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Diversity of Allosteric Regulation in Proteases

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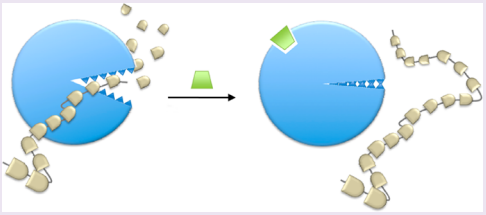
ABSTRACT: Allostery is a fundamental regulatory mechanism that is based on a functional modulation of a site by a distant site. Allosteric regulation can be triggered by binding of diverse allosteric effectors, ranging from small molecules to macromolecules, and is therefore offering promising opportunities for functional modulation in a wide range of applications including the development of chemical probes or drug discovery. Here, we provide an overview of key classes of allosteric protease effectors, their corresponding molecular mechanisms, and their practical implications.



Proteases catalyze the hydrolysis of peptide bonds, thereby performing critical functions in all living organisms. It has been recognized that proteolytic events are essential steps during most biological processes. Prominent examples include key roles in apoptosis, inflammation, protein quality control, or blood coagulation.[1] The participation of proteases in such astonishingly diverse processes is possible because different protease types with unique functional and structural properties have been evolved by nature.[2] This evolutionary process has resulted in the generation of five major protease classes that are classified according to their active site residues and are thus referred to as aspartate, cysteine, serine, threonine, and metallo proteases. Bioinformatic and structural analyses of their evolutionary relationships and three-dimensional folds, respectively, is further used to group them into different clans and families (commonly known as the MEROPS protease classification that has emerged as the standard classification system of proteases).[3] Although a substantial number of protease clans and families are known, some protease families feature more members than others. For example, genome analyses have revealed that more than 550 proteases are encoded in the human genome (the exact number of proteases is still unclear and differs from article to article, depending on the selection criteria).[4] Among these, 178 are serine proteases, while only 21 aspartate or 28 threonine proteases are found. Strikingly, 138 of the 178 serine proteases belong to the S1 family of trypsin-like proteases.[4−6] This uneven distribution of proteases among different clans and families may suggest that some protease families such as the S1 family feature structural peculiarities that are better suited to perform different functions than others.[2,5]

Due to the inherent protein-destructive potential of proteases and their critical biological roles in living organisms, the enzymatic activity of proteases has to be strictly regulated and occurs in a tissue-specific manner on the transcriptional as well as protein level.[1] The latter is thereby not limited to protein abundance but also includes regulation of protease localization, post-translational modifications with particular importance of zymogen (pro-protease) cleavage, and compartmentalization of the active sites coupled to sophisticated substrate selection and delivery mechanisms (as for example realized in the ubiquitin-proteasome system). Besides these mechanisms, recent results demonstrate however that allosteric interactions also play critical roles in the functional regulation of several proteases.[7−9] Allostery is a fundamental biochemical mechanism based on a functional modulation of a site by a distant site and can be caused for example *via* ligand binding to the distant site.[10,11] Such ligands are also often referred to as allosteric effectors. Their binding therefore results in either activation or inhibition of the proteolytic activity of proteases.

Proteases thus join a growing list of proteins for which allosteric regulation has already been shown.[10,12] In fact, the phenomenon of allostery has been studied for decades and resulted in several theoretical concepts such as the textbook Monod−Wyman−Changeux (MWC) model that in its original verbalization represented the first conceptual framework for allostery in oligomeric proteins.[13] Nowadays, allostery is recognized as an intrinsic property of most dynamic proteins.[14] In some cases, the underlying allosteric mechanism is subtle and is based "only" on a change of protein dynamics.[15] In most cases, however, allostery is associated with a conformational change. The coupling of a conformational rearrangement occurring upon ligand binding at an allosteric site is thereby generally explained by two models: (i) The model of an "induced fit" in which the ligand binds to the dominant conformation that induces a conformational switch in the protein.[16] Without directly referring to this model, this concept is often applied to explain the conformational rearrangements seen in structural studies of allosteric effector binding. (ii) The "population shift" model, also known as the "conformational selection" model, which is conceptionally built on the MWC model of allostery (Figure 1).[13] NMR spectroscopy as well as computer simulations indicate that dynamic proteins can adopt

