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The role of allostery in the ubiquitin-proteasome system

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Abstract

The Ubiquitin-Proteasome System is involved in many cellular processes including protein degradation. Degradation of a protein via this system involves two successive steps: ubiquitination and degradation. Ubiquitination tags the target protein with ubiquitin-like proteins, such as ubiquitin, SUMO and NEDD8, via a cascade involving three enzymes: activating enzyme E1, conjugating enzyme E2, and E3 ubiquitin ligases. The proteasomes recognize the ubiquitin-like protein tagged substrate proteins and degrade them. Accumulating evidence indicates that allostery is a central player in the regulation of ubiquitination, as well as deubiquitination and degradation. Here, we provide an overview of the key mechanistic roles played by allostery in all steps of these processes, and highlight allosteric drugs targeting them. Throughout the review, we emphasize the crucial mechanistic role played by linkers in allosterically controlling the Ubiquitin-Proteasome System action by biasing the sampling of the conformational space, which facilitate the catalytic reactions of the ubiquitination and degradation. Finally, we propose that allostery may similarly play key roles in the regulation of molecular machines in the cell, and as such allosteric drugs can be expected to be increasingly exploited in therapeutic regimes.

Introduction

The Ubiquitin-Proteasome System (UPS) (Hershko, 2005) plays a central role in cell life and death. It is involved in numerous pathways, and is a key component of the cellular network. Since cell health depends not only on protein synthesis, but also on its degradation, malfunctioning of the UPS is linked to diseases, such as cancer (Mani and Gelmann, 2005) and AIDS (Klinger and Schubert, 2005). Understanding the mechanism of a machine like the UPS, which is fundamental to cell survival, is important not only on its own; but in particular, it is also expected to facilitate drug discovery. Degradation via the UPS of a malfunctioning or un-needed protein in the cell involves two steps. In the first ubiquitination step, the target protein is labeled with ubiquitin-like proteins (UBLs), such as ubiquitin, SUMO and NEDD8. The pathway initiates by the UBLs' linkage to activating enzyme E1, followed by their transfer to conjugating enzyme E2, and finally, E2 tags them unto target substrate proteins via E3 ligases (Capili and Lima, 2007). In the second step, polyubiquitin

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chain-labeled target proteins by the UPS head to the proteasome for degradation. The proteasome recognizes UBL-labeled target proteins, deubiquitinates them, and proceeds to degrade them (Nalepa et al., 2006) (Figure 1).

The UPS machinery is highly complex; and available data point to allostery as playing a major role in its regulation. Experimental and computational evidence, including crystal (Zheng et al., 2002, Duda et al., 2008, Duda et al., 2011, Das et al., 2009), electron microscopy (Pathare et al., 2012, Lasker et al., 2012, Lander et al., 2012), and atomic force microscopy structures (Gaczynska and Osmulski, 2011), molecular biology and biochemical experiments (Sanjo et al., 2010), and molecular dynamics simulations (Liu and Nussinov, 2009, Liu and Nussinov, 2010a, Liu and Nussinov, 2010b, Liu and Nussinov, 2011, Karaca et al., 2011, Papaleo et al., 2011, Papaleo et al., 2012), all demonstrated allosteric regulation in all UPS steps, including ubiquitination, deubiquitination and degradation. For example, a number of crystallography reports pointed to the presence of distance gaps between the active sites of E1 and E2 (Huang et al., 2005, Souphron et al., 2008), E2 and E3 (Das et al., 2009), and within E3 (Zheng et al., 2002, Duda et al., 2008), raising the question of how efficient catalytic reactions which transfer a UBL from one site to the other can span such apparent tens of Angstroms distances. Conformational changes have been reported for the different steps of the ubiquitination cascade following non-covalent or covalent binding events, and interestingly also caught in the same asymmetric unit cell in crystal structures (Duda et al., 2008), suggesting that all are allosterically regulated. Structural and functional analysis showed that the E2-RING finger-mediated ubiquitination is allosterically activated by the E3 gp78 (Das et al., 2009, Li et al., 2009); NF-kappaB-inducing kinase (NIK) and TNFR-associated factors 3 (TNFAF3) E3 ligases are allosterically regulated by the lymphotoxin-beta receptor (Sanjo et al., 2010); and polyubiquitin substrates allosterically stimulate their own degradation (Bech-Otschir et al., 2009). Molecular dynamics simulations also suggested that cullin-RING E3 ubiquitin ligase (Liu and Nussinov, 2009, Liu and Nussinov, 2010a, Liu and Nussinov, 2010b, Liu and Nussinov, 2011), Cdc34-like E2 enzymes (Papaleo et al., 2011, Papaleo et al., 2012), and sumoylation (Tozluoglu et al., 2010, Karaca et al., 2011) are all allosterically regulated. Recently, an electron microscopy structure of the proteasome regulatory particle was reported showing large conformational rearrangements (Lander et al., 2012), suggesting allosteric regulation of the deubiquitination and degradation process. More and more evidence indicates that the entire UPS is allosterically regulated.

