Published in final edited form as:

Mol Biosyst. 2010 August ; 6(8): 1431–1443. doi:10.1039/c003913f.

Allosteric regulation of protease activity by small molecules

Aimee Shen*

Department of Pathology, Stanford School of Medicine, 300 Pasteur Drive, Stanford, California 94305, USA

Abstract

Proteases regulate a plethora of biological processes. Because they irreversibly cleave peptide bonds, the activity of proteases is strictly controlled. While there are many ways to regulate protease activity, an emergent mechanism is the modulation of protease function by small molecules acting at allosteric sites. This mode of regulation holds the potential to allow for the specific and temporal control of a given biological process using small molecules. These compounds also serve as useful tools for studying protein dynamics and function. This review highlights recent advances in identifying and characterizing natural and synthetic small molecule allosteric regulators of proteases and discusses their utility in studies of protease function, drug discovery and protein engineering.

Introduction to proteases and allostery

Proteases are involved in the control of myriad physiological processes, ranging from cell death to cellular proliferation.¹ By virtue of their ability to irreversibly hydrolyze peptide bonds, the activity of proteases is tightly regulated. Mechanisms for controlling protease function include their production as zymogens (inactive forms of the enzymes that require proteolytic cleavage for activation), segregation from substrates, association with cognate proteinaceous inhibitors, and regulation by cofactors (such as ions, proteins, or sugars).² In addition to these classical mechanisms, it has become increasingly apparent that allosteric mechanisms can control protease activity.

Allosteric regulation is observed when effector binding to a site distinct from the active site alters protein function. The textbook example of allostery is the cooperative binding of oxygen to hemoglobin: binding of oxygen to one subunit of hemoglobin enhances binding of oxygen to neighboring subunits through conformational shifts in quaternary structure. Although allostery was originally defined as being restricted to quaternary proteins, it is now clear that allostery is an intrinsic property of *all* dynamic proteins (reviewed in ref. 3–6). This new updated view is based on the understanding that proteins exist as an ensemble of conformers in dynamic equilibrium rather than as a static structure. A given protein consists of a population of conformers that are in constant flux: some conformers are active, while others are inactive. Events such as ligand binding events, mutation, covalent modification, and changes in environment can shift the conformational equilibria towards an active (in the

case of allosteric activators) or inactive state (in the case of allosteric inhibitors) (Fig. 1). This shift generally involves structural changes in the protein, but this is not an absolute requirement.⁷

Although allosteric regulation *via* ligand binding is frequently observed in metabolic enzymes, there are surprisingly few examples for proteases; most of these mechanisms involve proteinaceous allosteric effectors (for review, see Hauske *et al.*).⁸ Nevertheless, there has been renewed interest in identifying and characterizing allosteric sites within proteases because these sites represent alternative targets for drug discovery over active sites. Since proteases within a given family exhibit remarkable conservation in their substrate binding pockets,⁹ active site inhibitors frequently lack selectivity.^{10,11} As a result, it has been proposed that inhibitors targeting allosteric sites will be more selective than active site inhibitors.^{12,13}

The feasibility of targeting allosteric sites within proteases is illustrated by the ability of small peptides that mimic natural allosteric proteinaceous effectors to modulate protease function. The dipeptide H-Ile-Val-OH, which resembles the activating N-terminus of trypsin, activates trypsinogen (the zymogen of trypsin).¹⁴ Likewise, hydrophobic peptides that resemble the C-terminal ends of misfolded outer membrane proteins activate DegS protease, which controls bacterial envelope stress responses.^{15,16} Although these molecules have provided insights into the activation mechanisms of these proteases, they have limited utility *in vivo* because peptidic compounds often exhibit poor pharmacokinetic properties.

Within the last few years, a number of non-peptidic allosteric effectors have been discovered for proteases, and their mechanisms of action are beginning to be characterized. Most of these modulators are specific for clan CD proteases, which have a common evolutionary origin based on the MEROPS database classification.⁹ In this review, we summarize recent developments in the identification and characterization of natural and synthetic small molecule allosteric effectors. We further discuss how these compounds can be used to study protease function and drive drug discovery and protein engineering efforts.

A natural allosteric activator of bacterial toxin proteases

The only example of a natural small molecule allosteric effector of a protease identified to date is the activation of Family C80 cysteine protease domain (CPD) by inositol hexakisphosphate (InsP₆, Fig. 2A).^{17–20} InsP₆ (or phytic acid) is a eukaryotic-specific small molecule that directly regulates enzymes involved in processes like DNA repair and RNA editing.^{21–23} The CPD is a bacterial protease found within the Multifunctional Auto-processing RTX-like (MARTX)²⁴ and large glucosylating (LGT)²⁵ toxin families. MARTX toxins are a newly recognized family of toxins that modulate the virulence of a number of *Vibrio* sp. pathogens,²⁴ including *Vibrio cholerae*,^{26–28} the causative agent of cholera, as well as the opportunistic pathogen *Vibrio vulnificus*.^{29,30} LGTs are cytotoxic toxins produced by *Clostridium* sp. pathogens^{25,31–33} and are the primary cause of *Clostridium difficile*-associated disease, a frequent and intractable problem in hospital settings.³²

Recent studies have shown that binding of the CPD to InsP₆ results in the autoproteolytic activation of MARTX and LGT toxin family effector domains.^{20,34–36} The *V. cholerae*

