Using Protein Motion to Read, Write, and Erase Ubiquitin Signals*
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Eukaryotes use a tiny protein called ubiquitin to send a variety of signals, most often by post-translationally attaching ubiquitins to substrate proteins and to each other, thereby forming polyubiquitin chains. A combination of biophysical, biochemical, and biological studies has shown that complex macromolecular dynamics are central to many aspects of ubiquitin signaling. This review focuses on how equilibrium fluctuations and coordinated motions of ubiquitin itself, the ubiquitin conjugation machinery, and deubiquitinating enzymes enable activity and regulation on many levels, with implications for how such a tiny protein can send so many signals.

Ubiquitin was first identified as a small, highly conserved, and heat-stable protein ubiquitously expressed in all eukaryotes and was later shown to be a central regulator of protein homeostasis by directing substrates to the proteasome (1). Since this seminal work in the late 1970s and early 1980s, the covalent modification of substrates with ubiquitin and ubiquitin chains has been shown to control myriad cellular processes (2) and has also been implicated in the pathology and potentially the treatment of numerous diseases (3).

Ubiquitin is typically attached to substrate proteins via an isopeptide bond between the flexible C terminus of ubiquitin and the ε-amino group of a substrate lysine, although substrate proteins can also be ubiquitinated at their N terminus. The initial modification can be differentiated by conjugation of additional ubiquitin molecules at any of ubiquitin’s seven lysine residues or its own N terminus, resulting in the formation of ubiquitin chains. These different chain linkages encode different signals, and tens of thousands of protein isoforms are ubiquitinated in human cells, implying a massive potential regulatory impact for the cell (4). Lys-48 polyubiquitination is the canonical ubiquitin signal marking substrates for degradation (5), although Lys-11 chains have also been shown to encode degradative signals, particularly in the regulation of mitosis (6). Lys-63 and linear chains are involved in driving substrates to specific signaling complexes, including those involved in NF-κB signaling (7), whereas Lys-6 chains have been implicated in autophagy (8, 9). Although all possible linkages have been detected in cells (10, 11), the functions of Lys-27, Lys-29, and Lys-33 chains are still emerging. The possibility of branched chains and chains of mixed linkages provides further complexity. Because the literature on ubiquitination is vast, we will direct the reader to more exhaustive reviews covering other aspects of ubiquitin signaling throughout this minireview.

Ubiquitination of substrates is controlled by the action of three families of enzymes: the ubiquitin activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). Ubiquitin modifications are edited or removed from substrates by deubiquitinating enzymes (DUBs).3 In humans, there are two E1s, 30–40 E2s, over 600 E3s, and over 100 DUBs, resulting in an abundance of regulatory possibilities.

Due to the importance of ubiquitin in eukaryotic biology (not to mention its extreme thermal stability and high solubility), ubiquitin is one of the most biophysically well studied proteins, and a growing body of literature has highlighted the importance of protein dynamics in its function. Here we define dynamics as the combination of the states a molecule can adopt, and the rates with which it samples those states. Protein dynamics are essential in ubiquitin recognition (12), the ubiquitin conjugation machinery exploits large conformational rearrangements to ubiquitinate substrates (13), and various DUBs have been shown to be allosterically regulated by ubiquitin (14) or to depend on the conformational fluctuations of ubiquitin chains (15). In this minireview, we highlight the conformational dynamics of ubiquitin, the dynamic structures of ubiquitin chains, and how the ubiquitination and deubiquitination machinery harnesses conformational dynamics. We have focused on systems in which experimental data provide a solid foundation for an understanding of the states and/or rates of conformational dynamics in the ubiquitin system.

Ubiquitin: The Poster Child for Protein Dynamics
Ubiquitin is a member of the β-grasp family of protein folds, where an α-helix is formed between two pairs of sequential β-strands (Fig. 1) (16). Ubiquitin’s compact fold allows for the dense presentation of sites for interaction with various protein partners, including eight potential covalent ubiquitination sites (seven lysine residues and the N terminus) as well as multiple non-covalent binding sites. Non-covalent engagement of ubiquitin is most frequently facilitated through two surface-exposed hydrophobic patches. The Ile-44 patch contains Ile-44, Leu-8, His-68, and Val-70 and is the ubiquitin surface most frequently engaged by binding partners, including the proteasome (17). The Ile-36 patch contains Ile-36, Leu-8, Leu-71, and Leu-73 and

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3The abbreviations used are: DUB, deubiquitinating enzymes; Ub, ubiquitin; Ubl, ubiquitin-like protein; RDC, residual dipolar coupling; UBA, ubiquitin-associated domain; cIAP1, cellular inhibitor of apoptosis protein-1; SMAC, second mitochondria-derived activator of caspases.
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is involved in intrachain interactions within ubiquitin chains and is also recognized by some binding partners (2, 18).

