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An Insight into Biomolecular Flexibility: Its Measuring, Modeling and Regulating on Function at Single Molecule Level

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Abstract: The protein structure-function paradigm implies that the structure of a protein defines its function. Crystallization techniques such as X-ray, electron microscopy (EM) and nuclear magnetic resonance (NMR) have been applied to resolve the crystal structure of numerous proteins, provided beautiful and informative models of proteins. However, proteins are not intrinsically in static state but in dynamic state, which is lack in crystal models. The protein flexibility, a key mechanical property of proteins, plays important roles in various biological processes, such as ligand-receptor interaction, signaling transduction, substrate recognition and post-translational modifications. Advanced time-resolved crystallography has been developed recent years to visualize and characterize the dynamic of proteins and reviewed in literatures. In the present review, we will focus on the single-molecule based techniques and theoretical methods in determining the flexibility of proteins, exhibit some interest examples of proteins and DNA molecular flexibility to their functions, and provide an insight in molecular flexibility from the biomechanics point of view.

Keywords: Molecular flexibility, single molecule, biomechanics, DNA.

1 Introduction

Protein flexibility is intrinsic and closely related to its function [Borges, Seraphim, Dores-Silva et al. (2016)]. In fact, 75% of signaling proteins and 40% of human membrane proteins contain at least one disordered and/or flexible segment longer than 30 residues [Borges, Seraphim, Dores-Silva et al. (2016); Kjaergaard and Kragelund (2017)]. The flexible protein regions connect the folded domains in a multi-domain protein with a preferred conformation, busy in inter-domain signal transduction, serve as the binding sites for interacting partners or the helpers in molecular recognition, work as activation/inhibition modules, and exhibit the post-translational modification sites [Borges, Seraphim, Dores-Silva et al. (2016)]. Enzymes generally are believed to be quite vulnerable structures sensitive to environmental changes [Zavodszky, Kardos, Svingor et al. (1998)]. This ability of an enzyme to move among closely related conformations is often the basis of its

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functionality [Fields (2001)]. Many enzymes undergo conformational changes, such that different structural components reorient themselves during catalytic cycle to form the appropriate geometry for binding substrate or cofactors, to bring chemically reactive species together, create a 'catalytic vacuole' with the appropriate physicochemical characteristics, separate from the surrounding medium, or release products after chemical catalysis has occurred [Fields (2001)].

In addition to protein, the flexibility of peptides, such as antimicrobial peptides (AMPs), is critical for their antimicrobial activity as well. AMPS, an innate immune component ubiquitous among plants and animals, are variously active against a wide range of pathogens, such as gram-positive or negative bacteria, fungi and protozoa [Liu, Fang, Huang et al. (2011)]. The antimicrobial activity of AMPs is believed to be relevant to their structural parameters, such as conformation, charge, hydrophobicity, amphipathicity and polar angle [Yeaman and Yount (2003); Dathe and Wieprecht (1999)]. However, the flexibility of AMPs is another important factor to their function. A hinge near the central position of an α-helical chain may be beneficial for peptide to span the lipid bilayer, and play essential roles in bacterial cell selectivity, and antimicrobial and antitumor activities [Oh, Shin, Lee et al. (2000); Lim, Kim, Park et al. (2005); Park, Yi, Matsuzaki et al. (2000)]. We demonstrated that the antimicrobial activity towards Candida albicans, Staphylococcus aureus or Escherichia coli may increase with Young's modulus for peptide HP(2-20) and its analogs [Liu, Fang, Huang et al. (2011)], and further exhibited that flexibility is a mechanical determinant of antimicrobial activity for amphipathic cationic α-helical AMPs [Liu, Fang and Wu (2013b)].