MARTX CPD processes the toxin at three sites to release discrete effector domains,³⁶ while *Clostridium* sp. CPDs cleave LGTs at a single site to release the glucosyltransferase domain.^{17,37,104} Although the CPD regulates the activities of both toxin families, MARTX and LGT toxins have divergent activities in host cells. *V. cholerae* MARTX toxin disrupts actin cytoskeletal dynamics in part by covalently crosslinking actin monomers together,³⁶ while *C. difficile* LGTs (TcdA and TcdB) covalently modify and inactivate small Rho GTPases through their glucosyltransferase activity (Fig. 2B).^{25,33}

Discovery of InsP₆ as an activator of Family C80 proteases

The discovery of the CPD as a protease was serendipitous, since it lacks sequence homology to known proteases. The Satchell group at Northwestern discovered the autoprocessing activity of *V. cholerae* MARTX CPD after transiently expressing the domain inside eukaryotic cells and observing that it was truncated.³⁵ This truncation was prevented upon mutation of putative catalytic residues, suggesting that the CPD itself harbored autoproteolytic activity. Interestingly, they showed that CPD activity could be induced by a small molecule present in boiled cytosolic extracts prepared from mammalian cells.

A similar observation was made by Reineke and Tenzer *et al.* in their studies of *C. difficile* LGT toxin TcdB processing.²⁰ Although *C. difficile* Tcds were known to be proteolytically processed within the host cell, how this processing event was regulated was unclear.³⁷ After observing that a heat-stable cytosolic component could also activate Tcd processing, Reineke and Tenzer *et al.* biochemically fractionated eukaryotic cytosolic extracts and screened fractions for TcdB processing activity.²⁰ Remarkably, they showed that the small molecule inositol hexakisphosphate (InsP₆) alone could activate TcdB cleavage. As with *V. cholerae* CPD, these results demonstrated that the proteolytic activity derived from the toxin itself. Subsequent analyses by the Aktories group conclusively demonstrated that a region with weak homology to the CPD characterized by the Satchell group could mediate toxin autoprocessing and bind InsP₆.^{17,38} Shortly thereafter, InsP₆ was shown to activate *V. cholerae* MARTX CPD autoprocessing^{18,19} and CPDs from related MARTX toxins.³⁶

Characterization of InsP₆ as an allosteric activator of Family C80 proteases

The observation that InsP₆ (rather than other inositol phosphates) was a highly potent activator of MARTX and LGT CPDs suggested an elegant mechanism for spatially and temporally regulating MARTX and LGT toxin function. Because InsP₆ is exclusively produced by eukaryotes and present at micromolar concentrations within the cytosol,^{23,39} the regulation of MARTX and LGT toxin activation by InsP₆ likely restricts toxin function until after toxin translocation across host cell membranes (Fig. 2B). This observation also implied that InsP₆, a small metabolite, might *allosterically* activate the CPD, a mode of regulating protease activity that had not previously been observed.

The crystal structure of InsP₆ bound to *V. cholerae* CPD provided key insights into this question, as it revealed that InsP₆ binds to a site distal from the active site.¹⁸ The InsP₆ binding site consists of conserved, basic residues engaged in electrostatic interactions with the negatively charged phosphates of InsP₆ (Fig. 3A).^{18,40} Using mutational, kinetic, and surface plasmon resonance assays, our group showed that these InsP₆ interacting residues

were critical for enzyme activity.¹⁸ We also determined that the InsP₆ binding site and substrate binding site are distinct but interdependent: although InsP₆ was needed for substrate cleavage, the affinity of InsP₆-bound CPD for its substrate (K_m) was constant regardless of InsP₆ concentration.¹⁸

Subsequent analyses have shown that InsP₆ binding to the CPD induces conformational changes. Using a fluorescent probe to monitor exposure of the CPD catalytic Cys, the active site Cys was determined to be more accessible to the probe in the presence of InsP₆.¹⁸ Limited proteolysis and thermal stability experiments have also shown that InsP₆ binding stabilizes a CPD conformer that is more resistant to proteolysis and heat denaturation.⁴⁰ Furthermore, iso-thermal calorimetry studies indicated that InsP₆ binding to the CPD has high negative enthalpy, suggesting that the CPD becomes more ordered in the presence of InsP₆.⁴⁰ These analyses suggest that InsP₆ binding causes an allosteric shift that permits formation of a catalytically competent enzyme.

As with many allosteric effectors, however, the molecular details of this shift remain elusive. In the absence of an apo-structure of the CPD, it has been difficult to study how the signal arising from ligand binding is propagated to the CPD active site. Nevertheless, structural and mutational analyses have implicated a three-stranded structure termed the β -flap as being critical for transducing the allosteric signal to the active site.^{18,40,41} Residues on one side of the β -flap interact with InsP₆, while residues on the flip side help form the substrate binding cleft (Fig. 3A, inset). When a conserved Trp residue in the β -flap was mutated to Phe, the mutant retained wildtype levels of InsP₆ binding but exhibited defects in protease activity.¹⁸ In contrast, when InsP₆ interacting residues were mutated, the mutant CPDs were impaired in both InsP₆ binding affinity *and* protease activity. The observation that β -flap mutations can uncouple InsP₆ binding from protease activity suggests that this region dynamically responds to InsP₆. In support of this hypothesis, limited proteolysis experiments by the Satchell group indicate that the β -flap becomes more ordered upon CPD binding to InsP₆.⁴⁰