The β-grasp family includes many ubiquitin-like proteins (Ubls) that can also covalently modify substrate proteins through the action of analogous E1, E2, and E3 enzymes (19). Ubl domains are also frequently found embedded in polypeptides involved in ubiquitin signaling. Despite the prevalence of structural homologs of ubiquitin and variation on the DNA level, the amino acid sequence of ubiquitin itself is extremely well conserved: only 3 out of 76 residues vary from yeast to humans. This implies that almost every residue plays some functional role that impacts evolutionary fitness. For example, recent work has suggested that the high stability of ubiquitin is necessary for efficient ubiquitin recycling upon proteasomal degradation of substrates (20). Ubiquitin’s extreme conservatism and stability have led many to consider it an immovable “rock,” overlooking the intrinsic conformational dynamics of ubiquitin that have been implicated in its ability to associate with many binding partners (12, 21, 22).

Although the C terminus of ubiquitin is unconstrained and flexible on a fast (i.e. nanosecond) timescale, a portion of ubiquitin’s core near the N terminus of ubiquitin’s α-helix has been known to be dynamic on the much slower microsecond to millisecond timescale since the early 1990s (23–25). Further characterization of these motions with spin relaxation experiments identified that this spatially clustered region fluctuates with a timescale of ~40–100 μs (25–29). This same region has also been observed to be mobile in a recent 1-ms molecular dynamics simulation of the native state of ubiquitin (30). As this region of ubiquitin is rarely contacted by binding partners, the functional significance of this motion remains unclear.

Microsecond conformational dynamics in a different region of ubiquitin were later identified through a hybrid computational and experimental approach using residual dipolar couplings (RDCs). By combining many RDC datasets, ensemble refinement showed that ubiquitin’s β1-β2 loop region was more mobile than previously appreciated. The dominant motion covered by this ensemble has been termed the pincer mode, which captures the opening and closing of the β1-β2 loop with respect to the C-terminal end of the α-helix, and seems to occur with a timescale of ~10 μs (31). Strikingly, the examples of the open and closed conformations observed for apo ubiquitin correlate with states recognized by ubiquitin’s binding partners, such that structures in complex with each binding partner lie along coordinates defined by the pincer (12).

Recognition of Monoubiquitin Relies on Its Conformational Flexibility

The observation that motions observed for apo ubiquitin cover the conformational space of ubiquitin in complex with partners argued in favor of a conformational selection mechanism, in which binding proceeds by picking competent conformations out of a pre-existing equilibrium distribution (12). However, several studies have stressed that interactions with ubiquitin also display characteristics of induced-fit binding mechanisms, in which partner binding triggers a subsequent conformational change (32–34). It is becoming increasingly clear that real protein-protein interactions rarely occur by purely conformational selection or induced-fit mechanisms (35). Despite continued debate about the mechanistic details of ubiquitin binding events, the conformational plasticity of ubiquitin is certainly involved.

The connection between ubiquitin conformational dynamics and binding events has been more directly shown by studies that mutationally alter dynamics. For example, point mutations in the hydrophobic core of ubiquitin (L69S and L69T) that decrease overall protein stability and increase the flexibility of the β-sheet-binding interface impair binding to one class of partner proteins (ubiquitin-interacting motifs (UIMs)) without affecting binding to partner proteins containing a UBA domain (36). These same point mutations were recently predicted to destabilize an “open” substate of the β1-β2 loop (37). If mutations that destabilize a partner-preferred state can negatively affect interactions, can mutations favoring a partner-preferred state strengthen interactions? We have used a combination of structure-based computational design and phage display to stabilize certain states and found several multisite mutants of ubiquitin with greater affinity and high selectivity for various ubiquitin signaling proteins. These variants, containing up to seven core mutations, are thermally stable but possess either altered structures or altered timescales of motion, with concomitant effects on cellular function (21, 22). The precise structures of sparsely populated “hidden states” have been described for wild type ubiquitin, but are unknown for any of the above mutants, and future work will hopefully shed further light on how transitions between particular substates affect function.

One Ubiquitin, Many Chains, Even More Conformations

As ubiquitin is often biologically active in a polymeric context, the structures and dynamics of ubiquitin chains could play key roles in the discrimination of ubiquitin-mediated signals. The eight potential linkage points between ubiquitin
monomers imply a wealth of higher-order interaction surfaces as chains grow in length and composition. Fluctuations between preferred conformations could also play a role in signal regulation as surfaces form and dissolve in response to partner binding, macromolecular context, or post-translational modification.

