Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
E-mail: jzus@zju.edu.cn

Review:

On the necessity of an integrative approach to understand protein structural dynamics*

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Received Mar. 15, 2019; Revision accepted Mar. 31, 2019; Crosschecked Apr. 12, 2019

Abstract: Proteins are dynamic, fluctuating between multiple conformational states. Protein dynamics, spanning orders of magnitude in time and space, allow proteins to perform specific functions. Moreover, under certain conditions, proteins can morph into a different set of conformations. Thus, a complete understanding of protein structural dynamics can provide mechanistic insights into protein function. Here, we review the latest developments in methods used to determine protein ensemble structures and to characterize protein dynamics. Techniques including X-ray crystallography, cryogenic electron microscopy, and small angle scattering can provide structural information on specific conformational states or on the averaged shape of the protein, whereas techniques including nuclear magnetic resonance, fluorescence resonance energy transfer (FRET), and chemical cross-linking coupled with mass spectrometry provide information on the fluctuation of the distances between protein domains, residues, and atoms for the multiple conformational states of the protein. In particular, FRET measurements at the single-molecule level allow rapid resolution of protein conformational states, where information is otherwise obscured in bulk measurements. Taken together, the different techniques complement each other and their integrated use can offer a clear picture of protein structure and dynamics.

Key words: Conformational dynamics; Integrative structural biology; Distance restraint; Ensemble averaging; Nuclear magnetic resonance (NMR)
https://doi.org/10.1631/jzus.B1900135 **CLC number:** Q51

1 Introduction

X-ray crystallography, nuclear magnetic resonance (NMR), and cryogenic electron microscopy (cryoEM) are the three main tools used in structural biology. In recent years, we have witnessed the increasing popularity of cryoEM, which can solve the structures of large biological macromolecules frozen in vitreous conditions. These structural biology techniques have provided us with numerous atomic resolution structures of proteins and other biological macromolecules. As of March 31st, 2019, 150000 structures have been deposited in the protein data bank (PDB, http://www.rcsb.org). However, a static picture of a protein may not provide sufficient information to understand how the protein works. Structural dynamics, more relevantly, shows enzymes, transporters, signaling proteins, and others in action (Henzler-Wildman and Kern, 2007).

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The structural dynamics of a protein arise from the vibrations and rotations of the chemical bonds occurring in femto-second timescale, and the fluctuation of the orientations of bond vectors occurring in pico-second to nano-second (ps–ns) timescale. These high-frequency dynamics can be coupled, giving rise to the collective movement occurring at micro-second to milli-second (μs–ms) or even slower timescales. Protein collective motion involves many residues, for example, the opening and closing of protein domains (Tang et al., 2007), and is intimately related to protein function (Bahar et al., 2010). A protein adopting a particular conformation is associated with a certain free energy (Bryngelson et al., 1995). At ambient temperature, the thermal kinetic energy allows the protein to overcome the energy barrier and interconvert between different conformational states (Fig. 1a).

The population of each conformational state is determined by its free energy, and the distribution is governed by the Boltzmann probability. As a result, the predominant lowest-energy conformational state is the most likely to be captured and visualized by X-ray crystallography. Traditionally, protein crystals are flash frozen in liquid nitrogen to avoid radiation damage during crystallographic data collection. As the protein is structurally fixed in the crystal lattice, only local conformational fluctuations of the protein are allowed. Such dynamics can now be studied using room-temperature X-ray crystallography (Fraser et al., 2011).

The kinetics of the interconversion between protein conformational states A and B are dictated by the energy barrier (Fig. 1a), as described by the Arrhenius equation. Thus, due to stochastic thermal fluctuations, it may only take a protein molecule μs–ms to overcome the large energy barrier. CryoEM deduces three-dimensional shapes from two-dimensional projection images and classifies the structure of the protein into a set of conformational states (Bai et al., 2015). However, upon freezing in liquid ethane, the energy landscape of the protein is likely perturbed, and the relative populations of the conformational states likely differ from those under ambient conditions. More critically, the interconversion dynamics are completely lost at −188 °C. Therefore, though powerful as a structural biology tool, cryoEM may only provide snapshots of the many alternative conformational states of proteins and protein complexes.