Since the UPS is allosterically regulated, allosteric inhibitors targeting the UPS become important subjects for rational drug design. Allosteric inhibitors such as proline- and arginine-rich peptides (Li et al., 2000, Gaczynska et al., 2003), chloroquine (Sprangers et al., 2008) and clioquinol (Mao et al., 2009), all aiming to inhibit the proteasome, have been extensively studied. Recently, a few allosteric inhibitors have been identified targeting also the ubiquitination pathways. In 2010, an allosteric inhibitor targeting Cullin-RING E3 ligase SCF(Cdc4) was synthesized and crystallized (Orlicky et al., 2010). In 2011, an allosteric inhibitor of the Cdc34 E2 enzyme was identified (Ceccarelli et al., 2011). Considering that the UPS is allosterically regulated, it is anticipated that more allosteric inhibitors will be identified in the near future.

Allostery is a key factor controlling cellular regulation, signaling and response to environmental changes (Nussinov et al., 2011, Ma et al., 2011, Gunasekaran et al., 2004, Leonard and Hurley, 2011, Cavanaugh et al., 2012); and recently allosteric drugs have been on the rise (Kar et al., 2010), and their potential in allosteric machines has been emphasized (Wood et al., 2011, Karagoz et al., 2011, Kenakin, 2012). Here, we propose to review the recent advances with the aim of highlighting how the UPS is allosterically regulated in the different steps and how these steps could be targeted by allosteric drugs. The following

topics will be covered in the review: 1) Allosteric regulation in ubiquitination; 2) Allosteric regulation in deubiquitination and degradation; 3) Allosteric inhibitors and drug design targeting the UPS. Accordingly, the review is divided into three parts addressing these topics.

Allosteric regulation in ubiquitination

E1 is allosterically regulated

UBLs are activated by ubiquitin-activating enzyme E1 in a three-step process. E1 first catalyzes the adenylation of the UBLs. In the next step, E1's catalytic cysteine forms a thioester bond with the UBLs to activate them. Finally, the UBL is transferred from the E1's catalytic cysteine site to the E2's catalytic cysteine site. Huang et al solved the crystal structure of a complex of heterodimeric E1 (APPBP1-UBA3) and E2 (Ubc12) (Huang et al., 2005). There is a ~35 Å gap between the E1 adenylation site and the catalytic cysteine. There is also a ~20 Å gap between the catalytic cysteine sites of E1 and E2. How does E1 bridge these gaps to facilitate the UBL transfer from E1 to E2? The conformation of E1 can be allosterically regulated by adenylation and by the E2 catalytic cysteine and this regulation can be inhibited allosterically. Crystallographic data pointed out that the E1 reaction may depend on a "thioester switch" mechanism (Huang et al., 2007, Schulman and Harper, 2009): when E1 is covalently linked to a UBL via the thioester bond, it induces a large conformational change which increases the E1–E2 affinity. When the UBL is no longer linked to E1, following its transfer to the E2 catalytic cysteine, the conformation of E1 changes again, reducing the E1–E2 affinity and releasing E2 from E1. Here, the thioester bond serves as an allosteric effector to regulate the UBL transfer from E1 to E2. Formation and cleavage of covalent bonds are allosteric effectors, as observed in allosteric post-translational modifications (Nussinov et al., 2012).