Importantly, the β -flap structure is also conserved in the crystal structure of TcdA CPD bound to InsP₆ recently published by the Lacy group at Vanderbilt.⁴¹ This structure reveals that *V. cholerae* and TcdA CPDs have a similar overall fold despite sharing only weak sequence homology (Fig. 3). In both proteases, InsP₆ binds to a highly basic site that is distal from the active site, while the N-terminus of the protein wraps around the core domain and extends into a hydrophobic substrate binding pocket. Likewise, TcdA CPD undergoes a conformational change within TcdA CPD detectable by limited proteolysis and 1D nuclear magnetic resonance (NMR) in the presence of InsP₆.^{38,41} Although further investigation is necessary, clostridal LGTs and MARTX CPDs likely share a common mechanism for communicating InsP₆ binding to the active site through ordering of the β -flap (Fig. 3, inset). Interestingly, this region is the primary site where bacterial CPDs diverge from other clan CD cysteine proteases.^{18,36} Clan CD proteases are characterized by a central β -sheet that is surrounded by α helices;^{42,43} however, in Family C80 proteases, two of the α helices have been replaced with the β -flap structure (Fig. 4). These observations suggest that the CPD may have evolved the ability to “sense” a eukaryotic-specific small molecule using a common clan CD structural scaffold.

Synthetic allosteric inhibitors of proteases

Identification and characterization of allosteric inhibitors of the caspases

An allosteric site was also discovered in another clan CD protease, the caspases (Family C14A). This well-conserved family of eukaryotic proteases cleaves specifically after Asp residues and regulates both cell death and survival.^{43–45} Apoptotic caspases control a proteolytic cascade that terminates in programmed cell death without inflammation (apoptosis),⁴⁴ while inflammatory caspases regulate a signaling cascade that can promote cell survival or induce cell death with inflammation (pyroptosis).^{46,47} Apoptotic caspases govern processes ranging from tissue remodeling during development to the regulation of immune responses,^{48,49,105} while inflammatory caspases govern processes ranging from direct cytokine activation to protection against microbial toxins and intracellular pathogens.^{47,105}

Because of their critical roles in these biological processes, caspases are synthesized as zymogens that typically require proteolytic cleavage in order to become activated.⁴³ Cleavage of the caspases results in removal of the pro-domain followed by intersubunit cleavage to produce monomers consisting of large α - and β -subunits. This activating event is mediated by dimerization on signaling scaffolds for the initiator caspases (*e.g.* caspase-1) or cleavage by initiator caspases for the executioner caspases (*e.g.* caspase-3, 6, and 7). Because of the key role that initiator and executioner caspases play in cellular processes, drugs that can selectively modulate the activation or activity of specific caspases will likely have therapeutic utility in a variety of disorders.¹¹

As with many active-site protease inhibitors, caspase inhibitor development has been complicated by issues of cross-reactivity because caspase family members exhibit similarities in substrate specificity and active site geometry.^{10,11,43,44} To circumvent this problem, the Wells group at Sunesis used a “tethering” approach to identify small molecule allosteric inhibitors of caspases.^{50,51} Tethering uses native or introduced cysteines on the surface of proteins to trap small molecules containing thiol groups.⁵² A tethering compound consists of a monophore, which directs specificity to the allosteric site, and a sulfhydryl group, which allows for conjugation of the small molecule to the protein of interest. Small molecules that become “tethered” to the protein *via* disulfide bond formation are rapidly identified using mass spectrometry; binders are assessed for their ability to modulate protein function. The Cys residue to which the small molecule is “tethered” is identified using peptide mapping, allowing the position of the novel allosteric site to be approximated in the absence of a crystal structure.

By screening a library of ~10 000 thiol containing compounds, Hardy *et al.* identified DICA and FICA as compounds that bind and block caspase-3 activity (Fig. 5A).⁵⁰ Peptide mapping of the modified Cys residue revealed that of the five surface-exposed Cys residues, DICA and FICA exclusively modified Cys264. Cys264 sits within a deep cavity at the dimer interface that is 14Å from the active site Cys. Notably, this central cavity is conserved in caspase-7, and both compounds modify caspase-7 at a structurally analogous Cys residue (Cys290, Fig. 5B).

Structural analyses of caspase-7 bound to either FICA or DICA revealed that the compounds induce identical conformational rearrangements in mature caspase-7, despite binding using different mechanisms (two FICA molecules bridge the central cavity in caspase-7, while two DICA molecules pack along the walls of the cavity.⁵⁰) In the presence of either FICA or DICA, (1) Tyr223 and Arg187 (which neighbors the catalytic Cys) no longer interact; (2) the neighboring Gly188 residue flexes like a hinge; and (3) the L2' active site loop is pulled downwards through a direct interaction with the allosteric inhibitors relative to the “on”-state of the mature enzyme (Fig. 5B). Remarkably, the conformation induced by FICA or DICA binding to mature caspase-7 strongly resembles that of procaspase-7.⁵⁰ Thus, similar to how active site inhibitors trap caspases in the “active” conformation,^{53,54} reaction with allosteric inhibitors appears to restrain caspase-7 in a “zymogenized” conformation. Consistent with this hypothesis, order of addition experiments indicate that *a priori* binding of active site inhibitors to caspase-7 precludes labeling by the allosteric inhibitors, and *vice versa*.⁵⁰

A more detailed understanding of how the allosteric signal is propagated to the active site has recently been provided by the Hardy group. By performing a systematic mutational analysis of residues that undergo conformational rearrangements upon allosteric inhibitor binding to caspase-7, they showed that the Gly188 hinge is absolutely critical for proper positioning of the catalytic Cys, while movement of the L2' loop is important for substrate binding.^{55,56} Thermal stability and structural analyses revealed that the L2' loop (Ile213 in particular) stabilizes the substrate binding groove even though it does not directly contact substrate.⁵⁶ By contrast, mutation of the interacting pair Arg187 and Tyr223 (also known as the “allosteric couple”) has minimal impact on enzyme activity, even though it disrupts the previously observed mutual exclusivity of inhibitor labeling at the active and allosteric sites.⁵⁵ Intriguingly, these analyses suggest that only a small set of residues are critical for transitioning between the active and inactive forms of the enzyme.