By using crystallography techniques, such as x-ray, NMR and EM, the informative models of proteins are resolved at atomic level. These models provide intramolecular interactions among amino acids, secondary and tertiary structures of proteins, as well as intermolecular interactions in protein complexes. Unfortunately, these models also give the impression that proteins in solution are static structures, rigidly waiting for ligands to approach and bind [Fields (2001)]. In fact, biological molecules such as protein and nucleic acids are dynamic instead of static structures [Schlessinger and Rost (2005); Flynn, Jagodzinski, Santana et al. (2013); Antunes, Devaurs and Kavraki (2015)]. Under physiological conditions, biological molecules experience everlasting motion or structural fluctuation in a wide variety of spatiotemporal scales because of their flexibility and uninterrupted external forces [Opron, Xia and Wei (2014, 2015)]. As an intrinsic property of the structure, molecular flexibility varies from molecule to molecule. In a given molecule, flexibility can be different from atom to atom, from residue to residue and from domain to domain [Opron, Xia and Wei (2014)]. Molecular flexibility, together with other molecular properties, such as geometry and electrostatics, determines molecular functions. However, the impact of molecular flexibility on function is often underestimated or even overlooked, which can be a major source of error [Opron, Xia and Wei (2014); Forrey, Douglas and Gilson (2012)].

To visualize and characterize protein dynamics, time-resolved crystallography was developed recent years, including small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), X-ray free electron laser (XFEL) and cyro-EM [Palamini, Canciani and Forneris (2016)]. Meanwhile, the theoretical analysis tools were developed as well. *B*-factor or temperature factor, which is linearly related to the mean square

displacement of an atom in protein crystal structure, is believed be able to calculate the protein flexibility, thermal stability and intrinsic disorder [Radivojac, Obradovic, Smith et al. (2004)]. We also proposed the peptide flexibility index (*F*-factor) as a weighted average of amino acid flexibility index profile over whole residue chain of the peptide. By applying F-factor analysis, we demonstrated that flexibility is a mechanical determinant in antimicrobial action of α-helical antimicrobial peptides [Liu, Fang and Wu (2013b)].

Conformational flexibility of a protein is essential in various biological events, and understanding the contributions of dynamics to its function is a fundamental aspect for the basic and applied biological research. In the present review, we will focus on single molecule techniques and theoretical methods on determining protein flexibility/rigidity and its role in protein function.

2 Single molecule techniques in characterizing molecule flexibility/rigidity

Recent years, many mechanical techniques have been employed to measure flexibility properties of biomolecules, such as DNA [Bryant, Oberstrass and Basu (2012)], RNA [Liphardt, Onoa, Smith et al. (2001)] and protein molecules [Kataoka, Iwaki, Hashimoto et al. (2002)]. In these experimental skills, the molecules are stretched by means of ultrasensitive force instruments, such as atomic force microscopy (AFM) [Grier (2003); Zhao, Davis, Sansom et al. (2004); Marshall, Sarangapani, Wu et al. (2006)], optical/magnetic tweezers [Grier (2003); Bryant, Oberstrass and Basu (2012)] and surface plasmon resonance (SPR) [Mayer, Hao, Lee et al. (2010)], and the force-extension curves of the molecules are applied to extract molecular flexibility parameters. Macromolecular flexibility is calculated quantitatively and visualized qualitatively from atomic resolution data with structural methodologies. Conventional structural technologies include X-ray crystallography, NMR and EM [Borges, Seraphim, Dores-Silva et al. (2016)]. But these structural approaches do not provide sufficient details concerning flexibility and dynamics. Therefore, new detectors, including solution X-ray scattering and cryo-electron microscopy (cryo-EM), appear to carry out time-resolved studies in order to overcome those limits to molecular flexibility information. Original from mechanical technologies and structural approaches, a numerical prediction method via steered molecular dynamic (SMD) simulation has been developed to calculate flexibility of protein or polypeptide [Liu, Fang, Huang et al. (2011)]. Based on all those methodologies mentioned above, a series of parameters are derived to characterize the molecular flexibility, such as spring constant [Marshall, Sarangapani, Wu et al. (2006)], bending rigidity [Fang, Wu, McEver et al. (2009)], *B*-factor [Liu, Fang, Huang et al. (2011)], F-index [Liu, Fang and Wu (2013b)] and Young's modulus [Liu, Fang, Huang et al. (2011)].