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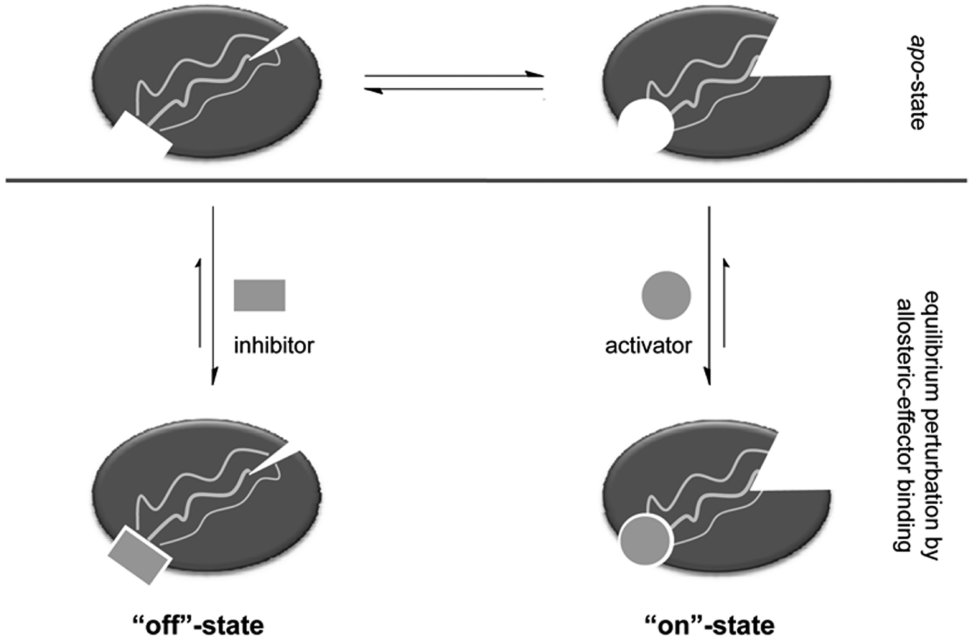

Figure 1. Schematic (and simplified) overview on allosteric regulation of protease activity. Allosteric effector binding either activates or inhibits protease activity. The corresponding active and inactive conformations are characterized by defined free energy landscapes that determine their level of population at the equilibrium state (and thus the level of enzyme activity in the absence of an allosteric effector). Ligand binding changes the corresponding free energy landscapes, resulting in a population shift.[11,18] The required signal propagation occurs *via* multiple, pre-existent intramolecular pathways (indicated by curly lines with dominant pathway in bold).[19]

different conformations that are characterized by distinct energy landscapes.[17] At equilibrium, these conformations are differently populated, often with one conformation being predominantly present. Ligand binding however perturbs the population equilibrium and results in a "population shift" of the different protein conformations as a result of the ligand's preference to interact with a distinct protein conformation (*i.e.*, conformational selection).[18,19] The allosteric signal is thereby transmitted through the protein by multiple, pre-existing pathways.[19]

In most cases, allostery is best described by the conformational selection model.[11,19] Nevertheless, calculations pinpoint that the induced fit model might be more appropriate for particular reaction conditions such as high ligand concentrations.[20] Indeed, kinetic analyses suggest that both reaction mechanisms might occur in parallel and their ratio can be evaluated by determining the "flux" through these pathways.[21]

Alongside its relevance in biology, it is important to note that allosteric control of protease activities may also offer new opportunities for designing small molecules for chemical biology and medicinal applications.[18,22] Proteases have a long-standing history as targets of small molecule probes and drugs.[1,23,24] Their relevance for drug discovery is exemplified by several drugs introduced into clinical practice, for example, for the treatment of HIV infections, cancer, hypertension, blood coagulation control, or type 2 diabetes. However, most of the currently known small molecule regulators target the active site of proteases. For those proteases that share highly homologous active site architectures, the development of protease-selective active site small molecule modulators is however a tedious and highly challenging task. As allosteric sites feature different binding site environments, these target sites might be better suited to achieve selective binding.[25] Furthermore, allosteric sites might present valuable binding sites for generating small molecule activators of proteases.[26] Consequently, small molecule targeting of allosteric sites in proteases could represent an alternative strategy to pharmacologically regulate protease function that unfortunately has so far been underexplored.

To highlight the potential and diversity of allosteric protease regulation for chemical biology research and drug discovery, we will provide an overview on the different classes of allosteric protease effectors as well as of the different conformational events triggered by their binding.