Notably, the deep central cavity observed in caspase-3 and 7 is also observed in other caspases, including caspase-1. Accordingly, the Wells group identified small molecules that bind a Cys residue adjacent to the dimer interface of caspase-1 using tethering, suggesting that this cavity functions as a common allosteric site in the caspases.⁵¹ By solving the structure of allosterically inhibited mature caspase-1, Scheer *et al.* demonstrated that Compound 34-bound caspase-1 resembles the ligand-free, inactive form of caspase-1, as was observed for caspase-7 (Fig. 5C). Relative to the “on”-state structure, binding of Compound 34 (1) causes the catalytic Cys to rotate ~5 Å away from its position in the activated enzyme, (2) re-positions the substrate binding loops so that they no longer interact with substrate, and (3) disrupts the Arg286–Glu390 salt bridge such that the substrate binding pocket is occluded. These structural alterations “trap” or “zymogenize” mature caspase-1 into an inactive conformation.

Scheer *et al.* noted that the Arg286–Glu390 salt-bridge is part of a network of 21 hydrogen bonds (from nine residues) that are altered in position between the two structures. A systematic mutational analysis of these residues by Datta *et al.* revealed that, as with caspase-7, only a small subset of these residues are critical for enzyme activity.⁵⁷ In particular, mutation of the Arg286–Glu390 salt bridge strongly affected the catalytic

efficiency of caspase-1, while mutations in Ser332 and Ser339 slightly reduced catalytic function. Crystallographic analyses of these mutants restrained in the “on” state (*via* modification by active site inhibitor) revealed that stabilization of the solvent pocket and salt bridge is important for caspase-1 to adopt the “on” state conformation. Thus, only a few residues are necessary to couple the allosteric site to the active site, forming what has been termed an “allosteric hotwire.”⁵⁷

Collectively, studies by the Wells group have identified a common allosteric binding site at the dimer interface of caspases that functionally reverses their activation. By trapping mature caspases in a zymogen-like state, the allosteric inhibitors reveal that even after zymogen activation, the mature enzyme interconverts between “on” and “off” states (Fig. 5D). This interconversion depends upon only a small subset of residues: the Arg286–Glu390 salt bridge in caspase-1 and the Gly188 hinge and L2' loop residue I213 in caspase-3/7. Notably, the allosteric site may be biologically relevant, since recent structural studies indicate that a Trp–Phe pair occupies a similar position to Compound 34 at the dimer interface of procaspase-1.⁵⁸

Identification and characterization of allosteric inhibitors of viral proteases

The Craik group at UCSF have similarly identified allosteric inhibitors that stabilize a zymogen-like state in herpesvirus proteases.⁵⁹ In contrast with the caspase allosteric inhibitors, the protease inhibitors identified by Shahian *et al.* disrupt dimer formation and prevent protease activation rather than inhibiting the mature enzyme. This class of enzyme is an important drug target, since all herpesvirus produce a structurally and functionally conserved serine protease that is required for capsid assembly during the lytic stage.⁶⁰ Herpesviruses cause a number of medically relevant diseases such as genital herpes (herpes simplex virus, HPV), retinitis (cytomegalovirus, CMV), and cancer (Kaposi's sarcoma-associated virus, KSHV).

To identify allosteric inhibitors of this class of enzyme, Shahian *et al.* targeted the dimerization interface of Kaposi's sarcoma herpesvirus protease (KSHV Pr). Pharmaceutical companies have had limited success at developing active site inhibitors of herpesvirus proteases.⁶¹ The Craik group reasoned that preventing dimerization, and thus activation, might be more effective than targeting the active enzyme. Dimerization is critical for herpesvirus protease activation^{62,63} and appears to occur only when high concentrations of protease are achieved during virus assembly of the pre-capsid.⁶⁴ A series of conformational changes induced by dimerization lead to proper positioning of active site residues ~15 Å away from the dimer interface, resulting in a disorder-to-order conformational switch.^{63,65,66}

Based on the observation that the KSHV Pr dimer interface is stabilized by two α -helices and that disruption of this interface by a 30 amino acid α -helix inhibits KSHV Pr function,⁶⁷ Shahian *et al.* screened a rationally designed library of 182 small molecule helical mimetics for compounds that could block KSHV Pr activity in a fluorogenic substrate assay.⁵⁹ This screen and subsequent SAR analyses of the hits identified a lead compound DD2 that inhibits KSHV Pr activity and disrupts dimer formation (Fig. 6). Kinetic analyses revealed that DD2 acts as a mixed-type inhibitor by preventing formation of a conformationally

stable active site (Fig. 6). The authors used chemical shift perturbation mapping to show that DD2 perturbs residues at the dimer interface and leads to peak broadening of active site residues. Importantly, these NMR analyses allowed the authors to visualize how allosteric conformational effects are *dynamically* propagated from the dimer interface into the active site. By combining NMR studies with mutational analyses, they identified a conserved Trp109 residue at the dimer interface as a key mediator of KSHV Pr dimerization.