E2 is allosterically regulated

The interaction of E2 with E1 elicits the covalent transfer of UBL from E1 to E2; next, E2 interacts with E3, HECT and/or the RING domain, which leads to the ubiquitination of the target protein. Recently, Das et al (Das et al., 2009) discovered that an E2 (Ube2g2) interacts not only with the RING domain of the RING-family E3 ubiquitin ligase gp78, but also with the G2BR domain of gp78. The crystal structure of the gp78 G2BR-Ube2g2 complex showed that G2BR forms a helix that binds the Ube2g2 "backside", which is far away from the RING domain binding site, and opposite to the catalytic cysteine (Figure 2). This seemingly irrelevant interaction not only allosterically induces the conformational change in the loops that surround the catalytic cysteine, but also allosterically increases the binding affinity between Ube2g2 and the E3 gp78 RING domains by nearly 50-fold. Thus this allosteric binding affects the E2 Ube2g2 function in at least two ways: First, when E3 gp78 binds at this allosteric site of E2, it induces conformational changes around the catalytic site to hinder the E1 loading ubiquitin to E2, thus signaling the end of the first step of the ubiquitination; Second, this backside binding allosterically enhances the interaction between the E2 and E3, thus signaling the start of the second ubiquitination step. In these two ways, this backside binding of E2 and E3 allosterically regulates the ubiquitination.

A key question is whether such allosteric regulation is a unique feature for E2 Ube2g2, or a common E2 strategy. Some observations suggest that the function of other E2s may be allosterically regulated in a manner similar to G2BR (Wang and Schulman, 2009). For example, in the E2 UbcH4/5 family, ubiquitin binds noncovalently to E2 via the backside binding site, similar to where Ube2g2 binds to G2BR. This noncovalent interaction helps the self-assembly of polyubiquitin chains in the BRCA1-directed ubiquitination (Brzovic et al., 2006). Another example is the E2 Ubc2. In addition to a RING domain, the E2 Ubc2 binds

to E3 Ubr1 at another basic residue region which increases E2–E3 binding affinity (Xie and Varshavsky, 1999). Smad7, an allosteric effector that regulates the binding affinity of E2 and E3, promotes binding of the E2 UbcH7 to the E3 Smurf2 HECT domain in a yet another example (Ogunjimi et al., 2005).

The interaction between E2 and E3 RING domain also allosterically regulates the E2 active site. Using coevolution and mutagenesis analysis, Ozkan et al. identified coevolved residues that link the E2–E3 interface to the E2 active site, indicating that the E3 interaction may allosterically activate E2 (Ozkan et al., 2005). Further, recently, NMR experiments provided the structural basis for this allosteric activation. An essential hydrogen bond at the E2–E3 interface has been identified as the linchpin for E2 allosteric activation (Pruneda et al., 2012). Dimerization of RING E3s has been reported to stabilize E2~ubiquitin interaction and position the ubiquitin for transfer (Dou et al., 2012b).

The allosteric effect can arise not only by the binding of protein partners; it behooves us to recall that phosphorylation (and all post-translational modification events) (Nussinov et al., 2012) may also serve in this role. One example of allosteric regulation of E2 function via phosphorylation comes from the casein kinase 2 (CK2) which phosphorylates E2 Cdc34-like enzymes (Cocchetti et al., 2008). Molecular dynamics simulations demonstrated that phosphorylation of two serine residues on E2 Cdc34-like enzyme serves as an allosteric, phosphorylation-controlled switch for the opening and closing of the $\beta 4\alpha 2$ loop proximal to the E2 catalytic cysteine, which allosterically regulates ubiquitin loading by E1 onto E2 as well as ubiquitin chain assembly (Papaleo et al., 2011).

Similar to ubiquitination, sumoylation can also be allosterically regulated (Tozluoglu et al., 2010, Karaca et al., 2011). Sumoylation is the covalent attachment of small ubiquitin-like modifier (SUMO) to a target protein. Unlike ubiquitination, the presence of E2 Ubc9 may be sufficient for the sumoylation of the target protein, and RanGAP1E3 RanBP2 is not absolutely required (Bernier-Villamor et al., 2002), even though in the presence of E3, sumoylation is more efficient (Reverter and Lima, 2005). Molecular dynamics simulations in the presence and absence of E3 showed that both are allosterically regulated: in the absence of E3, the Leu65-Arg70 region of SUMO allosterically assisted in the sumoylation, whereas in the presence of E3, E2 Ubc9 loop2 allosterically enhances the sumoylation (Karaca et al., 2011).