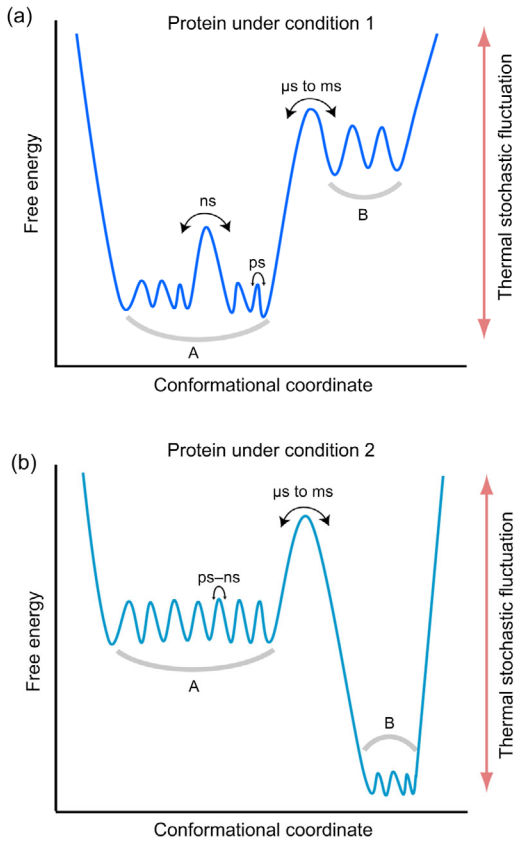

Fig. 1 An illustration of a protein energy landscape
(a) The conformational state (A or B) with the lowest free energy is the most populated. Yet all the states are stochastically accessible at ambient temperature. (b) Different cellular conditions, including but not limited to post-translational modifications, local pH, macromolecular crowding, and liquid droplet formation, can all lead to altered energy landscapes of protein conformational states

The interconversion among different conformational states allows the protein to perform specific functions. Indeed, a protein must undergo structural changes for catalysis, ligand binding, and signal transduction (Henzler-Wildman and Kern, 2007). More importantly, the energy landscape of the protein can be altered under different cellular conditions (Fig. 1b) and upon post-translational modifications. For example, ubiquitin, a 76-residue signaling protein, mainly adopts a relaxed state with a common ubiquitin fold, and rarely samples a C-terminal β-strand-retracted state even at 45 °C (Gladkova et al., 2017). However, phosphorylation by ubiquitin kinase PINK1 at residue Ser65 increases the population of the retracted state by more than 100-fold. Moreover, the relative populations of relaxed and retracted states of

the phosphorylated ubiquitin can change with pH, as the phosphorylation-enriched retracted state becomes more populated at a slightly basic pH (Dong et al., 2017). In recent years, molecular crowding and the formation of liquid droplets have been increasingly recognized as factors that perturb protein structural dynamics and enable certain protein functions (Kuznetsova et al., 2014; Elbaum-Garfinkle et al., 2015). Importantly, the concentration of the phase-separated protein in the droplet can reach 400 mg/mL or higher (Brady et al., 2017). Such high protein concentrations would promote fleeting protein–protein interactions with binding affinities in the millimolar range (Xing et al., 2014). Hence, protein liquid droplets may only form with multi-valent and weak interactions between proteins and protein domains (Li et al., 2012). Taken together, structural dynamics involves an ensemble of structures adopted by a protein or protein complex over time.

2 NMR is inherently integrative

NMR has been used to characterize proteins and other biological macromolecules in solution. Unlike X-ray crystallography and cryoEM, solution NMR is uniquely suited to characterize protein structural dynamics at near physiological conditions. In recent years, NMR has also been used to determine protein structures in the cell (Sakakibara et al., 2009; Luchinat and Banci, 2016; Pan et al., 2016).

It has been more than 40 years since the first protein structure was determined by NMR (Wagner and Wüthrich, 1978). Yet, the key concept of NMR structure determination remains largely the same. This is because NMR is inherently integrative, and can always incorporate additional experimental data as a new energy term. In NMR structure determination, the structure is optimized to make the calculated values based on the structural model agree best with the experimental inputs. The goodness of the fit is evaluated with a pseudo-energy term, which is minimized with simulated annealing.