The allosteric inhibitor identified by the Craik group is the first small molecule inhibitor of a herpesvirus protease to select for the partially unfolded zymogen.⁵⁹ In identifying a “monomer trap”, the authors illustrate how the conformational mobility that underlies enzyme activation can be exploited for inhibitor development. Notably, DD2 was also active against the related CMV protease, suggesting that DD2 and related compounds could broadly inhibit the replication of herpes-virus proteases. While the efficacy of these compounds during viral replication remains to be tested, their study has provided crucial insights into herpesvirus protease activation and validated the dimer interface as a site for inhibitor development.

Identification of selective allosteric inhibitors of γ -secretase

Instead of inhibiting protease activity completely, Shelton *et al.* sought to identify allosteric inhibitors that block cleavage of selective substrates by γ -secretase, while leaving its activity against other substrates intact.⁶⁸ γ -Secretase is a multi-protein membrane-bound complex that cleaves a number of type I membrane proteins such as amyloid precursor protein (APP) and Notch1 within the membrane.⁶⁹ Thus, γ -secretase is an attractive drug target for treating Alzheimer's disease (AD) and cancer. Cleavage of APP by γ -secretase generates neurotoxic peptides A β ranging from 37 to 46 amino acids in length that are associated with AD.⁷⁰ The ratio of A β peptides seems to control the development of AD, since A β 40 plays a potentially neuroprotective role, while A β 42 is associated with disease progression.⁷⁰ This observation, combined with the fact that blocking γ -secretase function altogether causes toxicity due to inhibition of Notch1 signaling,⁷¹ suggests that developing inhibitors that block A β 42 production, but not A β 40, production would be a more effective therapeutic strategy.

By screening a library of ~200 000 compounds, Shelton *et al.* identified dicoumarin compounds that selectively reduced A β 42 production over A β 40 by about 4-fold but did not inhibit Notch cleavage by γ -secretase. Kinetic analyses revealed that the dicoumarin compounds act as non-competitive inhibitors that affect $V_{\max}$ but not K_m . In contrast with previous γ -secretase inhibitors that target the APP substrate, the lead compound CS-1 identified by Shelton *et al.* alters the substrate specificity of γ -secretase. Using a series of novel biotinylated, photo-activatable inhibitors, they showed that CS-1 alters the reactivity of probes with γ -secretase at the S1 and S2 sub-sites, but not at S3' and S1' sites. While it is unclear how the compounds allosterically inhibit γ -secretase, they retain their selectivity for reducing A β 42 production inside cells and thus suggest a new strategy for developing AD therapeutic agents.

Synthetic allosteric activators of procaspases

While there are a number of examples of synthetic allosteric inhibitors, only one example of a synthetic allosteric activator has been described to date. In a landmark study, the Wells group recently identified the first synthetic small molecule activator of a proprotease.⁷² Wolan *et al.* reasoned that, just as allosteric inhibitors trap the “off”-state, small molecules might trap the “on”-state of caspases. Using a high throughput screening approach, they identified a small molecule (1541) that directly activates the apoptotic executioner caspases, procaspase-3 and procaspase-6 (Fig. 7A). Remarkably, 1541 can bypass initiator caspase activation and directly induce apoptosis in a caspase-3-dependent manner without requiring apoptotic stimuli or upstream factors. It should be noted that another small molecule PAC-1 has been reported to slightly induce procaspase-3 activation, but it apparently acts by chelating inhibitory zinc ions in assay buffers rather than through a direct activation mechanism.^{72–75}

1541 activates procaspase-3 through an unusual mechanism: it both increases the proteolytic susceptibility of procaspase-3 *and* increases its affinity for its substrates (Fig. 7B). By enhancing autoproteolysis, 1541 induces a highly cooperative feedback activation process in which 1541-stimulated production of mature caspase-3 accelerates the autoproteolytic activation of neighboring caspases. Although 1541-activated procaspase-3 is significantly less efficient at turning over substrate (as measured by k_{cat}) than mature caspase-3 in fluorogenic substrate assays, 1541-activated procaspase-3 exhibits increased affinity for its substrates (as measured by K_{m}) relative to mature caspase-3. Notably, the interaction between 1541 and procaspase-3 and 6 is specific, since procaspase-7 and procaspase-1 are not affected by 1541, and modifications of 1541 around the coumarin ring alter its selectivity for procaspase-3 over procaspase-6 (1541B, Fig. 7A). Thus, it should be possible to optimize 1541 for specificity and potency in the future.

Intriguingly, the dose–response curve for 1541 is bell-shaped: 1541 stimulates procaspase-3 activity at an EC_{50} of 2.4 μM and inhibits procaspase-3 activity at an IC_{50} of 34 μM through either a competitive or mixed non-competitive inhibitory mechanism. Consistent with the former mechanism, Wolan *et al.* identified suppressor mutations in the active site region that render procaspase-3 insensitive to 1541-mediated activation. Mutation of residues Ser198, Thr199, or Ser205 to Ala prevents 1541-mediated activation of procaspase-3 without affecting the intrinsic activity of the enzyme, suggesting that 1541 binds near the active site. Although further investigation is necessary, the suppressor mutations presumably alter procaspase-3 binding to 1541 and/or susceptibility to auto-proteolysis. Importantly, these residues are also conserved in procaspase-6, which is sensitive to 1541-mediated activation.