Thus, current evidence suggests that E2 function is allosterically regulated, either by protein-protein interaction, or by phosphorylation, or through inherent stimulation by certain structural motifs. Alternate E2 allosteric regulation strategies may also exist. The mechanistic basis of E2 regulation emphasizes the potential success of allosteric drugs targeting E2. One of the examples is the discovery of a small-molecule allosteric inhibitor of the CDC34 ubiquitin-conjugating enzyme (E2) (Ceccarelli et al., 2011), which we will discuss in a later section.

E3 is allosterically regulated

The UBLs are transferred from E2 to the target proteins via E3 ligases. HECT and RING/U-box E3 ligases are the two major classes of E3 ligases, with the existence of some less abundant E3 families such as the RING-between-RING (RBR) protein family (Wenzel and Klevit, 2012). Cullin-RING E3 ligases (CRL) comprise the largest E3 subclass (Jackson and Xiong, 2009). CRL has four components: RING-box (Rbx) proteins, Cullin proteins, Cullin adaptor proteins, and target (or substrate) binding proteins. Each component protein includes two or more domains connected by linkers (Fig. 2). The crystal structure of CRL (Zheng et al., 2002) indicates that there is a 50~60 Å gap between the UBL and the target binding sites. How is this gap bridged for ubiquitination to take place?

Molecular dynamic simulations demonstrated that the flexible linkers of the different components of E3 ligases may provide clues to this distance gap enigma (Liu and Nussinov, 2009, Liu and Nussinov, 2010a). The substrate binding protein (Figure 3) has two domains, the box domain and the substrate-binding domain. These two domains are connected by a linker, either a short four residue-loop, or a complex structure which may include helices and loops. All linkers share a common feature: in the unbound state they serve as allosteric hinges, allowing the substrate binding domain to not only rotate with respect to the box domain; but to preferentially turn toward the E2-ubiquitin (Ma et al., 2011). When the substrate binding proteins are bound to the cullin adaptors, such as Skp1 or Elongin C, the flexible inter-domain linker is locked.

Comparing the crystal structures of the Rbx1-Cul5 complex prior and following binding and thioester bond formation between NEDD8 and Cul5, it can be seen that NEDD8 allosterically dramatically changes the conformation of Cul5 and Rbx (Duda et al., 2008). The linker between the Cul5 WHB and the α/β domains, and the Rbx1 linker between the RING domain and cullin binding domain illustrate large conformational changes upon NEDD8 binding (Liu and Nussinov, 2010b). Thus collectively, 1) flexibility appears an intrinsic common feature in RING-box and cullin proteins; 2) the flexible linkers are locked during the formation of E3 ligases, restricting the motion of the RING domain; and 3) NEDD8 binding allosterically reorients the flexible linker (Ma et al., 2011), activating the CRL.

It has long been believed that cullin is rigid; yet, our molecular dynamics simulations (Liu and Nussinov, 2011) showed that the cullins are flexible with conserved hinges in the N-terminal domain, allowing them to adjust the E2-substrate distance, to allosterically regulate polyubiquitination. These observations led us to propose a “flexible two-arm machine” model to explain how the CRL machine facilitates mono- and poly-ubiquitination (Liu and Nussinov, 2011) (see Figure 3). The substrate binding protein constitutes one arm, and the Rbx1 serves as the other. Both arms are flexible; this allows the initiation, and the chain elongation of the ubiquitination. The cullin, which connects the two arms, serves as the flexible scaffold. Cullins have two domains, C-terminal domain (CTD) and N-terminal domain (NTD). Both are flexible. The flexibility of CTD, together with that of the Rbx1, which is regulated allosterically by neddylation, helps to juxtapose the E2-substrate active sites for monoubiquitination. At the same time, the NTD flexibility suggests that there exists an ensemble of cullins’ conformations ensuring that sufficient space is available for polyubiquitination. Thus, the flexibility of the two arms, which contain the substrate binding- and the Rbx proteins, with the help with the cullin C-terminal domain, facilitate the transfer of the first ubiquitin from the E2 to the substrate. The population shift of the conformational ensemble of the cullin, and of the two arms, following the ubiquitination events, accommodates the poly-ubiquitination process, allowing a long poly-ubiquitin chain. The cullin ensemble plays a key role in maintaining the distance at a certain range – neither too small nor too large – so as to ensure the catalytic efficiency of the ubiquitination and not to hinder the ubiquitin chain elongation. Of interest, each cullin has a distinct flexibility which allows it to accommodate specific adaptors, substrate binding proteins, and substrates: cullins, together with the substrate binding proteins and Rbx, allosterically regulate both mono- and poly-ubiquitination. Cullin flexibility has been validated by experiments (Figure 4). Examination of the Cul1-Rbx1 scaffold by single-particle electron microscopy imaged two conformations (personal communication, see below). The Cul1-Rbx1 coordinates from the molecular dynamics simulations fit perfectly these two conformations providing further validation that flexibility is indeed an intrinsic feature of the cullin scaffold.

