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Topical Perspectives

Ubiquitin: Molecular modeling and simulations

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ABSTRACT

The synthesis and destruction of proteins are imperative for maintaining their cellular homeostasis. In the 1970s, Aaron Ciechanover, Avram Hershko, and Irwin Rose discovered that certain proteins are tagged by ubiquitin before degradation, a discovery that awarded them the 2004 Nobel Prize in Chemistry. Compelling data gathered during the last several decades show that ubiquitin plays a vital role not only in protein degradation but also in many cellular functions including DNA repair processes, cell cycle regulation, cell growth, immune system functionality, hormone-mediated signaling in plants, vesicular trafficking pathways, regulation of histone modification and viral budding. Due to the involvement of ubiquitin in such a large number of diverse cellular processes, flaws and impairments in the ubiquitin system were found to be linked to cancer, neurodegenerative diseases, genetic disorders, and immunological disorders. Hence, deciphering the dynamics and complexity of the ubiquitin system is of significant importance. In addition to experimental techniques, computational methodologies have been gaining increasing influence in protein research and are used to uncover the structure, stability, folding, mechanism of action and interactions of proteins. Notably, molecular modeling and molecular dynamics simulations have become powerful tools that bridge the gap between structure and function while providing dynamic insights and illustrating essential mechanistic characteristics. In this study, we present an overview of molecular modeling and simulations of ubiquitin and the ubiquitin system, evaluate the status of the field, and offer our perspective on future progress in this area of research.

1. Introduction

Eukaryotic proteins have the potential to undergo a variety of post-translational modifications (PTMs), which greatly broaden their functional and biochemical diversity. Phosphorylation, glycosylation, methylation, redox-related modifications, lipid modification, and ubiquitination are considered to be the major PTMs [1,2]. This review is devoted to ubiquitin (Ub), the molecule that mediates ubiquitination [3,4].

1.1. Ubiquitin and ubiquitination

Ub is a highly stable protein that is evolutionarily well-conserved among the Eukaryota (except for three conservative changes) but absent from members of the other two superkingdoms [5]. This protein is composed of a 76-residue polypeptide and characterized by a compact β-grasp fold that includes a β-sheet composed of five strands packed against a single α-helix (Fig. 1). Most of the core residues of Ub are rigid, although its β1/β2 loop and C-terminal tail exhibit conformational flexibility and therefore may interact with different Ub–binding proteins [6,7]. Ub possesses seven lysines (K6, K11, K27, K29, K33, K48, and K63) that, together with the N-terminus, serve as attachment sites for polyubiquitin chain assembly. These lysines, which cover the surface of Ub, are oriented in distinct directions; therefore, Ub chains with different lysine linkages display different orientations (for in-depth reviews, see [8–10]).

Protein ubiquitination is an energy-dependent, multi-step enzymatic process that operates in all eukaryotic cell types and leads to the modification of substrates by Ub. Single Ubs can be attached to a protein substrate, and chains of Ub can also be formed by

Abbreviations: AFM, atomic force microscopy; DUB, deubiquitinating enzyme; MD, molecular dynamics; PTM, post-translational modification; RDC, residual dipolar coupling; Ub, ubiquitin; UBC, Ub-conjugating domain.

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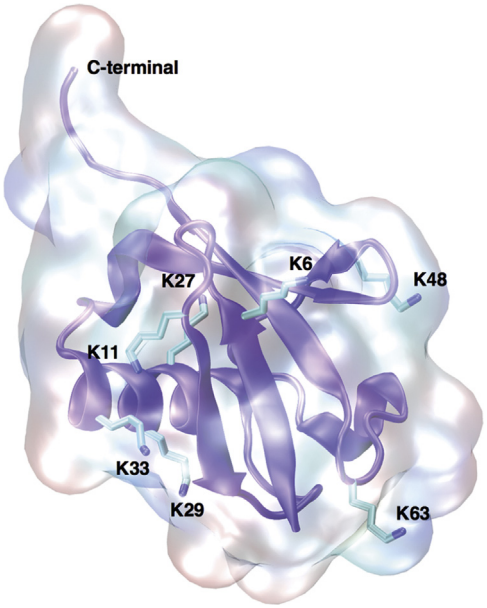

Fig. 1. Diagram of the overall structure of Ub (PDB code 1UBQ). An electrostatic potential surface surrounds the backbone atoms of Ub, which are depicted in a purple cartoon representation, whereas its seven lysines are shown in a licorice representation.

connecting a lysine of one Ub with the C-terminus of another Ub. As a result, ubiquitination includes a diverse set of modifications ranging from monoubiquitination of a target protein to the assembly of poly-Ub chains with varying linkages and lengths. The different Ub–Ub linkages most likely help to dictate the fate of the modified substrate [11–13].

Ubiquitination is achieved through an enzymatic cascade comprised of E1, E2, and E3 enzymes (Fig. 2). These enzymes functionally coordinate to link the C-terminal glycine of the Ub to the target protein through an isopeptide bond, which is an amide linkage outside the main protein backbone [14,15]. The cascade begins when the C-terminal carboxylate of Ub is linked to an active site cysteine of the Ub-activating enzyme (E1), forming a thioester bond in an ATP-dependent manner, and producing an activated Ub [16]. Ub is then transferred to the active site cysteine of a Ub-conjugating enzyme (E2) [17]. In the final step of the ubiquitination cascade, an isopeptide bond is formed between the C-terminal glycine of Ub and an ε-amino group of a lysine residue on the substrate (however, linkages can also form with the amino group at the N-terminus of the substrate). The process of isopeptide bond formation is facilitated Ub ligase (E3), which brings the

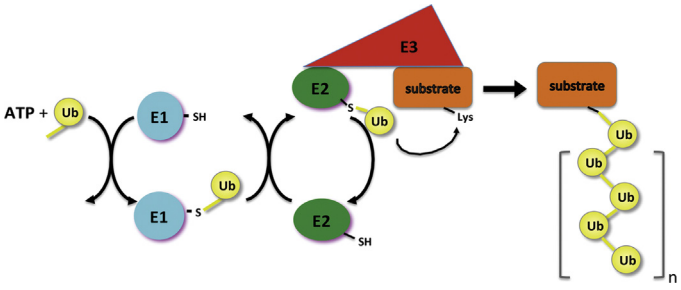

Fig. 2. The Ub-conjugating pathway. Ub is linked, in an ATP-dependent reaction, to an active site cysteine of the E1, forming a thioester bond. Ub is then passed to a cysteine of an E2. Subsequently, attachment of Ub to a substrate, usually to a lysine moiety, is achieved either directly by a RING-type E3 or after intermediate transthiolation to a cysteine moiety of a HECT-type E3. Arrows indicate transfer of Ub. The Ub chain is constructed by repeating the process several times.

substrate and E2 together [18,19]. Because there are dozens of E2s, which in turn can bind with hundreds of E3s in a hierarchical way, the modification of many proteins is highly specific and regulated.