Typical inputs for NMR structure calculation include nuclear Overhauser enhancement (NOE) cross-peaks, chemical shift values, scalar-coupling constants, and residual dipolar couplings. They can be readily measured and used in structure calculation software, such as Xplor-NIH (Schwieters et al., 2018). In addition, with a paramagnetic probe site-specifically attached to an otherwise diamagnetic protein, paramagnetic NMR parameters, exemplified by paramagnetic relaxation enhancement (PRE), can be measured (Iwahara et al., 2007; Clore and Iwahara, 2009). With the latest NMR developments, more and more energy terms from different types of experimental data have been incorporated. For example, small angle X-ray scattering (SAXS) and small angle neutron scattering (SANS) can provide an overall silhouette of the protein in solution (Schwieters and Clore, 2014). Probability of protein dihedral angles from the statistics of high-precision structures can also be used as a knowledge-based potential (Bermejo et al., 2012).

3 Distance matters

When a protein exists in a particular conformation, each atom, residue, and domain of the protein is separated from one another by certain distances. When the protein changes conformation, a new set of distances emerge. Just like protein dynamics are hierarchical, the distance between the domains can also be considered as the sum of inter-residue and interatomic distances (Fig. 2a). On the other hand, if all the distances can be measured, the particular protein structure or the ensemble of protein structures can be deduced. Yet, it should be noted that protein structure determination based on NMR restraints is an underdetermined problem, as the structure solution accounting for all the experimental data is unlikely to be unique. Thus, Bayesian inference has been applied for NMR structure determination, which assigns a statistical probability to the structural model (Rieping et al., 2005; MacCallum et al., 2015).

Many distance measurements in NMR and in other biophysical methods are based on dipolar interactions that follow the $<r^{-6}>$ distance relationship. The NOE effect arises from the dipolar interactions between two protons separated by less than 6 Å. The PRE effect arises from dipolar interaction between a proton and an unpaired electron in the site-specifically attached paramagnetic probe. Since an electron has a much larger magnetic dipole moment than a proton, the PRE can provide a distance relationship of up to

40 Å (Iwahara et al., 2003; Liu et al., 2016). Fluorescence resonance energy transfer (FRET) also arises from dipolar interactions between two fluorophores which have been site-specifically attached to a protein or protein complex, and can provide distance information in the range of 20 to 80 Å. The fluorescence signal has a much lower detection limit than the NMR signal, and as a result, FRET can be measured at the single-molecule level (Lerner et al., 2018). How single-molecule FRET (smFRET) can aid the analysis of protein structural dynamics will be discussed in the next section.

Methods have also been devised to obtain the distance restraints for protein structure calculation without introducing an NMR or fluorescent probe. For example, chemical cross-linking coupled with mass spectrometry (CXMS) allows the identification of pairs of residues closer than the lengths of the cross-linkers used (Yang et al., 2012). It should be noted that the cross-links may only "crawl" along the protein surface; therefore, solvent accessible distances instead of Euclidean distances should be calculated and used as distance restraints (Matthew Allen Bullock et al., 2016; Ding et al., 2017). Using only the distance restraints from cross-linking, it is now possible to obtain de novo protein structures (Brodie et al., 2017). On the other hand, with genomics information becoming available for different species, internal residues that are in contact can also be deduced from evolutionary couplings (Tang et al., 2015). Co-evolution of proximal residues assumes that mutations are correlated to minimize perturbations to the protein structure. Taken together, the distance relationships from various types of experimental data are multi-tiered and hierarchical, and the accurate measurement of these distances lays the foundation for structural characterization of proteins and protein complexes (Fig. 2a).