This elegant study has uncovered a highly unusual mechanism for heterologously activating executioner caspases. The activating small molecules appear to both reorganize the active site for catalysis and enhance the accessibility of procaspase-3 cleavage sites. The authors propose a model in which 1541 binds near the active site of one procaspase-3 monomer and inhibits its activity. Unlike most inhibitors, however, this binding event simultaneously stabilizes a conformation in the neighboring subunit that promotes self-cleavage (Fig. 7B). This new conformation may be an “on”-state conformation that differs from that of mature

caspase-3 or an “off”-state conformation that exhibits increased susceptibility to proteolysis. This model implies that 1541 functions as an allosteric activator, since binding of 1541 to one subunit affects the activity of the unoccupied subunit. This notion is supported by their observation of positive cooperativity in 1541-stimulated procaspase-3 autoproteolysis. Structural studies of 1541 bound to procaspase-3 will undoubtedly provide insight into its precise mode of action and may reveal previously undetected, naturally occurring conformational intermediates. Further mutational analyses should also illuminate how 1541 binding in one subunit is communicated to the neighboring subunit.

Current and potential applications of small molecule allosteric effectors

The identification of natural and synthetic small molecule allosteric regulators of proteases has opened up many avenues of inquiry. For example, the allosteric sites within CPDs, KSHV Pr, and the caspases present new opportunities for drug discovery.^{12,59} Allosteric inhibitors of *C. difficile* CPDs will likely be more efficient than active site inhibitors at preventing the autoproteolytic activation of Tcd toxins, since active site inhibitor binding is largely dependent on activation of the CPD by InsP₆ and thus must outcompete a substrate that is physically linked to the protease. By analogy, the dimerization inhibitors identified by the Craik group illustrate how preventing protease activation may be more efficient than blocking activated KSHV protease.¹³ The authors also note that allosteric inhibitors of viral protease dimer interfaces may resist the development of drug-resistant mutations, since natural variation at the dimer interface of HIV is very rare⁷⁶ and mutations in this region are often deleterious.^{62,63} Further more, allosteric inhibitors of these enzymes may exhibit more drug-like properties than compounds that target the active sites of proteases, which can often be highly charged (as for caspases)^{77,78} or hydrophobic (as for CPDs).³⁶

Allosteric sites are ripe for protein engineering, as they can be used to design proteins with switch-like behavior.^{4,79} Using an iterative domain insertion approach, Guntas *et al.* created an allosteric switch that responds to maltose by fusing the ligand binding domain of *E. coli* maltose-binding protein to TEM1 β -lactamase (output domain).^{80,81} A similar approach could be used to create InsP₆-responsive switches using bacterial CPDs as a starting point.^{79,82}

Alternatively, the CPD could be engineered to respond to a synthetic small molecule that would allow for regulated auto-proteolysis of proteins to which the CPD is fused, a property that may be useful in eukaryotic conditional expression and purification systems. To the former point, a fusion tag consisting of the engineered CPD domain (CPD*) followed by a degradation domain (DD) could be added to target proteins. In the absence of the synthetic activator, the CPD*–DD tag would result in the rapid degradation of the fusion protein. Addition of the synthetic small molecular activator would result in autocleavage of the CPD*–DD tag and stabilization of the now untagged-target protein. In theory, this could produce a rapid, reversible, and titratable method for regulating protein levels in eukaryotic systems, analogous to the ligand-dependent conditional expression system created by the Wandless group.⁸³

To the latter point, as a proof-of-concept, we have exploited the switch-like nature of the *V. cholerae* CPD to develop a simplified one-step purification system for bacterially expressed proteins.⁸⁴ In this system, target proteins are expressed as a fusion construct with an affinity-tagged CPD. Following affinity purification from *E. coli* lysates, addition of InsP₆ to the fusion protein releases the untagged target protein into the supernatant. If a mutant CPD that responds to a synthetic small molecule is developed, it could serve as a platform for the purification of target proteins from eukaryotic cells, analogous to the bacterial expression system we have developed.⁸⁴

The discovery of allosteric activators of procaspase-3 activation is particularly exciting because it allows for mechanistic studies of caspase-3 in regulating apoptotic and nonapoptotic processes, such as cellular remodeling and stem cell differentiation.^{48,49} This class of compounds may also be useful as selective chemotherapeutic agents to treat cancers that are resistant to apoptosis due to proapoptotic lesions or elevated levels of procaspase-3.^{85–88} As proof-of-principle, Wolan *et al.* showed that a cancerous B cell line is more sensitive to apoptosis induced by 1541B than other cell lines.⁷² In general, allosteric activators will be useful in allowing the biological sufficiency of a given protease in a specific pathway to be assessed *in vivo* and for interrogating protease activation mechanisms.

Similarly, allosteric inhibitors can uncover new principles in protease activation and inhibition. By stabilizing the inactive conformations of *mature* caspases, allosteric inhibitors have provided insight into the conformational pathways used by activated caspases to transition between “on” and “off” states. In a similar vein, the Craik group has used allosteric inhibitors and NMR to elegantly probe how conformational changes are linked to enzyme activation. Mutational studies arising from studies of both these protease families have identified allosteric circuits through which the signal is propagated.

Lastly, allosteric inhibitors can also be used in conjunction with active site inhibitors to select for conformation-specific antibodies that recognize either the “off” or “on” state of a given protease.⁸⁹ The Wells group demonstrated the feasibility of this approach by using phage display followed by negative selection to screen for Fabs that specifically recognize the inactive and active forms of caspase-1. This study outlines a general strategy for generating conformation-selective antibodies to biomolecules, which can be then used to control protein function and monitor active enzyme populations by immunostaining.