The diversity of the processes regulated by ubiquitination is remarkable, and the consequences of ubiquitination may vary according to the nature of the Ub chain that is attached to the substrate. In the most common scenario, when a K48-linked poly-Ub chain attaches to a substrate protein, the Ub-substrate complex is sent to the 26S proteasome, a large multi-subunit protease complex, where the substrate is degraded into small peptides; the Ub molecule is then recycled (comprehensively discussed in [20,21]). This Ub-mediated degradation process is extremely important for maintaining proper protein homeostasis in cells. However, it was found that ubiquitination has a role in non-proteolytic regulatory mechanisms as well, such as DNA repair [22], iron deficiency signaling [23], cell cycle control [24], and complex cell signaling networks [25].

1.2. Molecular modeling and molecular dynamics simulations

It is not news to the readers of this journal that proteins are not static since their functions are ultimately governed by their dynamic nature [26,27]. X-ray crystallography is commonly used to determine the three-dimensional structures of proteins, yet these structures lack motion. Nuclear magnetic resonance (NMR), Förster (also referred to as Fluorescence) resonance energy transfer (FRET), and other experimental biophysical methods provide valuable information on the dynamics of macromolecules, but are technically limited in terms of sensitivity, applicability and timescales [28,29].

Molecular modeling and molecular dynamics (MD) simulations are computer-aided methods and methodologies used to study the dynamics, structure and interactions of biomolecules. These simulations allow the exploration of the conformational energy landscape accessible to biomolecules, and they allow researchers to connect between a protein's three-dimensional structure, mainly obtained *via* X-ray crystallography and NMR, and its dynamics [30–33]. Increasing computing power, modern hardware [34] and software [35,36], and sophisticated models for interactions between atoms are now extending the timescales of MD simulations to the microsecond range. These simulations can address biological questions and solve problems that may not be accessible to experimental scientists [37,38]. Additionally, enhanced sampling computational techniques facilitate the proposal of mechanistic explanations and provide atomic insights into the studied systems [39–41].

The first MD simulation of a protein was reported in 1977 and illustrated a 9.2-ps trajectory of a small protein in vacuum [42]. More than 35 years after this pioneering simulation, we are now successfully applying molecular modeling and MD simulations to study nearly every type of macromolecule (*e.g.*, proteins, nucleic acids, lipids, carbohydrates), and particularly cellular biomolecular protein-mediated functions such as synaptic vesicle fusion [43], the ribosomal translational machinery [44], action mechanisms of membrane [45] and motor proteins [46], and even protein folding [47].

The roots of molecular modeling and MD simulations began with the notion that molecular geometry, energy, and various molecular properties can be calculated from mechanical-like models that reflect basic physical forces. Accordingly, a molecule is represented as a mechanical system in which the particles, which are its atoms, are connected by springs that correspond to the bonds between the atoms. To assume the molecule's conformation in three-dimensional space, the molecule can vibrate, rotate, and act in response to the intermolecular and intramolecular forces

Table 1
Popular MD simulation packages.

MD simulation package	Website	License	Official description by the developers
Amber [187]	http://ambermd.org	Academic/non-profit/government: $400. Industrial (for-profit): $20,000 for new licensees, $15,000 for licensees of Amber 11 (the previous version and not the updated one)	A collection of numerous programs that work together to setup, perform, and analyze MD simulations, from the preparation of the necessary input files, to the analysis of the results
CHARMM [35]	http://www.charmm.org	Academic users can obtain CHARMM by contacting The CHARMM Development Project. For-profit companies should contact Accelrys Inc.	A versatile and widely used molecular simulation program with broad application to many-particle systems, which has been developed with a primary focus on the study of molecules of biological interest
Desmond [188]	http://www.deshawresearch.com/resources_desmond.html	Desmond and its source code are available without cost for non-commercial use by universities and other not-for-profit research institutions. A license for the commercial use of Desmond is available from Schrödinger, LLC.	A software package developed at D. E. Shaw Research to perform high-speed MD simulations of biological systems on conventional commodity clusters
GROMACS [36]	http://www.gromacs.org	GROMACS is free software, available under the GNU General Public License	A versatile package to perform molecular dynamics, *i.e.* simulate the Newtonian equations of motion for systems with hundreds to millions of particles
NAMD [189]	http://www.ks.uiuc.edu/Research/namd	NAMD is distributed free of charge with source code, yet a special license is needed for commercial use	A parallel MD code designed for high-performance simulation of large biomolecular systems

exerted upon it. The forces between the atoms of the studied system are described by a simplified empirical potential that attempts to mimic the real world underlying interaction potential, referred to as a force field. Simply, a force field is a collection of physical parameters that represent both bonded and non-bonded interactions in the system. It includes a sum of harmonic-like terms (from Hooke's law) for bond length and bond angle deviations from reference equilibrium values, a cosine expansion for torsion angles, as well as Lennard–Jones and Coulomb terms for non-bonded interactions. Using this potential function, the forces (the derivative of the potential with respect to the position) on all atoms in the system of interest are calculated and used to solve classical Newtonian equations of motion.