Because ubiquitin is a globular molecule with a flexible C terminus, ubiquitin chains might be thought of as beads on a string. The crystal structures of Lys-63-linked and linear ubiquitin dimers do exhibit such relatively extended conformations, with most surfaces of each monomer accessible to solvent. However, because the various faces of ubiquitin have affinity for one another, covalently linked ubiquitins can also self-associate with surprising variety (Fig. 2). For example, Lys-48 chains pack closely through their Ile-44 patches (38, 39), implying that Ile-44-binding recognition partners either actively remodel this arrangement or take advantage of transient openings in the chain. Lys-6 and Lys-11 chains also adopt compact conformations, but these are distinct from each other and from Lys-48 chains. The Ile-44 face is exposed on both monomers in Lys-48 dimers, whereas Lys-6 chains adopt an interesting arrangement with one Ile-44 face exposed and the other buried at the dimer interface (40–43).

Although static snapshots of ubiquitin chains have provided insight into how each linkage type differs, these images only hint at the variety of accessible conformations. This is especially true for Lys-63-linked and linear ubiquitin chains, which form extended structures whose precise arrangement probably reflects crystallographic trapping rather than a preferred intersubunit orientation. Solution-based measure-

ments have instead shown that the extended chains can adopt many states or lack distinct conformations altogether (44, 45).

Even the more compact chain types are relatively dynamic, such that Lys-48-linked and Lys-11-linked chains sample open and closed states or flex against one another at equilibrium or in response to environmental factors such as pH (38, 41, 46, 47). Because ubiquitin polymers can grow very long and even contain mixed linkages, it is even possible that in a cellular context, a given chain may exhibit local conformational heterogeneity relative to the bulk properties of the entire chain. It should be noted that flexibility does not imply a loss of information, and effectors can distinguish even conformationally labile chains from one another.

Binding partners and chain remodeling enzymes can directly affect the structure of ubiquitin polymers. For example, because Lys-48 polymers bury their Ile-44 hydrophobic face between ubiquitin monomers but many effectors engage this same surface, recognition requires some kind of conformational rearrangement (for review, see Ref. 48), and the observed intrachain mobility may allow partner proteins access to their recognition sites (46). Single molecule FRET measurements illuminated these conformational distributions previously only measured in bulk and have shown that Lys-48-linked dimers do sample a sparsely populated open state. Binding to a deubiquitinase favors an open state, consistent with stretching of the dimer across the active site, although it is unclear whether this structure is identical to the pre-existing open state. Partner binding affects even the structural distribution of relatively extended Lys-63 and linear dimers. These data imply that ubiquitin-binding proteins can remodel existing structures and/or recognize rarely sampled conformations, and that differential affinities for various chain types could in part stem from the energetic penalties implied therein (15).

**Conformational Dynamics Harnessed by the Ubiquitination Machinery**

The machinery to ubiquitinate substrates and assemble ubiquitin polymers is itself dynamic, with large conformational rearrangements involved in the process of ubiquitination. Broadly speaking, during ubiquitination, an E1 first generates an activated ubiquitin, which is handed off to an E2 conjugator, and ubiquitin is finally directed to specific substrates by an E3. E1s use a multistep mechanism to transfer ubiquitin to an E2 (for review, see Ref. 49). Ubiquitin is first adenylated via ATP, and a large domain rotation presents the catalytic cysteine of the E1 for ligation to ubiquitin via a thioester bond, releasing AMP (50). A subsequent rearrangement induced by the binding and adenylation of an additional ubiquitin moves the conjugated ubiquitin more than 30 Å out of the E1 active site toward the E2. The newly exposed E1-ubiquitin bond is now ready to hand off ubiquitin to the E2, and the remaining adenylated ubiquitin is primed for another round of thioesterification (51).

The E2 enzymes bridge the activity of the E1 and E3 enzymes and catalyze the transfer of the conjugated, donor ubiquitin to various acceptor molecules (for review, see Ref. 52). Although E2s have often been thought of as simple carrier proteins, recent studies have demonstrated that E2 activity can be more
complexly regulated. For example, E2s can be involved in the selection of target residues via weak side-chain interactions (53, 54).