SMALL MOLECULES

Two major types of small molecule allosteric regulators of proteases are known. Most allosteric regulators act *via* reversible, noncovalent interactions; however, chemically reactive, covalently modifying protease regulators are also known. Although functional assays provide a straightforward approach to determine if small molecules act as allosteric effectors, the elucidation of the underlying allosteric regulation mechanism, including its structural basis, often represents a major hurdle. One of the most famous examples for such a case is the trypsin-like serine protease thrombin, for which the underlying allosteric mechanism has been studied for more than four decades.[27] Among several other allosteric effectors, the small cation Na^+ was identified as an allosteric effector already in 1992.[28] On a molecular level, Na^+-binding induces a switch between two well-defined conformations: In the Na^+-free form, thrombin adopts a conformation termed as "fast" because it efficiently cleaves procoagulant substrates. In contrast, the "slow" Na^+-bound conformation exhibits a different proteolytically activity, preferentially cleaving protein C, which results in activation of an anticoagulant pathway.[29] Consequently, the allosteric regulator Na^+ plays an important role in hemostasis. It has however taken numerous biochemical studies, supplemented by mutational and structural approaches to identify the Na^+ binding site, the residues involved in the

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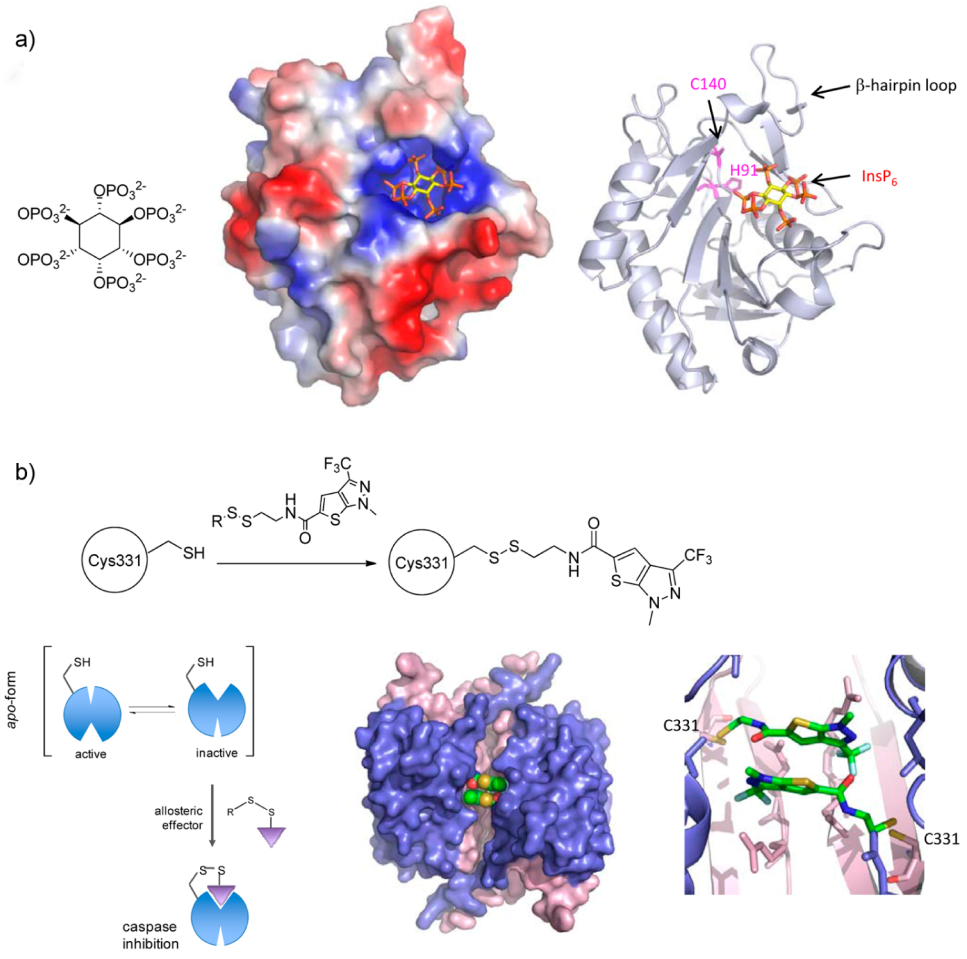