Practically, in explicit, all-atom MD simulations, thousands to millions of individual atoms representing, for example, all the atoms of a protein and surrounding water molecules, move in a series of short (*e.g.*, 2 fs), discrete time steps. At each step, the forces on each atom, which are determined from the force field, are computed, and the atoms' position and velocity are updated according to Newton's laws of motion. This process is repeated billions of times to provide a continuous atomic trajectory of all atoms in the system and their positions in time. Dynamic, structural, and thermodynamic properties of the simulated system can be calculated using this trajectory. Detailed descriptions and explanations of the method, together with its present limitations, have been widely reviewed (*e.g.*, [30,31,48–51]) and are well-presented in excellent chapters and textbooks [52–54]. A list of the most popular and powerful MD simulation packages can be found on Table 1.

The usage of MD simulations and modeling as computational techniques for investigating the Ub system in atomic detail has been rapidly growing. The relatively high, and constantly increasing, number of structures of Ub and Ub-related proteins deposited at the Protein Data Bank [55] (PDB; more than 1300 due to May 2013) and the accumulation of data regarding the biochemical principles of ubiquitination have both facilitated the application of computational approaches. Of course, modeling and simulations are most effective when performed in close conjunction with experiments on protein function, which play an essential role in validating and improving the simulations [51]. Accordingly, many Ub studies present combined comprehensive research efforts, involving both experimental and computational methods.

1.3. Text limitation and organization

Obviously, the Ub system is very broad in range, while the current review is limited in scope. Given the massive amount of scientific data and the ample number of reviews about the Ub system, we focused our attention on a narrow portion of the vast subject of Ub complexity; specifically, we chose to examine molecular modeling and MD simulations. It should be noted that this text does not survey computer-aided approaches to protein research but rather reflects the implementation of computerized methodologies to Ub system research. Herein, we introduce the interface between Ub research and molecular modeling and simulations; specifically, we describe the contribution of computational tools to the study and understanding of the Ub protein and its counterparts.

In the next section, we describe the dynamics and folding of Ub, and in section three, we discuss the interactions between Ub and other proteins. Studies of the E2s, the E3s, and their interactions are described in the fourth section. Finally, in the fifth section, we provide our view on the exertion of molecular modeling and simulations in this field, and we speculate about what the future may hold.

2. Protein dynamics

Proteins, being the workhorses of the cells, are dynamic biomolecular entities. Hence, understanding proteins' function at atomic level resolution requires addressing their internal fluctuations and motions. The structural flexibility of proteins is thus a key determinant of their biological functionality. It may vary from subtle motions to large conformational changes, and enables the existence of complex biomolecular mechanisms. For example, even the relatively minor internal dynamics of breaking a few van der Waals contacts or hydrogen bonds in a protein, which contains hundreds to thousands of such interactions, can catalyze a chemical reaction or stimulate a signaling cascade [26,27].

Protein dynamics (particularly at the domain level) is important in every cellular process, from catalysis, cell signaling, and allosteric regulation to cellular locomotion [56,57]. In the recent years, the notion that proteins can sample an ensemble of conformations around their average structure as a result of thermal energy has become more widely accepted. Thus, a detailed description of a protein inevitably necessitates probing its multi-dimensional energy landscape that defines the relative probabilities of the various conformational states and the energy barriers between them [58]. Consequently, the well-known concept of the structure–function relationship of proteins is being extended to include dynamics as well [59]. Since time is hidden behind the term dynamics, then frozen snapshots of proteins do not necessarily cover their entire biological wealth. Despite the importance of protein dynamics, the current experimental biophysical methods are pretty limited in their abilities to account for inherent motions of protein. However, MD simulations and other computer-aided approaches can successfully cope with protein dynamics and provide useful insights [60].

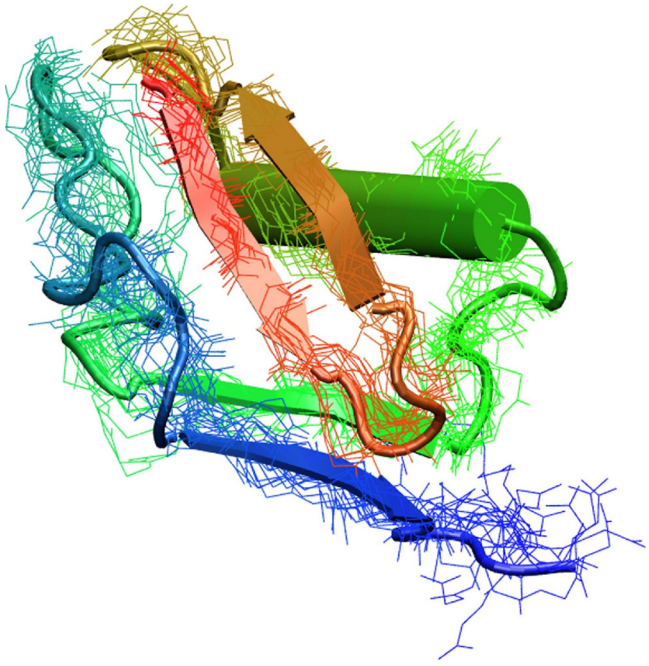

Fig. 3. Ensemble of conformations of Ub. The conformations, which were constructed from MD simulations restrained by Φ and Ψ values, may represent the intermediate states of Ub. The structures were clustered using pairwise Cα RMSD metrics and colored from the N-terminus to the C-terminus with a red-green-blue gradient.

Reprinted from [72], J Mol Biol, 377(2), P. Várnai, C.M. Dobson, M. Vendruscolo, Determination of the transition state ensemble for the folding of ubiquitin from a combination of Phi and Psi analyses, pp. 575–588, © (2008), with permission from Elsevier.

2.1. Ubiquitin dynamics and folding

The process of protein folding has intrigued researchers for decades, and understanding the mechanism by which proteins fold into unique three-dimensional structures is considered the "Holy Grail" of computational biology since Anfinsen determined that proteins fold to a unique structure [61]. Ub's relatively small size, plethora of solved X-ray crystallographic and NMR structures, stability, and the wealth of biochemical information about its function, have made it an attractive model target for studying protein folding and dynamics. Accordingly, MD simulations of Ub were used to scrutinize the validity of different force fields when compared with experimental NMR data [62], and to estimate possible systematic errors in NMR measurements [63].