Cullin-RING E3 ubiquitin ligases are not the only E3s that exhibit flexibility. E3 ubiquitin ligase Ltn1 is also flexible, as demonstrated by the single-particle EM (Dr. Dmitry Lyumkis,

Scripps Institute, personal communication; Dmitry Lyumkis, Selom K. Doamekpor, Mario H. Bengtson, Joong-Won Lee, Tasha B. Toro, Matthew D. Petroski, Christopher D. Lima, Clinton S. Potter, Bridget Carragher, and Claudio A. P. Joazeiro, submitted). The overall architecture of Ltn1 is reminiscent of multi-subunit cullin-RING ubiquitin ligase complexes (CRLs) despite being composed of distinct constituent motifs. Phosphorylation-dependent activation of E3 was recently reported by Dou et al. These authors showed that phosphorylation of a conserved tyrosine residue (Tyr371) in E3 c-Cbls can induce the conformational change, which brings E2 into proximity of the substrate binding site, and facilitates ubiquitination of the substrate (Dou et al., 2012a).

An example of allosteric regulation of the E3 ubiquitin ligase is provided by the lymphotoxin- β receptor (LT β R), which regulates the Ubiquitin:NIK (NF- κ B inducing kinase) E3 ligase (Sanjo et al., 2010). Ubiquitin:NIK E3 ligase is comprised of three subunits: TNFR-associated factors TRAF3 (TNF receptor-associated factor 3), TRAF2, and cellular inhibitor of apoptosis (cIAP). The substrate NIK interacts with TRAF3, whereas TRAF2 binds to cIAP. The allosteric regulator LT β R competitively displaces the substrate NIK and redirects the E3 ligase to ubiquitinate the TRAF3 instead. In this way, LT β R successfully rescues the NIK so that the accumulated NIK can bind IKK α (I κ B kinase α) to activate the NF- κ B2 transcription factors, p100 and RelB. The LT β R-driven substrate-switching from NIK to TRAF3 changes TRAF3 from being part of the E3 ligase to a substrate. Thus, effectively, in this case the allosteric regulator alters the substrate specificity by liberating the original substrate and converting a component of the E3 ligase to a new substrate and in this way prevents further degradation of the original substrate.

The activity of E3 can also be regulated by small compounds. In 2000, Turner et al reported that E3 Ubr1 has two allosteric sites besides the substrate binding sites for substrate Cup9 (Turner et al., 2000). Binding to dipeptides in these allosteric sites can allosterically activate the ubiquitination of the Cup9 substrate. This work for the first time linked E3 to the presence of an environmental signal through an allosteric interaction with a small compound, thus presenting attractive targets for allosteric drug discovery (Pickart, 2000).

Unlike E1 and E2, most E3 ligases do not form a covalent bond with UBL, but productively mediate the transfer of the UBLs from the E2 to the substrate. Thus, most E3s can be considered as allosteric effectors, which increase the population of reaction-favored conformational states. Kinetic analysis of the sumoylation showed that E3 RanBP2 is entropy driven (Truong et al., 2011). A favorable entropic activation is consistent with substrate binding to E3 via a conformation selection mechanism and the observation that many E3s are flexible (Tatham et al., 2005). E3s which do not form covalent bonds with UBLs may also help by creating an enzymatic environment favorable for the catalytic action of UBL transfer.