E3 ligases come in three distinct flavors. RINGs do not covalently engage ubiquitin and instead bring together E2 and substrate (reviewed in Ref. 55), HECTs transfer ubiquitin from the E2 to their own active site cysteine and then onward to the substrate (reviewed in Ref. 56), and RBRs use a mechanism that is a hybrid between HECTs and RINGs and includes covalent attachment to ubiquitin (reviewed in Ref. 57).

Although E2−Ub conjugates are inherently unstable, partner E3s of the RING class can increase the rate of discharge, even to substrates that lack a specific interaction with the ligase (58). Structures of E2−RING−E3 complexes have shown that the binding interface is distant from the E2 active site (59), suggesting that RING E3−induced activation of the E2−Ub thioester bond is an allosteric effect (58, 60). Measurement of the rotational diffusion of ubiquitin conjugated to an E2 with and without a RING E3 indicated that the presence of the ligase restricts the mobility of ubiquitin, likely by increasing the population of a “closed” conformation where ubiquitin contacts the E2 (61). Indeed, such a closed conformation has been captured crystallographically in two E2−Ub−RING−E3 complexes (62, 63). In these structures, the E2−conjugated ubiquitin makes contact with both the RING E3 as well as the E2, optimally orienting the ubiquitin moiety for nucleophilic attack through non-covalent interactions. Hence, the long-range allosteric activation of an E2 by RING E3s may originate in the enrichment of catalytically productive orientations from a conformational ensemble.

HECT E3 ligases differ from RINGs in that ubiquitin is transferred from the E2−Ub to the active site cysteine residue of a HECT before transfer to substrate. HECTs are also known to engage E2−Ub conjugates by a different mechanism than RING ligases. In an E2−Ub−HECT complex, both the E2 and ubiquitin are engaged in extensive non-covalent interactions with the HECT domain, inducing conformational changes in the HECT domain that bring the flexibly linked HECT C-lobe into contact with ubiquitin to orient transfer to the active site cysteine of the HECT (64). After transfer of the ubiquitin to the HECT active site cysteine, the C-lobe remains associated with ubiquitin and rotates with respect to the N-lobe to deliver the ubiquitin to its substrate (65, 66).

Conformational rearrangements of particular E3 ligases are also utilized in the regulation of their activity. Several ligases in the RBR family including Parkin, HOIP, and HHARI are autoinhibited through mechanisms that include conformational restriction (67–70). In particular, Parkin structures reveal that its active site cysteine and E2-binding site are occluded by a dense network of intradomain interactions that presumably must unravel to allow full activity (Fig. 3a) (71–73). PINK1, a kinase known to accelerate Parkin ubiquitination, can phosphorylate Ser-65 of the Ubl domain (74–76) or ubiquitin itself (77–79), which may in turn allosterically activate Parkin; however, the precise interplay between PINK1, Parkin, and ubiquitin is still
Deubiquitinases Are Surprisingly Flexible Scissors

DUBs are specialized proteases that cleave the isopeptide or peptide bond between two ubiquitins in a chain or between ubiquitin and a substrate. There are ~100 human DUBs that are separated into five structural classes: USP, UCH, JAMM/MPN, OTU, and MJD. These enzymes regulate many biological processes, including cell proliferation (USP7, USP22), DNA damage response (USP1), ubiquitin biogenesis and recycling (USP5, UCHL1, UCHL3), proteasomal regulation (USP14, UCH37), membrane trafficking (USP8), and immune signaling (OTUD5) (reviewed in Ref. 85).

Most DUBs are modular, containing several domains in addition to their catalytic center, and the nature of these domains implies a substantial layer of regulation (reviewed in Ref. 86). A promiscuous and highly active DUB would be dangerous indeed, with the capacity of erasing a cell’s hard-earned ubiquitin-mediated information content. DUBs are fundamentally proteases, and their activity must also be strictly constrained to ubiquitin modifications. Accordingly, structural and biochemical studies have revealed that many DUBs adopt unproductive states in the absence of ubiquitin or activators and transition to active conformations in the holoenzyme. The molecular nature of the inactive apo state is heterogeneous, and DUBs have adapted various ways to harness protein motion to restrict or trigger activity in a manner that ensures appropriate specificity.

In some cases, the active site of an apo DUB is misaligned so that the catalytic cysteine is not engaged by an activating histidine (87, 88). Proper arrangement of the catalytic residues can be accomplished in multiple ways. UCHL1 is activated by ubiquitin itself, such that binding of the globular portion of ubiquitin realigns the enzyme’s active site around ubiquitin’s C terminus (89). The catalytic center of USP7 also rearranges in the presence of ubiquitin to both tighten around ubiquitin’s C-terminal tail and form a competent active site (88). USP7 can further be allosterically activated by distal regions of the protein to greatly increase activity, possibly by rearranging a “switching loop” that hosts several of the catalytic residues (90). Allostery additionally regulates USP5, with binding of a zinc finger domain to the free C-terminal diglycine of ubiquitin necessary for full activity, possibly as a means to discriminate the enzyme’s preferred substrate of unanchored chains from substrate-linked chains (14). Unfortunately, structural information on the inhibited state of USP5 is lacking, and so the molecular mechanisms of repression and activation are unclear.