2.1 Evaluation of the spring constant through atomic force microscopy

In single-molecule experiments of biomechanics, the molecular elasticity is usually measured from the deformation in response to a controlled applied force. Generally, spring constant is obtained from the slope of the force-extension curve approach (stretch method). Differently, we suggested an alternative method based on a recently developed thermal fluctuation theory (thermal method) [Marshall, Sarangapani, Wu et al. (2006); Wu, Fang, Yang et al. (2005)]. The principle is to measure the variation in thermal fluctuations of the

AFM cantilever tip when it is coupled with a rigid surface via the soft molecule. This new method was testified by flexibility measurements of L- and P-selectin complexed with its ligands P-selectin glycoprotein ligand-1(PSGL-1), which was showed highly consistent with the measurements from the slope of the force-extension curves through stretch method. And, the spring constants of L- and P-selectin were measured as approximately 4 and1 pN/nm, respectively [Marshall, Sarangapani, Wu et al. (2006)].

2.2 Extension and twist studies with magnetic tweezer

Magnetic tweezer used as a single-molecule manipulation tool started in 1992, when Smith, Finzi and Bustamante firstly applied it to measure the elasticity of single DNA molecule [Smith, Finzi and Bustamante (1992)]. Briefly, one end of a dsDNA molecule was fixed on a glass surface and the other end was immobilized to a magnetic bead which was stretched by magnetic force. In this way, force-extension curve was measured for individual DNA with force of up to ~150 pN. When force in excess of 60 pN was applied to DNA, unexpected overstretching of the dsDNA molecule by about 70% was observed [Cluzel, Lebrun, Heller et al. (1996)]. Technically, the extension of the DNA is determined from the distance between the bead and surface. The force is calculated with a thermal fluctuation technique [Strick, Allemand, Bensimon et al. (1996)]. There is an approximate calculation valid for extensions greater than half of the DNA contour length, and is an exact calculation that leads the same result without making the approximation of small fluctuations [Yan, Kawamura and Marko (2005)]. Then, force-extension curve is plotted to study flexibility properties. Recently, magnetic tweezers approach was developed as to measure freely fluctuating twist and extension simultaneously, which further shed light on the structural dynamics of large nucleoprotein complexes [Bryant, Oberstrass and Basu (2012)].

2.3 Extraction of B-factor and F-index from X-ray crystal structure

Benefited from *X*-ray diffraction technology, the *B*-factor can be derived from crystal structure to quantitate temperature-dependent vibration from average positions. *B*-factor includes complicated information about molecular flexibility, crystalline disorder, discrepancy between model and data [Borges, Seraphim, Dores-Silva et al. (2016)]. In general, *B*-factor can be used to define thermal motion of individual atom, which describe the uncertainty of atomic positions in crystal structure. The low *B*-factor of the atom indicates ordered and steady property of atom. While the large *B*-factor of the atom means disordered and flexible feature of atom. Therefore, mutant of residues in protein or peptide with lower *B*-factor can significantly increases its thermal stability. For analyzing the conformational stability and their flexibility of amino acid residues and associated proteins, one will convert the *B*-factors of all the atoms to the corresponding *B*-factors value of amino acid residues. In this way, we used *B*-factors to rate antimicrobial peptides (AMPs) flexibility, and further demonstrated the activity-flexibility relationship of AMPs [Liu, Fang and Wu (2013b)]. For the short peptide, the way to calculate the flexibility with *B*-factor is suitable, but for the case of long peptide (or protein), the flexibility difference among various peptides/proteins will be counteracted because of its low weight in long peptide. To address this issue, we defined a flexibility index (*F*-index) as a weighted average of amino acid flexibility index profile over whole residue chain of the protein [Liu,

Fang and Wu (2013b)]. A sliding hat-shaped window with length of n (n=1, 3 or 5) was used to evaluate the values of F-index [Liu, Fang and Wu (2013b); Smith, Radivojac, Obradovic et al. (2003)], considering the neighbor effects on the amino acid flexibility. This flexibility index was better in scaling the peptide flexibility, a reflection of mechanical properties such as tensile and flexural rigidities [Liu, Fang and Wu (2013b)]. It further demonstrated that flexibility index is closely relevant to antimicrobial activity of AMPs, same as other structural parameters such as hydrophobicity and positive charge as well.