Conclusions and perspectives

To date, there are only a handful of examples of small molecules allosterically regulating protease activity. The discovery of these compounds undoubtedly portends the identification of other small molecule effectors that will facilitate mechanistic studies of allostery and protease function. In particular, such compounds will allow for the *in situ* regulation of specific proteases in biological processes and in disease, and studies of the mode of action of these compounds will provide further insights into allosteric mechanisms of regulation. In the case of the synthetic allosteric caspase and KSHV Pr inhibitors, the molecules “trap” the “off”-state of the protease, apparently by disrupting allosteric networks intrinsic to the

protein that permit the enzyme to interconvert between “on” and “off”-states. It will be interesting to determine whether newly identified allosteric compounds will also stabilize naturally occurring, low-energy conformations, such as zymogen-like or active protease conformations, rather than entirely new conformations. Such a finding would lend further support to the emerging view that allostery involves a shift in conformational equilibria between *pre-existing* conformations.^{3–6}

Identifying new allosteric sites and regulators

Given the obvious utility of allosteric small molecule regulators, it will be important to identify methods for discovering such compounds and elucidating their mode of action. Thus far, high throughput screening (HTS),^{68,72} phage display,⁹⁰ tethering,^{50,51} biochemical fractionation,²⁰ rational design based on proteinaceous allosteric effectors,^{14,15,59,91} and computational methods⁹² have been useful in discovering allosteric regulators of other proteins (see ref. 4, 12, and 93 for review).

Although allosteric sites have been serendipitously discovered using HTS and tethering, it has been possible to rationally target allosteric sites by designing inhibitors that mimic natural ligands. Examples include the design of α -helical mimetics for KSHV protease,⁵⁹ peptide mimics of OMP C-terminal tails for DegS,⁹¹ and adenosine triphosphate analogs such as Gleevec for Abl Tyr kinases. These mimetics act as “conformational traps”¹³ that restrain the conformational mobility of target proteins to either the “on” or “off”-state.

In these examples, an understanding of the conformational dynamics of the protein was known prior to performing focused or high-throughput screens. In order to identify regions that alter conformational dynamics in response to allosteric effectors, mutational analyses can identify protein regions that are important for enzyme activity beyond the active site. *In silico*-based approaches such as COREX,⁹⁴ covariance analyses,⁹⁵ and statistical coupling analysis (SCA)⁹² can also be applied to identify conserved allosteric sites. This latter approach tends to require large number of sequences in order to generate predictions of which residues are involved in transducing the allosteric signal.

Developing a screen that specifically detects alterations in natural allosteric signaling pathways rather than enzyme activity alone presents an additional challenge to identifying allosteric effectors. A promising approach is the use of LiReC (ligand-regulated competition),⁹⁶ a fluorescence polarization assay that uses fluorescently labeled peptide probes to compete with binding of a regulatory domain to the catalytic domain. Tethering methods can also specifically target putative allosteric sites by using natural or engineered Cys situated within predicted allosteric pathways.⁵² These examples highlight the importance of designing screening assays to enrich for small molecule modulators, whether the approach involves HTS, or focused libraries. As with any screening approach, however, even though hits may display the appropriate characteristics initially, they may lack selectivity, cell permeability, non-toxicity, and other desirable properties once subjected to secondary screening.

Characterizing allosteric effectors

Once allosteric effectors are identified, their mode of regulation must be characterized. Kinetic assays have been particularly useful in understanding how small molecule effectors modulate protease function by determining whether effectors selectively alter k_{cat} and/or V_{max} and act non-competitively (in contrast with active site competitors). Active site probes^{18,77,97} have also served as useful tools for examining the accessibility of the active site in the presence of allosteric effectors.^{18,36,68}

Crystallographic studies of the compounds bound to the protease are critical for identifying the allosteric site and the residues involved in mediating the allosteric transition. Ideally, structures of both the apo-form and effector-bound form are needed to visualize the conformational changes that are induced by allosteric effectors. Once these structures are solved, mutational analyses are essential for validating predictions made from the structure, by measuring the contribution of specific residues in recognizing allosteric ligands and/or propagating the signal. An elegant way of validating models arising from these studies is to engineer disulfide bonds that exogenously stabilize regions proposed to form in response to allosteric effector binding.⁶⁶ Similarly, the isolation of suppressor mutations that confer insensitivity to the allosteric effector can yield information on sites where the ligand binds as well as the sites within the protein that are disrupted by the effector.⁷²

Ultimately, mapping the pathways through which the allosteric signal travels requires the ability to visualize protein dynamics in real time. While crystallographic studies that compare bound and unbound states have considerable utility, static structures do not allow direct observation of the signaling pathway. Indeed, the NMR experiments performed by Shahian *et al.* illustrate how NMR techniques allow the effect of allosteric regulators on enzyme conformational dynamics to be directly observed.⁵⁹ A more precise technique for visualizing protein dynamics is NMR relaxation dispersion, which measures rates of interconversion between relative populations in real-time.^{98–102} These two techniques have been recently combined by the Alber group to demonstrate how both crystallographic and NMR approaches can be used to identify functional minor protein conformations.¹⁰³

As general principles regarding the underpinnings of allostery emerge, it may be possible to computationally predict allosteric sites and pathways in the future. Small molecule modulators will likely facilitate these studies by trapping specific conformers within a conformational ensemble. Given that the rate of allosteric site discovery and techniques for studying allostery have improved, it seems likely that considerable progress will be made in identifying and characterizing new small molecule allosteric effectors of proteases in the coming years. The identification of these sites will dramatically expand the possibilities for drug discovery and design of sensitive switch-like proteins.