Figure 2. (a) The natural small molecule inositol hexakisphosphate ($InsP_6$, left panel) acts as a molecular activator of the cysteine protease domain (CPD) of *Vibrio cholerae* multifunctional autoprocessing RTX-like (MARTXs) toxins *via* binding into a highly basic (indicated by blue regions in the electrostatic surface presentation, middle panel) allosteric site. Propagation of the allosteric activation signal (right panel) from the allosteric site to the active site residues Cys140 and His91 (in magenta) that are located on the backside of the allosteric site critically relies on a *β*-hairpin loop.[32,34] (b) In caspase-1, allosteric inhibition is achieved *via* a covalent reaction of Cys331 with a thiol-reactive disulfide (upper panel). This compound selectively reacts with Cys331 in the inactive zymogen-like conformation, thereby switching the conformational equilibrium to the inactive species (lower panel, right). Cys331 is located in a central cavity between both subunits (lower panel, middle; the allosteric effector is drawn in spheres). A closer inspection of the binding mode (lower panel, right) reveals that the allosteric effector binds in a symmetrical fashion to both Cys331 residues from the two subunits.[38]

conformational change, and the underlying structural basis of this transition.[29−31]

While Na^+ due to its low molecular mass and high abundance in living organisms is a rather unusual allosteric effector, the natural small molecule inositol hexakisphosphate, $InsP_6$, is a more classical example for a reversibly acting small molecule effector. $InsP_6$ has been identified as a noncovalent allosteric regulator of family C80 cysteine proteases.[32] The corresponding cysteine protease domains (CPDs) are for example elements of the multifunctional autoprocessing RTX-like (MARTXs) toxins occurring in certain bacterial *Vibrio* species in which they act as a virulence factors.[33] Binding of $InsP_6$ to the allosteric site of *apo*-CPD triggers proteolytic activation of CPD that subsequently catalyzes an autoproteolytic activation of MARTX toxin family effector domains.[32,34] As $InsP_6$ is a metabolite exclusively occurring in eukaryotic cells, this allosteric activation mechanism represents a regulatory event that triggers activation of the bacterial CPD exclusively in eukaryotic host cells. The co-crystal structure of $InsP_6$ complexed to *V. cholerae* CPD provided insights into the underlying structural mechanism (Figure 2a).[32,34] It revealed an allosteric binding site built up from mainly basic amino acid residues that communicates with the proteolytic active site *via* a *β*-hairpin region. Interestingly, structural studies with the *Clostridium difficile* large glucosylating toxins (LGTs) TcdA and TcdB revealed, despite only weak sequence homologies, a structurally similar CPD domain that is also allosterically controlled by $InsP_6$ binding.[35,36] Moreover, the allosteric and active sites are connected by a similar allosteric circuit with a *β*-hairpin region as an essential element, revealing a conserved feature of the C80 family of cysteine proteases.[37]

In contrast to the reversible allosteric effector $InsP_6$, studies from the Wells group on human caspases revealed a set of allosteric small molecule effectors that inhibit this family of cysteine proteases *via* a covalent mechanism.[38,39] The identified small molecule effectors modified the thiol functionality of selected cysteine moieties located in a central cavity at the interface of both subunits. Their covalent modification in caspases-1, -3, and -7 *via* a thiol exchange reaction at the disulfide-based small molecules induces an allosteric switch