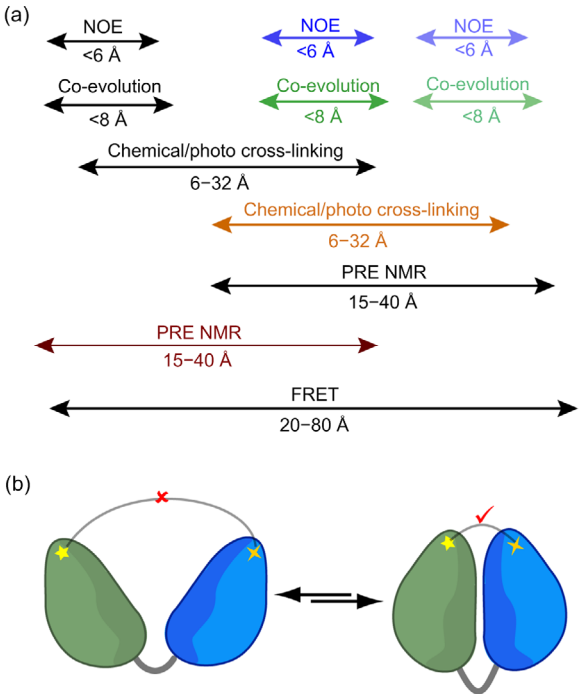

Fig. 2 Distance information between protein domains, residues, and atoms, which can be obtained from a multitude of experimental inputs

(a) The distance restraints with different length-scales are hierarchical and can help to reconstruct a complete structural picture of the protein. (b) With protein dynamics present, the distance restraints are ensemble-averaged, and sometimes manifest as the most compact conformation(s). This scheme illustrates that chemical cross-linking may only occur when this two-domain protein adopts a closed conformation, in which the distance between cross-linked residues is shorter than the length of the cross-linker. NOE: nuclear Overhauser enhancement; PRE: paramagnetic relaxation enhancement; FRET: fluorescence resonance energy transfer

4 Resolving multiple conformational states with multiple methods

Proteins are dynamic. As a result, the distance restraint obtained may not arise from a single conformation, but is likely an average from multiple conformational states adopted simultaneously by the protein (Fig. 2b). For a typical bulk measurement, such as NMR and SAXS/SANS, the data are collected for many billions of protein molecules, and are therefore ensemble-averaged over all the conformational states adopted by the protein. Thus, it is an ill-posed inverse problem to decompose experimental data and to assign the relative contributions to each conformational state. In particular, this is the case for PRE NMR measurements. Owing to the $\langle r^{-6} \rangle$ distance dependence, a proton in a conformation with a short distance to the paramagnetic probe experiences a disproportionally large effect when compared to the overall observed PRE data, since the PRE effect rapidly tapers at longer distances. As a result, the characterization of protein dynamics based on PRE NMR data alone can be biased towards the most compact conformational state (Clore and Iwahara,

2009). Therefore, it is important to refine the ensemble structures against different types of experimental data. Such an integrative approach will not only yield a more accurate and complete picture of protein structure and dynamics, and the various types of data with different information content and different ways of ensemble averaging can also provide cross-validation (Fig. 3).

Unlike bulk measurements, single-molecule techniques inspect only one molecule at a time. Therefore, single-molecule detection is not ensemble-averaged, and from the smFRET profile the number of interconverting conformational states and their relative populations can be readily obtained. Equipped with such information, bulk measurements, like PRE NMR, can be defined without resorting to complicated calculations. For example, the cross-links identified in CXMS represent the compact conformational state(s) of the protein, in which the separation between the cross-linked residues is shorter than the length of the cross-linker (Gong et al., 2015; Ferber et al., 2016; Ding et al., 2017). SAXS data, on the other hand, contain averaged shape information of all the conformational states. Liu et al. (2018) used smFRET in conjunction with SAXS and CXMS data to rapidly characterize the three interconverting structures of K63-linked di-ubiquitin.

5 Along came the time dimension

Characterization of a set of interconverting conformational states or ensemble structure of a protein provides information on their conformational dynamics. Here, only the snapshots and their relative populations are visualized. To fully understand protein structural dynamics, we also need to know the timescale of the dynamics and the pathway of the dynamic interconversion.

NMR is uniquely suited to characterize protein dynamics since it is inherently integrative, and a plethora of techniques exist that can define timescale information of protein dynamics (Sekhar and Kay, 2013). By examining the relaxation properties of NMR spins, and using relaxation dispersion and off-resonance spin-lock methods, ps–ns and μs–ms timescale dynamics can be identified. To analyze protein dynamics with slower timescales, chemical exchange saturation transfer, *Z*-axis exchange experiments, and real-time two-dimensional spectroscopy data can be collected.