2.4 Estimation of molecular bending rigidity with electron microscopy

Flexibility of a molecule not only includes extensional elasticity, which represents the ability for a molecule to resist elongation along its long axis, but also contains bending rigidity, which represents the ability for a molecule to resist lateral deflection away from its long axis. Fang et al. [Fang, Wu, McEver et al. (2009)] reported the first bending rigidity measurements for *P*-selectin and PSGL-1by means of analyzing the curvature distribution of an ensemble of molecules imaged with electron microscopy. The diversity of bent shapes of the adhesion molecules should be determined by thermal fluctuations and follow the Boltzmann distribution. Therefore, the bending rigidity can be calculated from the following equation $EI=k_BT/\lambda^4a^2$, which describes the relating bending rigidity EI to vibration amplitude a and the corresponding eigen values λ, where k_B is the Boltzmann constant and T is the absolute temperature [Gittes, Mickey, Nettleton et al. (1993)]. In this study, the bending rigidities of P-selectin and PSGL-1 were reported to be the order of magnitude of 100 pN/nm^2 [Fang, Wu, McEver et al. (2009)].

2.5 Evaluation of the spring constant of the peptide with SMD simulation

Not only from the wet experimental measurements, mechanical property of a protein or polypeptide can be extracted efficiently from dry numerical prediction via steered molecular dynamic (SMD) simulation as well. In SMD simulation, the tensile force F and extension x of each of the peptides are detected when the peptides are stretched gradually from their initial states. Then the variation of F versus x are recorded, and k, the spring constant of the peptide, is read from the slope of F-x curve with the use of Hook's Law $F=k\times x$. The Young's modulus (E) of the peptides are obtained by the equation, $E=kL/A$, when the peptides are assumed as circular rods with original contour length of L and the cross-sectional area of A. Using this method, the molecular spring constants of a series of amphipathic cationic α-helical antimicrobial peptides were determined, and be proved that the antimicrobial activity is rigidity-enhanced, meaning that a harder peptide has stronger antimicrobial activity [Liu, Fang, Huang et al. (2011)].

3 Flexibility is critical for molecular functions

3.1 Protein flexibility and its function

As mentioned above, numerous proteins contain flexible regions mediating their functions. In the present review, we will focus on how the flexible regions of mechanical sensitive proteins (such as selectins, integrins and Von Willebrand factor) participate in their functions. Selectins and integrins are adhesive molecules mediating the adhesion of leucocytes to inflammatory site [McEver and Zhu (2010)]. Lou et al. [Lou, Yago, Klopocki

et al. (2006)] demonstrated that eliminating a hydrogen bond increased the flexibility of an inter-domain hinge in *L*-selectin and reduced the shear threshold for adhesion by affecting either the on-rate which increases tethering or the off-rate which strengths rolling through augmented catch bonds with longer lifetimes at smaller forces. Integrins are heterodimers of non-covalently associated α and β subunits [Campbell and Humphries (2011)]. Regulation of integrin affinity is thought to be allosteric [Kong, Garcia, Mould et al. (2009)]. Integrins are maintained in the bent, low-affinity state through weak interactions of the transmembrane and cytoplasmic domains of α and β subunits. Signals disrupting these interactions leads to the legs of the α and β ectodomains to separate resulting unbent conformation. Binding of the flexible β tail to talin and kindlin further activates integrin to a high-affinity conformation [McEver and Zhu (2010); Campbell and Humphries (2011)]. Wei et al. [Chen, Lou and Zhu (2010)]demonstrated that stabilization and better orientation of the flexible αA domain ligated to the βA domain in integrin LFA-1 substantially increase on-rate, facilitate conformational changes, and further induce downstream signaling.