Allosteric regulation in deubiquitination and degradation

The fate of ubiquitinated proteins depends in part on the length and linkage type of the ubiquitin chain. In general, substrates with four or more ubiquitin moieties linked via Lys48 are targeted for degradation by the 26S proteasome. Rather than marking for degradation, Lys63-linked ubiquitination is involved in regulation of endocytosis (Dupre and Haguenaue-Tsapis, 2001), mitochondria inheritance (Fisk and Yaffe, 1999), ribosome function (Spence et al., 2000), post-replicative DNA repair (Hofmann and Pickart, 1999, Ulrich and Jentsch, 2000), and kinase activation (Lamothe et al., 2007). Attachment of a single ubiquitin moiety functions in endocytosis of a number of plasma membrane proteins, protein sorting, and subnuclear trafficking. Deubiquitinating enzymes (DUBs), the enzymes responsible for the removal of ubiquitin by cleaving ubiquitin-protein bond, are responsible

for ubiquitin recycling and regulate ubiquitin-dependent metabolic pathways. DUBs can be classified into five main superfamilies (Komander et al., 2009), one of which is the ovarian tumor (OTU) superfamily (Rual et al., 2005). OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) binds to the E2 UBC13-ubiquitin thioester to inhibit ubiquitination at Lys63 (K63Ub) (Balakirev et al., 2003). Recently, Wiener et al discovered that the OTUB1 inhibition mechanism of E2 is allosterically regulated (Wiener et al., 2012). OTUB1 has two ubiquitin binding sites, one proximal the other distal. The proximal site binds to the donor ubiquitin of E2-ubiquitin complex, whereas the distal binds to a free ubiquitin. The binding of a free ubiquitin at the distal site allosterically triggers a conformational change in the OUT domain and increases the OTUB1 binding affinity to the donor ubiquitin, thus promoting the binding of OTUB1 and donor ubiquitin and disrupting the E2-ubiquitin interaction.

The final step of the ubiquitin-proteasome pathway is the degradation of polyubiquitin-tagged substrate proteins. The 26S proteasome degrades polyubiquitinated proteins by an ATP-dependent mechanism (Varshavsky, 2011). Here too, allostery appears to play an essential role in activation of the degradation process. The 26S proteasome is composed of a 19S regulator complex and the 20S core proteasome (da Fonseca and Morris, 2008). The 20S proteasome has a cylindrical structure with a central catalytic chamber where the substrate protein is broken into amino acids. The 19S regulator has two subunits, the lid and the base subcomplexes. The lid recognizes the polyubiquitin labeled substrate and removes the ubiquitin chain, whereas the base subcomplexes bind to the 20S proteasome. The lid consists of nine proteins including the deubiquitination enzyme. Electron microscopy studies on the 19S regulatory particle found that the lid is bound to the holo-DUB side and interacts with both the base and core particle (Lander et al., 2012). When comparing the structure of the unbound lid with the one bound to the holoenzyme, large conformational changes can be observed pointing to allosteric regulation of the DUB deubiquitination activity.

Not only the deubiquitination, but protein degradation is also activated allosterically. Bech-Otschir et al reported that polyubiquitinated substrates allosterically activate its own degradation (Bech-Otschir et al., 2009). They observed that a polyubiquitinated substrate binding to the 19S regulator not only stabilizes the gate opening of the 20S proteasome; but it also induces conformational changes in the proteasome, which facilitate the channeling of the substrate to the active sites, thus allosterically activating degradation.

Allosteric inhibitors and drug design targeting the UPS

As an extremely important pathway in the cell, the UPS has been a drug target for many diseases, including cancer (Yang et al., 2009), AIDS (Klinger and Schubert, 2005), cardiovascular diseases (Herrmann et al., 2004), chronic neurodegenerative diseases (Ciechanover and Brundin, 2003), immune and inflammatory disorders (Wang and Maldonado, 2006), metabolic diseases (Paul, 2008), and more. The UPS is allosterically regulated; therefore allosteric inhibitors targeting the UPS become important areas for rational drug design. Compared to orthosteric drugs, allosteric drug design has several advantages. Orthosteric drugs block the protein active site; in contrast, allosteric drugs act as a dimmer switch, allowing up- or down-regulation of a protein, and the complex it is involved in. More importantly, allosteric drugs are in general more selective, because they bind elsewhere on the surface. Because active sites are typically highly conserved in protein families, orthosteric drugs can have many side effects, which is generally not the case for allosteric drugs which bind at non-conserved regions of the surface.

Allosteric inhibitors targeting the proteasome, such as proline- and arginine-rich peptides (Li et al., 2000, Gaczynska et al., 2003), chloroquine (Sprangers et al., 2008), and clioquinol

(Mao et al., 2009), have been extensively studied. Unlike the orthosteric inhibitors that bind at the active site of the proteasome, allosteric inhibitors bind away from the catalytic center. These allosteric inhibitors may overcome some forms of drug resistance by active site mutations which appear to persist in response to orthosteric inhibitors. However, they may encounter mutations elsewhere in the structure, which can hamper their action. To date, no allosteric inhibitors have been advanced into clinical trials (Ruschak et al., 2011). Eventually, a combination therapy may have better prospects.