The smFRET can also provide information on the timescales of protein dynamics. It allows the identification of multiple interconverting conformational states, and provides kinetic information for the exchange between any two of the conformational states (Kalinin et al., 2010). Thus, smFRET can chart the pathway of conformational dynamics, that is, how the protein preferentially switches from one state to another (Peulen et al., 2017). Such information can be difficult to obtain using NMR alone.

With more experimental data on protein structure and dynamics becoming available, physics and/or knowledge-based computational simulation methods have also greatly improved over the past 10 years, and are increasingly used along with experimental analysis (MacCallum et al., 2015). Based on Newtonian laws, simulations can recapitulate the essential dynamics of protein systems (Dror et al., 2012). Moreover, simulations can predict structural outcomes which can then be experimentally validated. Simulations can also provide information on the detailed interconversion process between protein conformational states, which may not be obtainable from experiments.

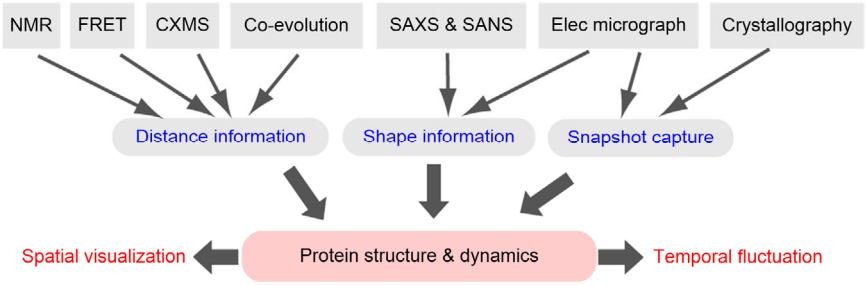

Fig. 3 Use of an integrative hybrid approach to fully characterize protein structure and dynamics
NMR: nuclear magnetic resonance; FRET: fluorescence resonance energy transfer; CXMS: chemical cross-linking coupled with mass spectrometr; SAXS: small angle X-ray scattering; SANS: small angle neutron scattering

6 Concluding remarks

Today's biological research systems are becoming increasingly complex. To tackle these problems and to determine the structures of these systems, researchers have resorted to the "non-conventional" methods in addition to X-ray crystallography, NMR, and cryoEM. Structures obtained using an integrative approach may not provide an atomic resolution picture, yet it still gives us mechanistic insights. Recently, a database called PDB-Dev (https://pdb-dev.wwpdb.org) was established for curating structures determined using integrative hybrid methods (Sali et al., 2015). However, the strength of using NMR and other biophysical methods jointly should go beyond determining a single structure of the protein, and can excel in providing an ensemble of protein structures with a time stamp. The various types of experimental and computational data complement each other, and as a result, a clear and complete picture of protein structural dynamics emerges.

Contributors

Both authors conceived the idea. Qing-fen YANG wrote the initial draft and Chun TANG revised the manuscript.

Compliance with ethics guidelines

Qing-fen YANG and Chun TANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by either of the authors.

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中文概要

题　目：整合方法研究蛋白质结构动态之必要性

概　要：蛋白质是动态的，在多种结构状态间转化。蛋白质的结构动态在时间和空间上跨越多个数量级，允许蛋白质执行特定生物学功能。细胞条件和细胞环境的变化会使蛋白质动态结构发生变化。因此，对蛋白质结构动态的全面表征可提供对蛋白质功能的机制的深入了解。在这里，我们综述了用于测定蛋白质系综动态结构和表征蛋白质结构动态变化的方法的最新发展。X 射线晶体学、冷冻电镜和小角散射等技术可提供关于蛋白质特定状态或平均的结构信息，而核磁共振、荧光共振能量转移（FRET）和化学交联质谱等技术则提供了蛋白质结构域、蛋白质残基和原子之间的处于不同结构状态时的距离波动信息。尤其要指出的是，单分子水平的 FRET 测量能对蛋白质构象状态进行快速区分，而这样的信息会在其它测量中被掩盖。总之，不同的生物物理技术互为补充，只有通过它们的综合运用，才能清晰可见蛋白质结构和动态。

关键词：结构动态；整合结构生物学；距离约束；系综平均；核磁共振