Upon stimulated, Von Willebrand factor multimers are secreted from endothelium cell at the site of vascular injury, mediating platelet adhesion by binding to the exposed collagen via its A3 domain and glycoprotein Iba (GPIbα) on platelet surface with its A1 domain [Wu, Lin, Cruz et al. (2010); Springer (2014)]. Von Willebrand factor is considered to be activated through a two-step conformational transition when blood flow alters during bleeding: first, elongation from compact to linear form, and consequently, a tension-dependent local transition to a state with high affinity for GPIbα on platelets [Fu, Jiang, Yang et al. (2017)]. Switch force occurs at about 10 pN, the receptor-ligand interaction follows the first state with slower off-rate or the second state with faster on-rate. Force increases the effects of mutations on Von Willebrand Disease [Kim, Hudson and Springer (2015)]. It has been suggested that the flexible mucin-like segments of GPIbα are likely to enhance VWF-platelet binding *in vivo* [Fu, Jiang, Yang et al. (2017)]. In comparison with VWF-A1 or VWF-A3 domain, VWF-A2 domain lacks a disulfide bond between its N- and C-termini, resulting in a vulnerable structure which is easily unfolded to expose the cryptic cleavage site (Tyr^{1605}-Met^{1606}) in flow [Zhang, Zhou, Zhang et al. (2009); Springer (2014); Wu, Lin, Cruz et al. (2010)]. Through molecular dynamics simulations for wild-type VWF-A1 and its eight gain of function mutants, we found that mutation-induced increase of local flexibility of A1 domain would enhance interaction of VWF and GPIbα [Liu, Fang and Wu (2013a)]. The enzyme ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13) cleaves the cryptic site to prevent aberrant platelet aggregation [Zheng (2015); Dong (2007)]. It has been recently demonstrated with EM and small angle X-ray scattering that ADAMTS13 converts from a "closed" conformation to an "open" one when the inter-domain interaction of the domain spacer and CUB is disrupted, resulting in approximately 2.5-fold increase of its activity [South, Luken, Crawley et al. (2014); Muia, Zhu, Gupta et al. (2014)]. Deforche et al. [Deforche, Roose, Vandenbulcke et al. (2015)] reported that the linker regions and the flexibility around the metalloprotase domain account for conformational activation of ADAMTS13.

In the swinging cross-bridge model of muscle contraction, the myosin cross-bridge performs work by structural change of an elastic element, such as the muscle fibers

[Lewalle, Steffen, Stevenson et al. (2008)]. The fibers are made of actin filaments, myosin, and microtubules etc. Knowledge of the stiffness of the actin and the myosin are essential for estimating of the working stroke. Actin polymerization in response to myosin contraction can act as a mechanical absorber which prevents accumulation of local tension that may disrupt the filament [Yu, Yuan, Lu et al. (2017)]. Formin can bind to cytoplasm membrane indirectly through flexible adapter proteins. Giant protein titin unfolds with force in a non-monotonic manner which regulates the passive elasticity of muscles [Yuan, Le, Yao et al. (2017)].

The activity of enzyme is highly dependent on the flexibility of its active sites. The active sites are considered to formed by relatively weak molecular interactions and hence are more flexible than other domains of the enzymes [Tsou (1993)]. In the action of substrates or other ligands, the conformation of an enzyme can switch from an equilibrium state to another, which is known as the induced fit model [Gianni, Dogan and Jemth (2014)]. Chemical or thermal denaturation can lead to rapid decrease in enzyme activity such as D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribonuclease A (RNase A).

3.2 Peptide flexibility and its function

Flexibility of antimicrobial peptide is associated with its activity towards the bacteria, such as Escherichia coli, Candida albicans, or Staphylococcus aureus [Liu, Fang and Wu (2013b)]. Bertocco et al. [Bertocco, Formaggio, Toniolo et al. (2003)] compared a fusion peptide derived from the influenza virus hemagglutinin with its conformationally restricted analog and found that the rigid analog was less potent in promoting lipid mixing, suggesting the ability of the peptide to sample a broader range of conformations is required for the peptide to destabilize membranes. Another study was carried out with the 22-mer AMP piscidin 1 also suggested that the rigid analog was shown to be less active [Kim, Lee, Shin et al. (2010)]. In contrast, Radchenko et al. reported that AMP activity may not change at all upon the purposeful rigidification of peptides [Radchenko, Michurin, Grygorenko et al. (2013)]. We found that rigidity enhanced antimicrobial activity for linear cationic α-helical peptide HP (2-20) and its four analogues [Liu, Fang, Huang et al. (2011)], and further demonstrated that flexibility, as a mechanical determinant of antimicrobial activity of the peptides, might enhance activity against *E.coli* for stiff clustered peptides or reduce activity against *E.coli* for flexible clustered peptides, and the optimum of flexibility index occurs at about -0.5 [Liu, Fang and Wu (2013b)], suggesting that the effect of flexibility on antimicrobial activity may be involved to the antimicrobial actions, such as stable peptide-bound leaflet formation and sequent stress concentration in target cell membrane, mechanically.