Other DUBs are seemingly restricted by occlusion of critical functional units. The active site of USP14 is well formed in the absence of ubiquitin, but two loops block a cleft that would hold a peptide (91). It is unclear how this blockade is relieved, because full-length USP14 is activated by binding to the proteasome but the catalytic domain is not (92). An additional regulatory module may play a role, potentially opening the cleft to accept ubiquitin’s C terminus (91). The active site residues of apo USP8 are also aligned for catalysis, but the same two blocking loops observed in USP14 likewise occlude the ubiquitin-binding cleft of USP8. Additionally, the “fingers” of apo USP8 that would hold the globular portion of ubiquitin are too closed to accommodate these contacts (93). The functional relevance of the active site blockade of USP8 is ambiguous, because the catalytic domain is quite active in isolation. Hence, instead of requiring a trans-factor, the obstructions of USP8 may dynamically sample open conformations that only allow productive binding of the ubiquitin substrate.

Other DUBs exhibit too little order in the unbound state, with regulatory interactions necessary to prop key elements in place to promote activity. The structure of apo UCHL3 exhibits both order and disorder, with one loop occluding a peptide-binding groove near the active site but possessing a completely disordered substrate specificity “crossover” loop (94). Binding of ubiquitin promotes clearance of the groove to accept ubiquitin’s C terminus and stabilization of the crossover loop to pack around same (95). OTUD5 (also known as DUBA) is an even more dramatic case. This enzyme is activated by phosphorylation, and large portions of the ubiquitin-binding site are extremely flexible in the unmodified apo state. Phosphorylation nucleates many intra- and intermolecular interactions between DUBA and ubiquitin to bring together functional elements and promote activity (96). Yeast Ubp8 (USP22 in humans), part of the SAGA transcriptional coactivator complex, also contains several disordered regions and misplaced structural elements that could restrict binding to inappropriate substrates and do gain order upon binding of ubiquitin. Intriguingly, other com-
ponents of SAGA tightly interweave with Ubp8, and the activity of DUB is greatly reduced in isolation, suggesting that formation of the complex helps "prop" the enzyme into its active conformation (97). Most recently, UCH37 has also been shown to adopt distinct conformations when bound to effectors, with opposite effects on catalytic activity. Apo UCH37 may exhibit some conformational flexibility, but binding of INO80G places the enzyme into an inactive state by both blocking the ubiquitin-binding site and disrupting alignment of the active site. By contrast, binding to RPN13 increases activity above that of unliganded UCH37 by constraining the enzyme's crossover loop and increasing affinity for ubiquitin (98, 99).

There are many DUBs and effectors for which the molecular mechanisms restricting activity to ubiquitinated substrates and chains remain tantalizingly vague. For example, USP7 can be stimulated by association of GMP synthase with the same distal regions of USP7 that intramolecularly promote catalysis (90). Also prominent among effector-mediated DUBs are USP1, USP12, and USP46, which are strongly activated by binding to the UAF1 WD40 repeat protein. Association with UAF1 could act like SAGA-Ubp8 to somehow properly align or constrain these DUBs into an active conformation. Future work will no doubt shed more light into how binding partners regulate the activities of the DUBs.

**Perspective**

Given that protein dynamics have been observed in almost every part of the ubiquitin pathway, from ubiquitin itself, to chains, ligases, and DUBs, one might be forgiven for thinking that the whole system randomly flops about like a ball of spaghetti. Instead, a picture emerges of controlled motion that has been exploited for biological effect. Because ubiquitin is used in huge numbers of cellular signaling processes, this may be a mechanism to squeeze every last bit of information content from a tiny polypeptide. Perhaps protein dynamics underlie the cell's ability to route disparate inputs through the ubiquitin machinery and maintain distinct outputs. The coupling of macromolecular motion, especially "invisible" dynamics between sparsely populated substates, to cellular phenotypes is an exciting and emerging area at the interface of biology and biophysics. Ubiquitin-mediated signaling is an outstanding system in which one might deeply understand how life harnesses a seemingly abstruse biophysical concept such as dynamics to drive biology.

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