The early MD simulations of Ub were aimed to explore its dynamic nature. These simulations lasted only a few nanoseconds [64], used a combined explicit-implicit treatment of water [65], or even focused on only one short peptide that was derived from Ub's N-terminal sequence and partly mutated to maximize its folding probability [66]. These studies, along with subsequent studies [67–71] analyzed at the native state dynamics of the protein, exploring its conformational space and accounting for its inherent fluctuations.

These efforts paved the way for much more elaborated and extensive MD studies that examined the folding process from various perspectives, primarily attempting to generate the folding (and unfolding) trajectory. Vendruscolo and co-workers performed a series of MD simulations in which the values of the Φ and Ψ angles of the protein were restrained, and therefore were able to illustrate the conformational space of Ub. They proposed that the obtained set of the possible conformations represents the intermediate folding states of Ub [72] (Fig. 3). The structural organization of Ub was examined under native as well as non-native conditions such as extreme temperatures [68,73–75], pressure perturbations [76], various pH values [68,75,77], or in the presence of organic solvents [68,75,77,78]. It was shown that the folding of Ub is a gradual process progressing in a defined pathway, in which a population of intermediate states appears after an initial hydrophobic collapse phase. The population of intermediate states was identified, and their dynamics was carefully evaluated [67,68,73,74,77–82]. Specifically, Shaw and co-workers probed several of the intermediate folding transition states of Ub by long-timescale MD simulations. These simulations allowed a sufficient number of folding/unfolding events to ensure proper sampling of the conformational space, from an unstructured conformation to a folded protein (Fig. 4) [83].

Given that Ub serves as a common prototype model protein for modeling and simulations, unique aspects of its structural dynamics have been explored. For example, water evaporation causes Ub to shrink in volume and minimize its hydration shell [84,85]; the Ub–Ub interaction, which produces di-Ub dimers, was shown to be characterized by a defined geometrical configuration as the two monomers accommodate each other [86]; confinement of Ub in reverse micelles enabled the study of its interactions with the micelles, expectedly demonstrating that the self fluctuations of the protein are reduced when it is entrapped compared to when it is present in bulk in a native state [87]; full quantum mechanical MD simulation of Ub demonstrated the importance of protein–water charge transfer effects [88]; and most recently, the driving forces of Ub binding to gold nanoparticles have been exposed [89].

Nevertheless, molecular modeling and simulations are obviously not the sole methodologies to address protein dynamics and folding, and a wide variety of experimental techniques were developed for these purposes. Accordingly, joint efforts for studying Ub dynamics and folding, involving both *in silico* and experimental methods have been undertaken. These efforts

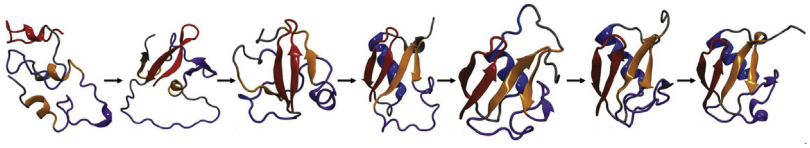

Fig. 4. Intermediate folding states of Ub. Representative structures that occur throughout the folding process of Ub, from a completely unstructured protein (left) to a native state structure (right). The structures were obtained by a cluster analysis of MD simulation trajectories and represent different folding conformers and transition states occurring during the folding of Ub.

Reproduced from [83], Proc Natl Acad Sci U S A, 110(15), S. Piana, K. Lindorff-Larsen, D.E. Shaw, Atomic-level description of ubiquitin folding, pp. 5915–5920, © 2013, with permission from The National Academy of Sciences, USA.

include joint studies of MD simulations that were performed together with circular dichroism (CD) spectroscopy [90–92], small-angle X-ray (SAXS) scattering [90], mass spectroscopy [91], and transient two-dimensional infrared (2D IR) spectroscopy [93]. These studies further investigated either the dynamics of the Ub [92] or characterized its intermediate states during the unfolding and refolding processes [90,91,93].

NMR relaxation is a prevalent experimental method used to quantitatively probe protein dynamics. The local globular structure of a small sphere around residue 45 of a mutant Ub was determined by NMR and subsequently refined by MD simulations [94], long-timescale MD simulations of Ub under denaturing conditions faithfully reproduced an array of NMR-related data [95], and MD simulations with NMR measurements indicated the presence of specific hydrogen bonds that account for Ub dynamics [96]. However, in contrast to the successful previous studies, attempts to reproduce the N–H rate constants measured by NMR by performing MD simulations of three Ub point mutants did not yield satisfactory results. It was concluded that the N–H rate constant cannot be properly obtained using MD simulations [97].

The sensitivity of NMR relaxation to protein motion is in the range of 50 μs to 50 ms; thus, the time window between the correlation time of proteins (which is 4 ns for Ub) and 50 μs is not usually explored by NMR. To uncover this hidden window, a specialized method that uses the residual dipolar couplings (RDCs) has been developed [98,99]. In a series of cooperative studies between the group of Grubmüller from the Max Planck Institute (MPI) for Biophysical Chemistry and experimental groups (as the groups of Griesinger from the MPI for Biophysical Chemistry, Blackledge from the Institute of Structural Biology (IBS, Grenoble), and Salvatella from the Institute for Research in Biomedicine (IRB, Barcelona)), Ub's internal dynamic fluctuations and conformational rearrangements have been extensively probed by MD simulations that were refined and integrated against experimental RDC data [98–102]. In addition to describing the dynamics of Ub, its interaction with other proteins has also been examined; it was suggested that the recognition of Ub is governed by the conformational selection paradigm rather than by the induced fit one (see Section 3 for more detailed descriptions of these paradigms).