3.3 DNA flexibility and its function

The mechanical properties of DNA are key factors to understand gene replication, repair and regulations. DNA can be described as a semi-flexible polymer, with a persistence length of about 50 nm [Xiao, Zhang, Johnson et al. (2011)]. The bending rigidity and twisting stiffness of the DNA double helix arise from the intrinsic molecular flexibility of the backbones and the bases. In the cell, the rigidity of DNA affects the interactions between DNA and functional proteins, such as architectural, regulatory, catalytic proteins.

When a protein binds to a DNA, there will be some mechanical change in DNA. For example, the force-extension behavior of a single bare DNA is different from a protein-DNA complex. Two DNA-bending proteins, histone-like protein from Escherichia coli strain U93 (HU) [van Noort, Verbrugge, Goosen et al. (2004); Xiao, Johnson and Marko (2010)] and various High-Mobility Group (HMG) proteins [Zhao, Peter, Droge et al. (2017)], can generate from 53° to 140° bends where they bind DNA. At around 10 pN, the extension of protein-DNA complex can be stretched to near the contour length of the original bare DNA. This means that the DNA-protein complexes are flexible. The sequence-independent binding and bending activities are consistent with their important architectural role in chromosome compaction. Moreover, HU-DNA binding has a special bimodal behavior. At low HU concentration, a compaction effect has been observed, whereas at high concentrations (~500 nM) the force curve shifts back to lower forces. A DNA will be stiffer if it contains bound proteins along its length. This bimodal behavior of a protein was not observed in bulk assays but with single-DNA methods.

Tension in the DNA is likely to slow down the binding since work needs to be done to achieve the transition state. Tension may also increase the unbinding of the protein-DNA complexes as indicated by the Bell model. When binding equilibrium is reached, the ratio of the on and off rates reflect the presence of the additional mechanical work. The work will fluctuate in a large scale when the DNA forms a loop or opens an existing loop.

4 Future perspectives

Numerous proteins are formed by more than one domain connected through unstructured or flexible regions, or they present disordered regions, or they can be intrinsically unstructured. These regions are important sits for regulation and usually mediate protein-protein interactions, and thus intrinsically linked to their cellular functions [Borges, Seraphim, Dores-Silva et al. (2016)]. Investigating the role of proteins flexibility to their functions advances our understanding on how proteins exert their function, and facilitates to drug design. Overlooking the role of flexibility may result in improper interpretations of protein structure and function. Modern crystallization techniques and theoretical tools have been developed to take protein flexibility into account as discussed above and other reviews [Palamini, Canciani and Forneris (2016)]. However, challenges are still needed to be overcome. Protein complex crystallization provides the information on proteins interactions in certain binding mode, but critical and valuable information on flexibility is completely absent [Fuentes, Dastidar, Madhumalar et al. (2011)]. Boosted by ever-increasing computational power, over the last decade a large number of methods have been developed to include protein flexibility in structure-based drug discovery (SBDD) [Buonfiglio, Recanatini and Masetti (2015)]. However, accounting for protein flexibility in SBDD is still a challenging task, especially because it would come additionally to the treatment of ligand flexibility. This increase in the dimensionality translates into an explosion of computational costs [Antunes, Devaurs and Kavraki (2015)]. In addition, no single method can address all the tasks on SBDD. Cryo-EM is a powerful tool to investigate the flexible region a protein, but it remains to be seen exactly how much information it can provide about the flexible region. The choice of the most appropriate experimental strategy to carry out the investigation must take into account the overall extent of conformational changes, and will likely involve the usage of multiple structural biology methods [Palamini,

Canciani and Forneris (2016)]. FRET techniques will be particularly useful in bridging the gap between high resolution structural techniques and native-like conditions, as it can even provide structural information in live cells. Another key challenge is to quantify the interaction of flexible region and its counterpart. For instance, some posttranslational modification sites locating at the flexible region of a protein cytoplasmic tail are needed to be exposed for modification, but how long does the exposure last? Signaling complexes enforces proximity between enzymes and their substrates, but how closely? This information is crucial for understanding the biological processes precisely. In summary, although exciting findings on protein flexibility have been increasingly reported, more comprehensive techniques and analysis tools are needed to be developed to provide more insights and contributions to biology, structural biology and pharmacology fields.

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