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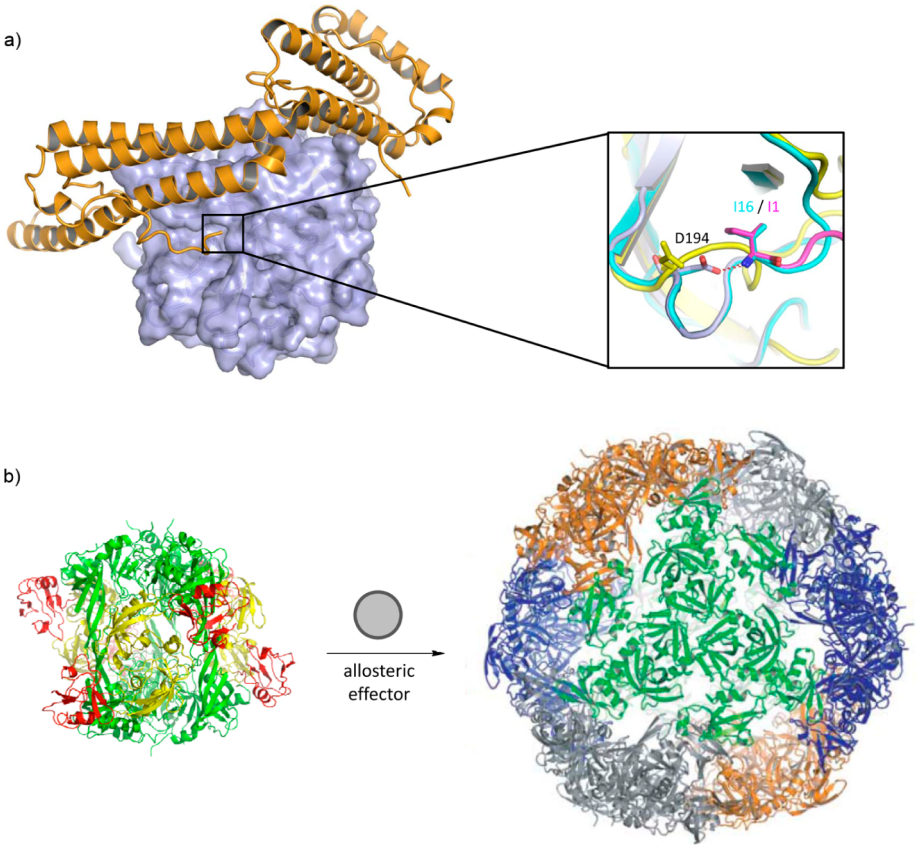

Figure 3. (a) Staphylocoagulase (orange) acts as a proteinaceous allosteric activator of prethrombin-2 (light blue, right panel). To this end, the N-terminus of staphylocoagulase (Ile1, magenta) mimics the well-established zymogen activation mechanism of serine proteases by establishing a salt bridge to Asp194 (light blue, chymotrypsin numbering) of prethrombin, thereby triggering a conformational rearrangement into an active, thrombin-like conformation. For comparison, this structure is superimposed with active α-thrombin (cyan) and the inactive zymogen structure of prethrombin-2 (yellow).[50] (b) Allosteric activation by a proteinaceous effector of the HtrA protease results in a change of oligomeric state from a resting hexamer (protease domain in green, PDZ domains in red and yellow, left panel) to proteolytically active higher ordered oligomers up to 24-mers (built up from trimeric units indicated by different colors, right panel).[64]

from the active protease conformation into a zymogen-like structure (Figure 2b). Structural analyses of the identified allosteric regulators in complex with caspase-1 or caspase-7 revealed that the small molecule modulators were bound symmetrically at the allosteric site, thereby forming an unexpected supramolecular dimeric structure (Figure 2b). Interestingly, mutational studies together with structural data indicate that coupling between the active site and the allosteric site in caspases seems to rely on only a small subset of residues. In caspase-1, a salt bridge between Arg286−Glu390 as part of a larger network of hydrogen bonds aptly termed as "allosteric hotwire" has been identified as critical for signal transmission.[40] A similar analysis for caspase-7 identified the Gly188 hinge residue as well as the L2′ loop as essential.[41] Interestingly, a recent high-throughput screening campaign resulted in the identification of allosteric caspase inhibitors based on copper complexes and thus featuring a different chemotype that accommodated the same allosteric site.[42] In contrast to the allosteric effectors from the Wells group, these compounds however were described as pancaspase inhibitors. Indeed, structural analysis of the binding mode of these compounds to caspase-7 revealed a similar binding mode in which the thiol group of the cysteine residue located in the allosteric site was involved in the formation of a copper complex. In contrast to the disulfide ligands, the copper complexes however also inhibited caspases such as caspase-2 that lack the corresponding cysteine residue at the central cavity. Although no structural model for this unexpected finding could be presented, biochemical studies indicated that caspase-8 inhibition is caused by a suppression of caspase dimerization. Finally, a recent X-ray structure of the zymogen procaspase-1 disclosed an occupation of this allosteric site by Trp294 and Phe295 that were arranged in a similar manner as the allosteric ligands. Again, this finding pinpoints that occupation of this allosteric site drives the conformation equilibrium to the zymogen conformation.[43]

