Chennubhotla, chakracs@pitt.edu. Pratul K. Agarwal, agarwalpk@ornl.gov.

The authors declare no competing financial interest.

REFERENCES

1 Fischer, E. Einfluss der Configuration auf die Wirkung den Enzyme. *Ber. Dtsch. Chem. Ges.* **1894**, *27*, 2985–2993.

2 Henzler-Wildman, K. A.; Lei, M.; Thai, V.; Kerns, S. J.; Karplus, M.; Kern, D. A Hierarchy of Timescales in Protein Dynamics Is Linked to Enzyme Catalysis. *Nature* **2007**, *450*, 913–916.

3 Hammes, G. G.; Benkovic, S. J.; Hammes-Schiffer, S. Flexibility, Diversity, and Cooperativity: Pillars of Enzyme Catalysis. *Biochemistry* **2011**, *50*, 10422–10430.

4 Ramanathan, A.; Agarwal, P. K. Evolutionarily Conserved Linkage between Enzyme Fold, Flexibility, and Catalysis. *PLoS Biol.* **2011**, *9*, No. e1001193.

5 Ramanathan, A.; Savol, A. J.; Langmead, C. J.; Agarwal, P. K.; Chennubhotla, C. S. Discovering Conformational Sub-States Relevant to Protein Function. *PLoS ONE* **2011**, *6*, No. e15827.

6 Senet, P.; Maisuradze, G. G.; Foulie, C.; Delarue, P.; Scheraga, H. A. How Main-Chains of Proteins Explore the Free-Energy Landscape in Native States. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 19708–19713.

7 Agarwal, P. K. Enzymes: An Integrated View of Structure, Dynamics and Function. *Microb. Cell Fact.* **2006**, *5*, 2.

8 Bowman, G. R.; Pande, V. S. Protein Folded States Are Kinetic Hubs. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 10890–10895.

9 Boehr, D. D.; McElheny, D.; Dyson, H. J.; Wright, P. E. The Dynamic Energy Landscape of Dihydrofolate Reductase Catalysis. *Science* **2006**, *313*, 1638–1642.

10 Savol, A. J.; Burger, V. M.; Agarwal, P. K.; Ramanathan, A.; Chennubhotla, C. S. QAARM: Quasi-anharmonic Autoregressive Model Reveals Molecular Recognition Pathways in Ubiquitin. *Bioinformatics* **2011**, *27*, I52–I60.

11 Ramanathan, A.; Savol, A. J.; Agarwal, P. K.; Chennubhotla, C. S. Event Detection and Substate Discovery from Biomolecular Simulations Using Higher-Order Statistics: Application to Enzyme Adenylate Kinase. *Proteins* **2012**, *80*, 2536–2551.

12 Boehr, D. D.; Nussinov, R.; Wright, P. E. The Role of Dynamic Conformational Ensembles in Biomolecular Recognition. *Nat. Chem. Biol.* **2009**, *5*, 789–796.

13 Kar, G.; Keskin, O.; Gursoy, A.; Nussinov, R. Allostery and Population Shift in Drug Discovery. *Curr. Opin. Pharmacol.* **2010**, *10*, 715–722.

14 Fraser, J. S.; Clarkson, M. W.; Degnan, S. C.; Erion, R.; Kern, D.; Alber, T. Hidden Alternative Structures of Proline Isomerase Essential for Catalysis. *Nature* **2009**, *462*, 669–673.

15 Boehr, D. D.; McElheny, D.; Dyson, H. J.; Wright, P. E. Millisecond Timescale Fluctuations in Dihydrofolate Reductase Are Exquisitely Sensitive to the Bound Ligands. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 1373–1378.

16 Akke, M. Conformational Dynamics and Thermodynamics of Protein-Ligand Binding Studied by NMR Relaxation. *Biochem. Soc. Trans.* **2012**, *40*, 419–423.

17 Homouz, D.; Sanabria, H.; Waxham, M. N.; Cheung, M. S. Modulation of Calmodulin Plasticity by the Effect of Macromolecular Crowding. *J. Mol. Biol.* **2009**, *391*, 933–943.

18 Mehmood, S.; Domene, C.; Forest, E.; Jault, J. M. Dynamics of a Bacterial Multidrug ABC Transporter in the Inward- And Outward-Facing Conformations. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 10832–10836.

19 Borreguero, J. M.; He, J. H.; Meilleur, F.; Weiss, K. L.; Brown, C. M.; Myles, D. A.; Herwig, K. W.; Agarwal, P. K. Redox-Promoting Protein Motions in Rubredoxin. *J. Phys. Chem. B* **2011**, *115*, 8925–8936.

20 Schiro, G.; Vetri, V.; Frick, B.; Militello, V.; Leone, M.; Cupane, A. Neutron Scattering Reveals Enhanced Protein Dynamics in Concanavalin A Amyloid Fibrils. *J. Phys. Chem. Lett.* **2012**, *3*, 992–996.