2.2. Mechanical unfolding

Computer-assisted mechanical deformation of proteins under a stretching force is a beneficial methodology for probing their unfolding process (*e.g.*, [103,104]). Although Ub is not naturally subjected to forced mechanical stress, this technique appears to be very effective for following its unfolding process and exploring its energy landscape. Similarly, single-molecule force-clamp atomic force microscopy (AFM) is an experimental tool that enables the tracking of protein unfolding under controlled conditions. From the pioneering experiments involving AFM pulling of titin immunoglobulin domains [105] until the present time, AFM has become a prominent mean of studying the mechanical properties of proteins (as detailed in [106]). Therefore, not surprisingly, these computational and experimental methods may complement each other, and are often performed in parallel.

The unfolding process of Ub was studied by computer simulations alone under various stretching forces and simulation methodologies [107–117], and by combined studies involving simulations and single-molecule force-clamp AFM spectroscopy measurements [118,119]. Taken together, these studies traced the Ub unfolding pathway from different perspectives (exploring the role of water, kinetics, thermodynamics, pulling geometry, and the stability of poly-Ub chains) and shed light on the unfolding process of the protein by highlighting its intermediate conformations at atomic level resolution. The emerging scenario implies that after an initial fast collapse of the folded Ub, a broad ensemble of conformations with partial and continuously changing secondary structures and side chain interactions exist simultaneously. This protein ensemble shares common characteristics with the well-known molten-globule state.

3. Protein-protein dynamics and interactions

Non-covalent protein-protein interactions are the central biochemical phenomenon underlying biological signaling and functional control at the molecular level [120]. X-ray structures of protein complexes provide a wealth of structural information yet present only a static snapshot of the structure, leaving the mechanism and dynamics of complex formation unaddressed. Moreover, given the inherent technical difficulties of the X-ray crystallography technique, there are only a small number of known protein complex structures when compared with the growing number of experimentally determined structures of unbound proteins, as reflected at the PDB [55]. This situation encouraged the development of computational methodologies for studying protein-protein interactions.

The earliest mechanistic model for protein-protein interactions was introduced by Emil Fischer at the end of the 19th century. According to this model, the interaction between a substrate and an enzyme resembles that of a lock and a key, as both the substrate and the enzyme assume specific complementary geometric shapes that fit exactly into one another [121]. This concept has been modified into the induced fit model, which was developed by Daniel Koshland. The induced fit model postulates that after the formation of a preliminary encounter complex, the interaction between the binding partners induces conformational changes in both partners until they fit one to the other [122]. Nevertheless, an increasing number of cases suggest a different scenario, in which the interaction between the partners is mediated by the conformational selection mechanistic model. According to this mechanism, binding is primarily enabled due to the conformational diversity of the unbound state of the proteins. The interactions between proteins in a complex shift the pool of protein conformers, which are found in the conformational space, to the direction of bound conformations. Hence, only specific conformers are preferentially selected from the population, reflecting changes in the free energy landscape of the proteins [123–125]. However, recent work indicates that elements from both the induced fit model and the

conformational selection model may co-exist in several cases [126,127]. Evidently, because Ub interacts with various proteins, both models may hold true, either alone or in a combination, depending on the binding partner and the conditions.

3.1. Ubiquitin–substrate protein interactions

Given that Ub may be attached to a large number of proteins, it can modulate many cellular functions. Research over the past several years has expanded our understanding of how Ub interacts with other proteins [128]. Most Ub–protein interactions occur at a few distinct areas on the surface of Ub that are recognized by its binding partners [129]. Surprisingly, only a few studies have dealt with Ub–protein interactions from a computational perspective.

The induced fit paradigm was favored when the interaction between Ub and ataxin-3 was studied. Using MD simulations and a combination of NMR measurements and enzymatic activity assays, it was suggested that the binding mechanism of Ub a to a specific ubiquitin-binding site of ataxin-3 obeys to the induced fit paradigm [130]. On the contrary, the conformational selection paradigm was found to be preferential in other studies. For example, the interaction between Ub and Ub-interacting motif (UIM) derived from hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) was studied using an *in silico* energy-based re-weighting approach involving MD simulations and a statistical distribution of the conformational states of Ub as a function of its distance from its binding partner. It was found that conformational selection holds true in the case of Ub interaction with Hrs-UIM. When the conclusions were broadened, it was determined that conformational selection largely suffices to explain the conformational heterogeneity of Ub in different Ub–protein complexes when the binding partner carries a UIM motif [131]. Further support for the preference of the conformational selection model over the induced fit model was reported when MD simulations of Ub crystal structures with more than 20 different binding partners were refined and integrated against experimental RDC data [98,101].

A mixed model, incorporating both the induced fit and the conformational selection paradigms, was proposed using simulations of Ub in complex with 11 different binding partners. The results of the MD simulations did not correspond to either the complete conformational selection model or to the induced fit scenario. Instead, they suggested a model of conformational restriction, extension, and shift, which describes the full range of observed binding effects. Particularly, a conformational selection model appeared to be appropriate for complex formation when considering the dominant backbone dynamics, whereas some localized differences between bound and unbound conformations were found near the binding interface and corresponded to the induced fit model [132]. A similar combined model of Ub binding blended the conformational selection scheme with the induced fit scheme. This model was based on an extensive structural analysis of 19 complexes of bound and unbound Ub conformations, and it followed the level of their residual induced fit after conformational selection during the binding process. Interestingly, this model succeeded in providing quantitative measures of the respective contributions of both the induced fit and the conformational selection hypotheses [69]. A coarse-grained model for Ub binding to partners with low binding affinity was developed as well. Although this model did not specify the preferred binding hypothesis (induced fit or conformational selection), its results suggest a blending of both paradigms [133].

Not all studies of Ub complexes have specifically dealt with the induced fit and conformational selection paradigms. A study combining X-ray crystallography, biochemical assays, and MD simulations described the structural details and identified key determinants of complexes of Ub and Ub-like proteins with the CHBP protein [134]. A joint study involving NMR experiments, site-directed mutagenesis, and MD simulations suggested that a generic Ub ligand binding process consists of initial localization of the ligand *via* long-range electrostatic interactions, followed by multiple short-range interactions leading to high affinity of the ligand for specific Ub residues [135]. A solely computational study consisting of MD simulations of K48-linked di-Ub in the presence of S5a, which is the proteasomal component that recognizes Ub, showed that binding of di-Ub to S5a reduces the structural mobility of the latter [136].