Besides targeting the proteasome, recently, a few allosteric inhibitors have been identified targeting the ubiquitination pathways. An allosteric inhibitor, SCF-12, targeting Cullin-RING E3 ligase SCF(Cdc4) was synthesized and crystallized. Orlicky et al (Orlicky et al., 2010) reported that the inhibitor is not at the substrate binding site, but at a site 25 Å away (Figure 5). This allosteric inhibitor exhibits high selectivity. It only inhibits Cdc4, but not Fbw7, even though the structure and substrate binding sites are highly conserved across Cdc4 and Fbw7.

Allosteric inhibitors targeting E2 were also extensively studied. In 2011, an allosteric inhibitor of the Cdc34 E2 enzyme, CC0651, was identified (Ceccarelli et al., 2011). Structure determination revealed that CC0651 inserts into a cryptic binding pocket on hCdc34 distant from the catalytic site, causing subtle but wholesale displacement of the E2 secondary structural elements. CC0651 analogs inhibited the proliferation of human cancer cell lines and caused accumulation of the SCF(Skp2) substrate p27(Kip1). CC0651 does not affect hCdc34 interactions with E1 or E3 enzymes or the formation of the ubiquitin thioester but instead interferes with the discharge of ubiquitin to acceptor lysine residues.

Considering that every step of the UPS is allosterically regulated, it is anticipated that more allosteric inhibitors will be identified in the near future. In our previous study on pVHL, a tumor suppressor protein and a component of E3 ubiquitin ligases, we discovered that conformational changes of pVHL could be allosterically regulated by rescue mutant design (Liu and Nussinov, 2008). We also developed a strategy for identification of allosteric sites that could regulate conformational changes of proteins (Goodey and Benkovic, 2008). This strategy may be applied toward identification of allosteric sites for different steps during the UBL transfer process. For example, preventing UBL from transferring from E1 to E2 to inhibit degradation of target proteins such as tumor suppressors could be a drug discovery target. Specifically, changes in residue-residue correlated motions for the catalytic cysteines, thioester bond, or the E1 hinge region, could be identified from covariance matrix maps generated from simulation trajectories. Residue sites with the largest correlated motions change could be a potential allosteric drug target site candidate. Pathological mutations at the identified drug target site candidates could be searched and compared in such a fashion.

Conclusions and outlook

Allostery is a physical phenomenon (Gunasekaran et al., 2004). It derives from the fundamental fact that biomacromolecules exist in a range of conformations, with certain distribution. The distributions are dynamic, and reflect snapshots in time. The distributions, re-distributions, and the barriers between conformers which need to be overcome during a re-distribution, play a key role in cellular function. A redistribution event, or a 'population shift', takes place following some perturbation, such as that arising from binding, covalent or noncovalent, change in temperature, pH, ion strength, and concentration (Tsai et al., 1999a, Tsai et al., 1999b, Ma et al., 1999, Kumar et al., 2000, Tsai et al., 2009). A population shift from one conformational state to another can take place if either the first state gets destabilized by e.g. the binding event; or the following state gets stabilized. Population shift takes place during the propagation of the allosteric event from the allosteric

to the active site, leading to a change in the active site conformation and its dynamics. Nature has exploited this fundamental phenomenon, and optimized it to regulate biological processes (Pan et al., 2010, Tsai and Nussinov, 2011).

Here we focus on allosteric regulation of the UPS system. We do not have high resolution structures illustrating changes in the active site conformations; however, we observe the outcome of the population shift through changes of the global conformations, and the dynamics. We illustrate how the allosteric effect works by distributions favoring conformations where the linkers are rotated in specific directions, to shrink (for the catalytic transfer reactions) or extend (to leave space for polyubiquitin chain) intermolecular distances. The UPS provides an example how evolution adopted the linkers, and employed them to control crucial multiple functional steps. We propose that this could be a general mechanism pursued in nature in large multimolecular machines. Employing flexible linkers (Ma et al., 2011) is advantageous: it can straightforwardly achieve large conformational changes. On the down side, large conformational changes typically present high kinetic barriers and thus longer time scales; however, depending on the linker sequence and length, this may not constitute a significant hurdle. In our simulations of the substrate binding proteins we observed that large rotations can be observed even already in fewer than 10 nanoseconds. Linker sequences can be pre-encoded, as in the case of the linkers in substrate binding proteins (Liu and Nussinov, 2009), such that barriers between successive states that are hierarchically populated are lower, achieving faster timescales even for large conformational changes.