MACROMOLECULAR AND SUPRAMOLECULAR EFFECTORS

While all effectors discussed so far belong to the small molecule realm, macromolecular regulators are also known. For example, the first structural insights on allosteric serine protease regulation by antibodies were reported recently.[44−47] In this context, the extracellular trypsin-like serine protease hepatocyte growth factor activator (HGFA) has been studied in particular, and two different antibodies acting as allosteric effectors have so far been reported. A first antibody termed Ab75 binds to the 60- and 99-loops of HGFA, but without inducing large structural rearrangements.[46] Nevertheless, the binding epitope corresponds to exosite II of thrombin, a related trypsin-like serine protease (*vide infra*), suggesting a related allosteric mechanism that might be conserved in serine proteases of the trypsin family. Nevertheless, the site on HGFA is decorated

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with residues chemically distinct from those found on thrombin's exosite II, and the so far obtained structural data did not reveal a precise mechanism of how Ab75 binding modulates the function of the active site. This was however achieved with a second antibody, Ab40.[44] Ab40 also targets loop99 of HGFA. In contrast to Ab75, however, binding resulted in a conformational change of the 99-loop that spatially minimized the S2 pocket and rearranged the S4 pocket. As a consequence of these structural deviations, HGFA's substrate binding site became incompatible with the native HGFA substrates. Remarkably, the antibody hH35, an allosteric inhibitor of the type II transmembrane serine protease hepsin, also bound to a loop region.[45] hH35 mainly interacted with a Phe-Tyr-motif found in the 170-loop that follows the α3-helix. This interaction resulted in pronounced structural movements, resulting among other reorientations in distortions of the catalytic triad, the oxyanion hole, and the substrate binding pocket.

In contrast to these "artificially raised" antibodies, natural proteinaceous effectors of proteases are known. The human pathogen *Staphylococcus aureus*, for example, secretes staphylocoagulase, a protein that acts as an allosteric activator of prothrombin.[48,49] The underlying structural basis of this activating effect was determined in 2003 when a co-complex structure of staphylocoagulase with prethrombin-2 was published.[50] This study demonstrated that staphylocoagulase mimics the zymogen-active protease transition of serine proteases (Figure 3a).[51] The zymogen of serine proteases is characterized by a "disordered" conformation, *i.e.*, structural flexibility. Upon proteolytic cleavage of the propeptide, the newly formed N-terminus protrudes into a distinct binding site termed activation domain where it forms a salt bridge with Asp194 (chymotrypsin numbering). This structural rearrangement is accompanied by an overall stabilization of the overall molecular structure (the ordered conformation) and, more importantly, the correct conformation of the active site residues, oxyanion hole, and substrate binding site. Interestingly, staphylocoagulase mimics this activation mechanism by protruding its N-terminus into the activation pocket of prethrombin-2, thereby triggering a conformational switch into the active (ordered) protease conformation.

A new type of allosteric activators of caspases that can best be described as "supramolecular" modulators has recently been described by the Wells group. This group originally identified a small molecule named 1541 that effectively triggered allosteric activation of procaspase-3 to caspase-3.[52] Intriguingly, subsequent studies revealed that this small molecule does not act as a "classical" small molecule modulator but self-assembles into nanofibrils that bind to procaspase-3 to promote activation.[53] In fact, these nanofibrils seem to act as allosteric signaling scaffolds that could resemble amyloid-like fibers in natural settings.[54] Therefore, 1541 seems to represent a new prototype of small molecule allosteric regulators that are based on a supramolecular assembly into a higher-ordered structure that subsequently interacts with its target protein.

■ ALLOSTERIC EFFECTOR-TRIGGERED CONFORMATIONAL CHANGES

While these examples demonstrate the chemical diversity of allosteric effectors, the following examples suggest that the triggered conformational rearrangements are also highly diverse, and in some particular cases, these rearrangements are even associated with a change of the oligomeric state of the enzyme.

In proteases, such a change of the oligomeric state has been observed into both "directions". Allosteric protease inhibition can, for example, be achieved by a small molecule disrupting the proteolytically active oligomers. Mechanistically, such allosteric inhibitors can be considered as inhibitors of protein−protein interactions (PPIs) that prevent formation of the active protease.[55] The Craik group has developed such an allosteric disruptor of the dimeric protease of human **K**aposi's **s**arcoma-associated **h**erpes**v**irus (KSHV).[56] In this viral enzyme, the proteolytically inactive monomer is present in a dynamic equilibrium with the active dimer. Binding of the allosteric effector to the monomeric form (and thus *via* a conformational selection) "traps" the inactive monomer, thereby shifting the equilibrium toward the proteolytically inactive species.