3.2. Ubiquitin interaction with E1, E2 and E3 enzymes

Molecular modeling and simulation studies of Ub–E1 or Ub–E3 complexes have not yet performed to the best of our knowledge. Additionally, the number of Ub–E2 computational studies is very limited.

3.2.1. Ubiquitin–E2 interactions

The E2s are responsible for the second step in the ubiquitination process, and hence take a key role in the Ub cascade. After the E1 protein activates Ub, the latter is transferred to an E2 protein. However, at the turn of the century, due to the inherent instability of the Ub–E2 thiolester, the structural details of the Ub–E2 complex intermediate at atomic resolution were not known. Using a synergistic approach combing NMR measurements, X-ray crystallography and molecular modeling, the first structural model of the Ub–E2 thiolester intermediate from *Saccharomyces cerevisiae* was constructed [137]. The model provided valuable insights into the Ub–E2 interaction and paved the way for subsequent Ub–E2 computational structural models.

hUev1a is a Ub E2 variant (UEV) that, together with the E2 enzyme hUbc13, forms K63-linked poly-Ub chains. The ternary complex of hUev1a, when non-covalently bound to Ub and hUbc13∼Ub, was subjected to MD simulations that showed a fine array of dynamic hydrogen bonds that affect the tetramer's structure and dynamics, and provided atomic insights to the catalysis process [138,139]. At the same time that the model of the ternary complex Ub-hUev1a-hUbc13∼Ub was published, the crystal structure of Mms2 (yeast UEV) with ubiquitin covalently bound to UBC13 (C87S) *via* an oxyester bond was determined [140]. Taken together, the model and the crystal structure both help to uncover the molecular details of K63-linked chain elongation.

3.3. Ubiquitin–DUB interactions

Deubiquitination is a fundamental mechanism in Ub-mediated signaling networks [141]. Deubiquitinating enzymes (DUBs) are proteases that process Ub or Ub-like gene products, reverse the modification of target proteins by a single Ub-like protein, and may alter poly-Ub-like chains on target proteins. On a structural level, DUBs cleave the isopeptide bond between a lysine residue and the C-terminus of Ub [142]. The human genome encodes nearly 100 DUBs with specificity for Ub in five gene families. The activity of most DUBs is hidden, and conformational rearrangements often occur during the binding of Ub [143,144]. DUBs with specificity for Ub contain insertions and extensions that can modulate DUB substrate specificity, cellular localization, and protein–protein interactions. Structural, biochemical, and genetic studies have revealed the function of DUBs as regulators of diverse processes in yeast and humans [145]. However, the number of computational studies of the DUBs is surprisingly scarce.

The first molecular description of the binding site on Ub for DUB was reported for the DUB UCH-L3. After determination of the binding site by NMR and site-directed mutagenesis, supplemental docking studies found that specific basic residues at the surface of Ub are in a close contact with particular acidic residues

on UCH-L3 [146]. Mass spectroscopy and mutagenesis, corroborated by MD simulations, identified key residues responsible for substrate hydrolysis at the DUB USP2 [147]. The structural conformations of the DUB PfUCHL3, with and without Ub, were studied by X-ray crystallography combined with MD simulations. The simulations allowed the identification of the mediating interactions of PfUCHL3 with Ub and a Ub-like protein; consequently, the dual specificity of PfUCHL3 was discovered at the molecular level [148].

4. E2 and E3 modeling and dynamics

4.1. E2 dynamics

The yeast and the human genomes encode tens of E2 proteins, which are classified into 17 families and govern multitude of cellular events [149]. The E2 proteins orchestrate the attachment of Ub to the substrate protein and are therefore the main mediators of protein ubiquitination [17]. There are currently over 100 three-dimensional structures for E2s, both alone and in complex with protein binding partners. These structures provide a wide range of information regarding how E2s are recognized by various proteins [150]. The structure of an E2 consists of an evolutionarily conserved catalytic core domain (Ub-conjugating domain, UBC) of ~150 residues, characterized by β/α fold in which the highly conserved catalytic cysteine is located in a shallow cleft interacting with the Ub C-terminal tail [137].

The E2s interact with a large number of different E3s to recognize target proteins and attach Ub to these proteins. Because several E2s have also been related to neurodegenerative disorders and cancer, increasing efforts are being devoted to the understanding of E2 regulation, structure-function relationship, dynamics, and binding to other proteins even in the absence of Ub.

Despite the conservation of the UBC domain fold of the E2s, many E2s contain sequence insertions. For example, the yeast Cdc34, a multi-domain E2 enzyme that catalyzes the formation of poly-Ub chains on several proteins and is involved in cell-cycle regulation, possesses a 12 residue insertion at the β4α2 loop of the UBC domain, known as the "acidic loop". By performing MD simulations combined with biochemical assays, the regulatory phosphorylation mechanism of the Cdc34 catalytic domain was revealed. According to these experiments, loop activation by phosphorylation of serine residues in the UBC domain is a mandatory step for efficient Ub-charging and may be responsible for the proper function of downstream events in the Ub pathway as well [151].

To shed light on the interaction between an E2 protein and its target protein, the interaction between the Tsg101 E2 protein and a nonapeptide was studied. Tsg101 bears an E2 variant domain, whereas the 9-residue peptide is found in the Ebola Vp40 and the HIV Gag proteins. By performing multiple MD simulations of the free and bound species and theoretical calculations, the overall loss of configurational entropy upon binding of the two molecules was evaluated [152].

4.2. E3 dynamics

E3 proteins can bind E2s and substrate, invoking a catalysis transfer of Ub from the E2 to the substrate [18]. There are hundreds of E3 proteins, which are classified into two types based on the presence of either a HECT (Homologous to the E6-AP Carboxyl Terminus) domain or a RING (Really Interesting New Gene) domain (or the closely related U-box domain). Transfer of Ub to substrate occurs in two ways: directly from E2 when catalyzed by RING domain E3s, or *via* an E3 when catalyzed by HECT domain E3s. In the latter case, a covalent E3–Ub intermediate is formed before transfer of Ub to the substrate protein takes place [153,154].