21 Markwick, P. R. L.; Bouvignies, G.; Blackledge, M. Exploring Multiple Timescale Motions in Protein GB3 Using Accelerated Molecular Dynamics and NMR Spectroscopy. *J. Am. Chem. Soc.* **2007**, *129*, 4724–4730.

22 Boehr, D. D.; Dyson, H. J.; Wright, P. E. An NMR Perspective on Enzyme Dynamics. *Chem. Rev.* **2006**, *106*, 3055–3079.

23 Lange, O. F.; Lakomek, N. A.; Fares, C.; Schroder, G. F.; Walter, K. F. A.; Becker, S.; Meiler, J.; Grubmuller, H.; Griesinger, C.; de Groot, B. L. Recognition Dynamics up to Microseconds Revealed from an RDC-Derived Ubiquitin Ensemble in Solution. *Science* **2008**, *320*, 1471–1475.

24 Ban, D.; Funk, M.; Gulich, R.; Egger, D.; Sabo, T. M.; Walter, K. F. A.; Fenwick, R. B.; Giller, K.; Pichierri, F.; de Groot, B. L.; Lange, O. F.; Grubmuller, H.; Salvatella, X.; Wolf, M.; Loidl, A.; Kree, R.; Becker, S.; Lakomek, N. A.; Lee, D.; Lunkenheimer, P.; Griesinger, C. Kinetics of Conformational Sampling in Ubiquitin. *Angew. Chem., Int. Ed.* **2011**, *50*, 11437–11440.

25 Yang, L.; Song, G.; Jernigan, R. L. Protein Elastic Network Models and the Ranges of Cooperativity. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 12347–12352.

26 Yang, S. C.; Park, S.; Makowski, L.; Roux, B. A Rapid Coarse Residue-Based Computational Method for X-Ray Solution Scattering Characterization of Protein Folds and Multiple Conformational States of Large Protein Complexes. *Biophys. J.* **2009**, *96*, 4449–4463.

27 Skliros, A.; Zimmermann, M. T.; Chakraborty, D.; Saraswathi, S.; Katebi, A. R.; Leelananda, S. P.; Kloczkowski, A.; Jernigan, R. L. The importance of slow motions for protein functional loops. *Phys. Biol.* **2012**, *9*, No. 014001.

28 Agarwal, P. K.; Alam, S. R. Biomolecular simulations on petascale: Promises and challenges. *J. Phys. Conf. Ser.* **2006**, *46*, 327–333.

29 Dror, R. O.; Dirks, R. M.; Grossman, J. P.; Xu, H. F.; Shaw, D. E. Biomolecular Simulation: A Computational Microscope for Molecular Biology. *Annu. Rev. Biophys.* **2012**, *41*, 429–452.

30 Kastner, J.; Senn, H. M.; Thiel, S.; Otte, N.; Thiel, W. QM/MM Free-Energy Perturbation Compared to Thermodynamic Integration and Umbrella Sampling: Application to an Enzymatic Reaction. *J. Chem. Theory Comput.* **2006**, *2*, 452–461.

31 Agarwal, P. K.; Geist, A.; Gorin, A. Protein Dynamics and Enzymatic Catalysis: Investigating the Peptidyl-Prolyl Cis-Trans Isomerization Activity of Cyclophilin A. *Biochemistry* **2004**, *43*, 10605–10618.

32 Leone, V.; Marinelli, F.; Carloni, P.; Parrinello, M. Targeting Biomolecular Flexibility with Metadynamics. *Curr. Opin. Struc. Biol.* **2010**, *20*, 148–154.

33 Leone, V.; Lattanzi, G.; Molteni, C.; Carloni, P. Mechanism of Action of Cyclophilin A Explored by Metadynamics Simulations. *PLoS Comput. Biol.* **2009**, *5*, No. e1000309.

34 Lange, O. F.; Grubmuller, H. Full Correlation Analysis of Conformational Protein Dynamics. *Proteins* **2008**, *70*, 1294–1312.

35 Romo, T. D.; Grossfield, A.; Pitman, M. C. Concerted Interconversion between Ionic Lock Substates of the beta(2) Adrenergic Receptor Revealed by Microsecond Timescale Molecular Dynamics. *Biophys. J.* **2010**, *98*, 76–84.

36 Bucher, D.; Grant, B. J.; Markwick, P. R.; McCammon, J. A. Accessing a Hidden Conformation of the Maltose Binding Protein Using Accelerated Molecular Dynamics. *PLoS Comput. Biol.* **2011**, *7*, No. e1002034.

37 Mincer, J. S.; Schwartz, S. D. Rate-Promoting Vibrations and Coupled Hydrogen-Electron Transfer Reactions in the Condensed Phase: A Model for Enzymatic Catalysis. *J. Chem. Phys.* **2004**, *120*, 7755–7760.

38 Davarifar, A.; Antoniou, D.; Schwartz, S. D. The Promoting Vibration in Human Heart Lactate Dehydrogenase Is a Preferred Vibrational Channel. *J. Phys. Chem. B* **2011**, *115*, 15439–15444.