An alternative allosteric event, termed "activation by oligomerization", is observed in other proteases, for example, in members of the bacterial HtrA serine protease family. These proteases feature a trypsin-like serine protease domain that is C-terminally linked to at least one PDZ domain.[57] Several members of this protein family are regulated by allosteric mechanisms. For example, the regulatory protease DegS that acts as a protein folding stress sensor in the periplasm of Gram-negative bacteria[58] is proteolytically activated upon allosteric effector binding to its PDZ domain.[59,60] A similar allosteric activation is also observed for DegP.[61] In contrast to DegS, however, this allosteric activation is associated by a switch of DegP's oligomeric state, *i.e.*, from an inactive hexamer to proteolytically active oligomers with up to 24 protomers (Figure 3b).[62−64] Interestingly, the underlying molecular mechanism of the large structural rearrangements seems to be conserved in several HtrA proteases and critically involves rearrangement of loop L3 caused by interaction with the allosteric effector bound at the PDZ binding site.[65] This repositioning is accompanied by several structural rearrangements in the protease domain, resulting in a restoration of the previously distorted active site geometry and the formation of higher-ordered oligomers.[65,66] Interestingly, the rearrangement of loop L3 as a molecular trigger for activation has also been found in the induced fit-activation of the human HTRA1 protease, indicating a conserved mechanism of activation.[66]

■ STRATEGIES FOR IDENTIFYING ALLOSTERIC EFFECTORS

As in "standard" drug discovery, screening, often in a high-throughput format, has evolved into a reliable method to identify allosteric effectors of proteases. These screening approaches might yield small molecules as well as proteinaceous ligands and seem to be particularly rewarding if proteases such as caspases or factor VIIa with a "conserved" allosteric switching mechanism are used.[52,67−70] However, unbiased screens against a target usually yield several hits of which only a limited number act in an allosteric manner. Therefore, methods for screens biased toward the identification of allosteric regulators have been developed. In the field of kinase drug discovery, the Rauh group for example introduced a method in which fluorophore-tagged target enzymes are used to monitor conformational rearrangements upon ligand binding.[71] This method has been used to identify allosteric inhibitors of the cSrc kinase *via* high-throughput screens, and it is tempting to speculate that an application of this methodology to proteases might successfully yield allosteric

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regulators.[72] Alternatively, technologies such as tethering might be useful to "direct" ligand binding to distinct allosteric sites.[73] In tethering, site-directed mutagenesis is used to introduce cysteine residues in binding pockets. Screening of a library of thiol-reactive compounds then increases the chances to identify ligands that bind to these allosteric sites with low affinities. Subsequent fragment-based drug discovery can be applied to develop these initial hits into more powerful small molecule effectors.

Alternatively, a screen for the identification of binders stabilizing the inactive conformations can be applied. For example, this approach has been used to identify peptides serving as allosteric inhibitors of caspase-6 by performing a phage-display screen against the proteolytically inactive zymogen of caspase-6.[74] Further characterization revealed that the identified peptides bound to the tetramerization interface of procaspase-6. Application of such "raised" peptides to caspase-6 leads to inhibition, potentially *via* tetramer sequestration. The peptides therefore seem to act as stabilizers of protein−protein interactions.[75] It should be noted, however, that binders of inactive conformations do not necessarily promote allosteric switching. For example, the Wells group has reported the development of monoclonal antibodies that selectively bind to either the inactive or active conformation of caspase-1, but without inducing a switch between both conformations.[76]

In addition, rational design, for example, *via* structure-guided drug discovery, may be suitable to develop allosteric effectors. This has been used to develop peptide-based small molecule modulators of the HtrA protease DegS.[77] Due to the so far relatively few structural studies addressing allosteric effector binding to proteases, this approach is currently still in its infancy. However, with the continuously increasing structural data, this approach will undoubtedly become more important in the future.

CONCLUSIONS

Recent years have witnessed an enormous increase in literature on allosteric regulation of proteases and have consolidated a view that allosteric regulation of proteases is highly diverse. Being of particular interest for chemical biology and drug discovery research, these studies highlight that allosteric regulation can be achieved with various classes of allosteric effectors. Therefore, targeting of allosteric sites in proteases with customized allosteric effectors represents a powerful alternative approach to perturb cellular proteolysis that warrants further efforts. These endeavors are justified as they may result in the elucidation of novel tools and drugs for protease modulation.

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Notes

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