The E3s outnumber the E2s, and this may explain why more computational studies have been conducted on E3s. The dynamics of the E3s has been computationally studied in isolation and in the presence of substrate proteins other than the E2s; in both cases, studies have been performed in the absence of Ub.

Only limited number of isolated E3 proteins has been computationally studied thus far. The X-ray structure of Uld, a domain of the E3 protein parkin that interacts with the 26S proteasome, was determined. MD simulations of the wild type Uld and five mutants followed the dynamics and flexibility of the domain. These simulations suggested that some mutations caused structural changes in the Uld domain that lead to its loss of functionality, whereas other mutations showed little influence on the structure of Uld [155].

MD simulations involving the RING domains of two E3 proteins, CNOT4 and c-Cbl, were performed. It was found that the structural stability of their interaction site with a partner E2 is a basic requirement for their RING domains to function as E3 ligases, whereas the interaction site itself is maintained by a hydrophobic core and a hydrogen bonding network. As a negative control, when the RING domain of p44 (a protein with no E3 ligase activity) was simulated, its putative interaction site was not stable and showed structural flexibility [156]. In another effort to characterize the interaction site of an E3 protein, the dynamics of the TIR1 E3 was studied by MD simulations, which proposed an important role for two phenylalanine residues located at its interacting interface [157].

Complementary studies focused on E3s and their binding partners. For example, the dynamics of TIR1 in complexes with auxin and different synthetic analogues and an auxin/indole-3-acetic acid (Aux/IAA) substrate peptide offered a mechanistic interpretation for complex formation [157]. A modeling and docking study of the E3 protein β-TrCP with 4 different phosphorylated peptides (HIV-1 encoded virus protein U (Vpu) fragment, β-catenin fragment, IκB-α fragment, and ATF4 fragment) identified the binding pockets for each of these ligands [158]. Molecular modeling, combined with immunofluorescence microscopy, generated the first structural model of the ASB2α protein, which is the specificity subunit of an E3 ligase complex, when bound to the substrate FLNa [159]. Molecular modeling and binding assays of the CUL4 E3 protein in a complex with the DDB1 the DCAF1 proteins showed that DCAF1 directly recognizes mono-methylated substrates, whereas point mutations in the binding pocket of the DCAF1 chromodomain abolished binding to mono-methylated substrates. This model presented an example of a novel methylation-dependent ubiquitination mechanism for the first time in atomic detail [160].

The Cullin E3 protein, like any other E3 protein, facilitates Ub transfer from an E2-conjugating enzyme to a substrate protein. Thus, Cullin E3 needs to accommodate its structure to different substrate proteins with varying shapes and sizes. To account for this, MD simulations of Cullin E3 in the presence of five different substrate proteins, with and without an adaptor segment that connects the substrate binding proteins to Cullin E3, were performed. It was shown that the adaptor is used as linker that bridges between the substrate binding domain and the E2 active site [161]. This was corroborated by a follow-up MD study with three different Cullin E3s, demonstrating that the flexibility of the linker region is vital. Thus, E3s are not simply inert scaffold proteins, but rather dynamic proteins that can allosterically regulate ubiquitination [162].

4.3. E2–E3 interactions

E3 was subjected to modeling and simulations not only in isolation or in the presence of target proteins, but also in the presence of an E2 protein. The interaction between a HECT domain E3 and the E2 UbcH7 protein was studied using MD simulations and protein modeling techniques, which served to identify specific residues responsible for the E2–E3 interaction and explain the dynamic

process of Ub transfer [163]. The interaction of a RING domain E3 with an E2 protein was investigated using a combination of yeast two-hybrid screening, site-directed mutagenesis, and molecular modeling. These methods were applied to a case involving two E2s (UBC13 and UBCH7) and two E3s (RNF11 and RNF103), indicating the key role of electrostatic interactions in stability and functionality [164]. This observation was corroborated by a study that investigated the interactions between two E2s (UbcH6 and UbcH8) and one RING domain E3 (TOPORS). By combining MD simulations, yeast two-hybrid screening, and site-specific mutagenesis, an internal salt-bridge network located at the rim of the E2–E3 interaction site was revealed. Specific salt bridges that control the balance between binding-competent and non-binding-competent structural orientations of the E2s were identified [165].

5. Future perspectives on Ub modeling and simulations

Almost 40 years have passed since Ub was first identified. Since this time, our knowledge concerning its structural characteristics, biological functions, biochemical network signaling, interactions with other proteins, regulation, catalytic system, and mechanism of action has dramatically expanded. Despite the constant stream of new findings, important pieces of the complex puzzle of ubiquitination are still missing. Due to the complexity of the Ub system and the vast amount of experimental data available, the application of computer-aided approaches is undoubtedly beneficial and highly appreciated in the field of Ub research. Collaborations between computational and experimental researchers dealing with Ub should be more frequently performed to study the molecular details of the system at atomic level resolution. Because biomolecular modeling techniques are becoming increasingly easier to use nowadays, hardware and software turn to be better and faster, and rapid progress is being made to cope with technical limitations, the future holds promise. We believe that molecular modeling and MD simulations will be extremely useful in defined Ub-related aspects, as detailed below.

5.1. Structural elucidation of protein complexes

In the ubiquitination cascade, E1 can bind dozens of E2s, which in turn can bind hundreds of E3s in a hierarchical way. Hence, the amount of possible complexes (such as E1–Ub, Ub–substrate, E2–E3, E3–substrate, E2–E3-substrate, and DUB–Ub) is enormous. Clearly, solving the three-dimensional structure of each of these complexes by X-ray crystallography is tedious and not currently practical. By deploying molecular modeling methodologies and MD simulations, one can predict not only the three-dimensional structure of each of these complexes but also their dynamics. Three-dimensional dynamic models of these protein complexes will boost our understanding regarding their precise biochemical function and mechanism of action at the molecular level.