To date, numerous studies have been carried out on the UPS system, along with its series of mechanistic steps, and its regulation. However, the key role of allostery in its control has not been fully appreciated. We believe that we have only scratched the surface. Unfortunately, much is still unknown; and while structural data is increasingly becoming available, there is still a paucity of structures. Structure determination has also been difficult due to protein disorder, probably largely due to the flexible linkers which lead the linked domains to broadly sample space with respect to each other. Low resolution structures of the assemblies, together with high resolution atomic scale data of the protein components, and their dynamic behavior, such as that obtained via molecular dynamics simulations, can be powerful in figuring out molecular mechanisms. Coupling with functional data can make inroads into the regulation of such molecular machines in the cellular environment. Here, based on currently available structural and biochemical data collected from the literature, and our own work over the last few years, we showed how allostery governs steps throughout the UPS cycle. While this does not imply that other cellular and environmental factors such as concentration and the presence of cofactors, do not play crucial roles, it does indicate that allostery should be considered in mechanistic studies, and consequently, in further future drug discovery efforts.

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Figure 1.

Overview of the Ubiquitin-Proteasome System (UPS). Two steps are involved. The first step is the ubiquitination. In this step, three enzymes: activating enzyme E1, conjugating enzyme E2 and E3 ubiquitin ligase. Ubiquitin first forms a covalent bond with E1, then transferred to E2. Then ubiquitin and E2 forms a complex with substrate and E3 to help ubiquitin transfer to substrate. This process repeats until substrate is labeled by a chain of ubiquitins. The second step is the degradation. The ubiquitin labeled substrate is released from the E3 ubiquitin ligase. It is recognized and deubiquitinated by proteasome. The substrate is degraded.

Figure 2.

Binding to E3 gp78 G2BR domain allosterically regulates the E2 Ube2g2 active site. The unbound Ube2g2 (PDB entry 2CYX) is superimposed on the Ube2g2-gp78 complex. The unbound and bound Ube2g2 structures are shown in yellow and blue, respectively. The gp78 G2BR domain is shown in red. The catalytic site Cys89 is shown in green. Large conformational changes of the loops surround Cys89, including residue Tyr103 (shown in yellow and blue), are induced by E3 binding

Figure 3.

The linkers (in brown) in Cullin-RING E3 ligases (CRL) allosterically regulate the ubiquitination. CRL consists four components, RING-box protein (RBX, cyan), cullin (yellow), adaptor (orange), and substrate-binding protein (SBP, blue). RBX binds to E2 (green) and SBP binds to substrate protein (S, pink). Ubiquitin (Ub) is transferred from E2 to target protein. CRL works as a flexible two arm machine. RBX and SBP are the two flexible arms, and the cullin serves as the flexible scaffold. They work together to adjust the E2-ubiquitin distance for the efficient ubiquitination.

Figure 4.

The flexibility of cullin from the molecular dynamics simulations is validated by electron microscopy. (a) The Cul1-Rbx1 components of SCF are displayed side-by-side with the average cryo-EM map of Ltn1. (b) The random-conical tilt (RCT) reconstruction of one conformer of Cul1-Rbx1 complex identified by negative stain EM at $\sim 40\text{\AA}$ resolution. (c) RCT reconstruction of the second conformer of Cul1-Rbx1 complex identified by negative stain EM at $\sim 40\text{\AA}$ resolution. The Cul1-Rbx1 complex structures from snapshots taken from the MD simulation are fit to the first (e) and second (f) EM conformer, respectively. (d) Overlay of the two conformers identified by EM, showing apparent flexibility in the vicinity of the region between the second and third cullin repeat. (g) The Cul1-Rbx1 crystal structure is overlaid on a simulated $40\text{\AA}$ electron density map, so as to show a more accurate comparison to the low-resolution EM maps. (Dr. Dmitry Lyumkis from the Scripps Institute,

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Figure 5.
An allosteric inhibitor for the Cdc4. The inhibitor (cyan) is not at the substrate binding site (purple), but at a site 25 Å away.