39 Camilloni, C.; Robustelli, P.; De Simone, A.; Cavalli, A.; Vendruscolo, M. Characterization of the Conformational Equilibrium between the Two Major Substates of RNase A Using NMR Chemical Shifts. *J. Am. Chem. Soc.* **2012**, *134*, 3968–3971.

40 Tantillo, D. J.; Chen, J. G.; Houk, K. N. Theozymes and Compuzymes: Theoretical Models for Biological Catalysis. *Curr. Opin. Chem. Biol.* **1998**, *2*, 743–750.

41 Kiss, G.; Celebi-Olcum, N.; Moretti, R.; Baker, D.; Houk, K. N. Computational Enzyme Design. *Angew. Chem., Int. Ed.* **2013**, *52*, 5700–5725.

42 Bhabha, G.; Lee, J.; Ekiert, D. C.; Gam, J.; Wilson, I. A.; Dyson, H. J.; Benkovic, S. J.; Wright, P. E. A Dynamic Knockout Reveals That Conformational Fluctuations Influence the Chemical Step of Enzyme Catalysis. *Science* **2011**, *332*, 234–238.

43 Fan, Y.; Cembran, A.; Ma, S. H.; Gao, J. L. Connecting Protein Conformational Dynamics with Catalytic Function As Illustrated in Dihydrofolate Reductase. *Biochemistry* **2013**, *52*, 2036–2049.

44 Loveridge, E. J.; Behiry, E. M.; Guo, J. N.; Allemann, R. K. Evidence That a `Dynamic Knockout' in Escherichia coli Dihydrofolate Reductase Does Not Affect the Chemical Step of Catalysis. *Nat. Chem.* **2012**, *4*, 292–297.

45 Eisenmesser, E. Z.; Millet, O.; Labeikovsky, W.; Korzhnev, D. M.; Wolf-Watz, M.; Bosco, D. A.; Skalicky, J. J.; Kay, L. E.; Kern, D. Intrinsic Dynamics of an Enzyme Underlies Catalysis. *Nature* **2005**, *438*, 117–121.

46 Hardy, J. A.; Lam, J.; Nguyen, J. T.; O'Brien, T.; Wells, J. A. Discovery of an Allosteric Site in the Caspases. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 12461–12466.

47 Erlanson, D. A.; Wells, J. A.; Braisted, A. C. Tethering: Fragment-Based Drug Discovery. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 199–223.

48 Wang, N. K.; Majmudar, C. Y.; Pomerantz, W. C.; Gagnon, J. K.; Sadowsky, J. D.; Meagher, J. L.; Johnson, T. K.; Stuckey, J. A.; Brooks, C. L.; Wells, J. A.; Mapp, A. K. Ordering a Dynamic Protein Via a Small-Molecule Stabilizer. *J. Am. Chem. Soc.* **2013**, *135*, 3363–3366.

49 Pieper, U.; Webb, B. M.; Barkan, D. T.; Schneidman-Duhovny, D.; Schlessinger, A.; Braberg, H.; Yang, Z.; Meng, E. C.; Pettersen, E. F.; Huang, C. C.; Datta, R. S.; Sampathkumar, P.; Madhusudhan, M. S.; Sjolander, K.; Ferrin, T. E.; Burley, S. K.; Sali, A. ModBase, a Database of Annotated Comparative Protein Structure Models, And Associated Resources. *Nucleic Acids. Res.* **2011**, *39*, D465–D474.

50 Muller, M. M.; Allison, J. R.; Hongdilokkul, N.; Gaillon, L.; Kast, P.; van Gunsteren, W. F.; Marliere, P.; Hilvert, D. Directed Evolution of a Model Primordial Enzyme Provides Insights into the Development of the Genetic Code. *PLoS Genet.* **2013**, *9*, No. e1003187.

51 Yang, S. C.; Blachowicz, L.; Makowski, L.; Roux, B. Multidomain Assembled States of Hck Tyrosine Kinase in Solution. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 15757–15762.

52 Furnham, N.; Blundell, T. L.; DePristo, M. A.; Terwilliger, T. C. Is One Solution Good Enough? *Nat. Struct. Mol. Biol.* **2006**, *13*, 184–5.

53 Sinko, W.; de Oliveira, C.; Williams, S.; Van Wynsberghe, A.; Durrant, J. D.; Cao, R.; Oldfield, E.; McCammon, J. A. Applying Molecular Dynamics Simulations to Identify Rarely Sampled Ligand-Bound Conformational States of Undecaprenyl Pyrophosphate Synthase, an Antibacterial Target. *Chem. Biol. Drug Des.* **2011**, *77*, 412–420.

54 Agarwal, P. K.; Schultz, C.; Kalivretenos, A.; Ghosh, B.; Broedel, S. E. Engineering a Hyper-catalytic Enzyme by Photoactivated Conformation Modulation. *J. Phys. Chem. Lett.* **2012**, *3*, 1142–1146.