5.2. Pharmaceutical applications

Understanding the structural mechanism and the molecular interactions that underlie Ub-mediated protein degradation offers a tremendous opportunity for the development of novel therapeutic approaches. Because Ub is known to be involved in cancer, neurodegenerative diseases, genetic disorders, and immunological disorders, the potential pharmaceutical implications of Ub system-targeted drugs are immense. Hence, it is not surprising that the Ub pathway has already been recognized as an emerging drug target for the treatment of cancer [166–169], neurodegenerative diseases [168], and immunological disorders [169,170].

To illustrate the therapeutic potential of Ub and Ub–related drugs, we believe that a series of appropriate three-dimensional models of Ub–related protein complexes will clearly facilitate the identification of drug targets, which is a critical step in the drug development process. Then, examining libraries of small molecules that are most likely to bind these drug targets will be performed by virtual screening. Subsequently, MD simulations of the target alone and in the presence of possible suitable drugs (or drugs' fragments) should be performed. Along with complementary computational chemistry tools, these simulations will enable to validate and finetune the virtual screening results, and also discover novel drug binding sites, including allosteric sites. Next, the chosen drugs will be computationally scored, using free energy ranking, and experimentally tested for their binding and biochemical influence. Obviously, this prototypic suggested procedure highlights only a few guidelines for the process in which modeling and MD simulations of Ub-related complexes can contribute to pharmaceutical efforts (for detailed descriptions, see [171,172]). A few studies that use computerized approaches to examine Ub-related pharmaceutical applications is presented hereinafter.

A slightly different protocol, based upon docking and MD simulations, was recently used to identify potential inhibitors of the SUMOylation process, an enzymatic cascade that is analogous to ubiquitination. After crude docking of nearly 80,000 compounds to the ATP binding site of SUMO E1 that its structure was solved by X-ray crystallography, re-docking of the top hits using a method that incorporates both ligand and protein flexibility was performed. Then, top ranking compounds from the re-docking stage were prioritized using MD simulations incorporating free energy binding calculations. Subsequently, an *in vitro* SUMOylation assay was successfully used to assure binding to the target protein [173]. A shorter protocol, which skipped the use of MD simulations, was performed to discover inhibitors of the DUB UCH-L1. Possible candidates for binding to UCH-L1 were identified by virtual screening of libraries, ranked, and top-scoring compounds were further screened and re-ranked (a different screening software was used at each round). To substantiate the results, an enzymatic assay was effectively conducted [174,175].

A different approach was used to identify a potential inhibitor of the 25K E2 protein. A series of tropical rainforest plant extracts were screened for their ability to inhibit the synthesis of poly-Ub chains of the 25K E2 protein. Biochemical assays found that vitexin, a plant-derived apigenin flavone glucoside, was the active component responsible for inhibition. Docking studies and free energy binding calculations were performed to characterize its binding site with the 25K E2 protein, suggesting that its mode of action involves binding to a known E1 interaction site. Next, NMR spectroscopy was used to confirm the computationally predicted binding site [176].

Academy–industry partnerships in drug discovery and development are currently intensively discussed [177,178], and hence we assume that Ub-related drugs will be developed, eventually, by joint academy-industry efforts. These cross-community academy-industry interactions are necessary to establish databases of Ub-related targets and drugs and map the pharmaceutical needs in a constructive manner. Such a fruitful collaboration has already allowed for the discovery of an E1 inhibitor, and its efficacy as a cancer therapeutic drug was demonstrated in mouse embryonic fibroblasts [179].

5.3. Biomolecular simulations of Ub are only the beginning

5.3.1. The ubiquitination process

The ubiquitination process is carried out by an enzymatic cascade comprised of E1, E2, and E3 enzymes. Like any other chemical enzymatic reaction, this process involves the formation and breakage of covalent bonds. However, in classic MD simulations, covalent bonds remain unchanged.

To account for this discrepancy, it is possible to use a hybrid approach in which the system is mainly simulated by classic MD simulations, except for a small part that is described by a quantum chemical (QM) methodology. This approach was developed by Warshel and Levitt [180], and has been successfully applied (for comprehensive reviews, see [181,182]). Until recently, special software packages were required for QM calculations (*i.e.*, GMAESS [183]), which needed to be fused to the classic MD simulations. QM was only lately implemented and introduced into the MD simulation packages [184], consequently facilitating the implementation of the hybrid approach. An alternative approach to address the formation and breakage of covalent bonds is the use of reactive force fields (ReaxFF) [185,186], which allow atom pairs to bond and detach. However, these force fields are not yet mature enough to cope with complex enzymatic reactions.

We predict that it will be feasible, either by using the above-mentioned approaches or different ones, to simulate the ubiquitination process. Having the ability to model and simulate the events occurring during the ubiquitination cascade will provide an unprecedented opportunity to follow the underlying mechanisms of an enzymatic reaction at atomic resolution.

5.3.2. *The entire Ub system and beyond*

The field of molecular modeling and MD simulations is gaining momentum. By extrapolating the efficiency increase in MD simulations by a factor of 10 every 5 years, it is speculated that we will be able to simulate an entire mammalian cell by the year ≈2056 [50]. Nevertheless, this prediction needs to be regarded only as a rough indication because computer power growth issues, methodological problems (such as proper sampling and accuracy of force fields), and the value of a detailed atomic description of a macroscopic system should be carefully taken into consideration and evaluated. Additionally, technological breakthroughs in computer sciences, biology and engineering may yield more accurate and better techniques than present-day molecular modeling and MD simulations. However, although this forecast is highly speculative, it is tempting to imagine that the entire Ub system and Ub-mediated degradation events, in their physiological cellular context, will eventually be modeled and simulated. This achievement will open a new era of Ub research, in which experimental and computational researchers join efforts to tackle as-yet unknown problems. Setting ambitious goals such as these may lay a solid foundation for future success.

Competing interests

The authors declare no competing interests.

Authors' contributions

AG reviewed the relevant literature and wrote the manuscript. YT and RW provided substantive editing and critical review. All authors read and approved the final manuscript.

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