References

1. Turk B. Nat. Rev. Drug Discovery. 2006; 5:785–799.
2. Bird PI, Trapani JA, Villadangos JA. Nat. Rev. Immunol. 2009; 9:871–882. [PubMed: 19935806]
3. del Sol A, Tsai CJ, Ma B, Nussinov R. Structure (London). 2009; 17:1042–1050.
4. Goodey NM, Benkovic SJ. Nat. Chem. Biol. 2008; 4:474–482. [PubMed: 18641628]

5. Gunasekaran K, Ma B, Nussinov R. *Proteins: Struct., Funct., Bioinf.* 2004; 57:433–443.
6. Tsai CJ, Del Sol A, Nussinov R. *Mol. BioSyst.* 2009; 5:207–216. [PubMed: 19225609]
7. Tsai CJ, del Sol A, Nussinov R. *J. Mol. Biol.* 2008; 378:1–11. [PubMed: 18353365]
8. Hauske P, Ottmann C, Meltzer M, Ehrmann M, Kaiser M. *ChemBioChem.* 2008; 9:2920–2928. [PubMed: 19021141]
9. Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. *Nucleic Acids Res.* 2008; 36:D320–D325. [PubMed: 17991683]
10. Berger AB, Sexton KB, Bogoy M. *Cell Res.* 2006; 16:961–963. [PubMed: 17117159]
11. Howley B, Fearnhead HO. *J. Cell. Mol. Med.* 2008; 12:1502–1516. [PubMed: 18298652]
12. Hardy JA, Wells JA. *Curr. Opin. Struct. Biol.* 2004; 14:706–715. [PubMed: 15582395]
13. Lee GM, Craik CS. *Science.* 2009; 324:213–215. [PubMed: 19359579]
14. Bode W, Huber R. *FEBS Lett.* 1976; 68:231–236. [PubMed: 10181]
15. Hauske P, Mamant N, Hasenbein S, Nickel S, Ottmann C, Clausen T, Ehrmann M, Kaiser M. *Mol. BioSyst.* 2009; 5:980–985. [PubMed: 19668863]
16. Sohn J, Grant RA, Sauer RT. *Structure (London).* 2009; 17:1411–1421.
17. Egerer M, Giesemann T, Jank T, Satchell KJ, Aktories K. *J. Biol. Chem.* 2007; 282:25314–25321. [PubMed: 17591770]
18. Lupardus PJ, Shen A, Bogoy M, Garcia KC. *Science.* 2008; 322:265–268. [PubMed: 18845756]
19. Prochazkova K, Satchell KJ. *J. Biol. Chem.* 2008; 283:23656–23664. [PubMed: 18591243]
20. Reineke J, Tenzer S, Rupnik M, Koschinski A, Hasselmayer O, Schrattenholz A, Schild H, von Eichel-Streiber C. *Nature.* 2007; 446:415–419. [PubMed: 17334356]
21. Hanakahi LA, Bartlett-Jones M, Chappell C, Pappin D, West SC. *Cell (Cambridge, Mass.).* 2000; 102:721–729.
22. Macbeth MR, Schubert HL, Vandemark AP, Lingam AT, Hill CP, Bass BL. *Science.* 2005; 309:1534–1539. [PubMed: 16141067]
23. Michell RH. *Nat. Rev. Mol. Cell Biol.* 2008; 9:151–161. [PubMed: 18216771]
24. Satchell KJ. *Infect. Immun.* 2007; 75:5079–5084. [PubMed: 17646359]
25. Jank T, Aktories K. *Trends Microbiol.* 2008; 16:222–229. [PubMed: 18394902]
26. Olivier V, Haines GK 3rd, Tan Y, Satchell KJ. *Infect. Immun.* 2007; 75:5035–5042. [PubMed: 17698573]
27. Olivier V, Queen J, Satchell KJ. *PLoS One.* 2009; 4:e7352. [PubMed: 19812690]
28. Olivier V, Salzman NH, Satchell KJ. *Infect. Immun.* 2007; 75:5043–5051. [PubMed: 17698571]
29. Lee JH, Kim MW, Kim BS, Kim SM, Lee BC, Kim TS, Choi SH. *J. Microbiol.* 2007; 45:146–152. [PubMed: 17483800]
30. Liu M, Alice AF, Naka H, Crosa JH. *Infect. Immun.* 2007; 75:3282–3289. [PubMed: 17438022]
31. Kelly CP, LaMont JT. *N. Engl. J. Med.* 2008; 359:1932–1940. [PubMed: 18971494]
32. Rupnik M, Wilcox MH, Gerding DN. *Nat. Rev. Microbiol.* 2009; 7:526–536. [PubMed: 19528959]
33. Voth DE, Ballard JD. *Clin. Microbiol. Rev.* 2005; 18:247–263. [PubMed: 15831824]
34. Barroso LA, Moncrief JS, Lysterly DM, Wilkins TD. *Microb. Pathog.* 1994; 16:297–303. [PubMed: 7968458]
35. Sheahan KL, Cordero CL, Satchell KJ. *EMBO J.* 2007; 26:2552–2561. [PubMed: 17464284]
36. Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia KC, Bogoy M. *Nat. Chem. Biol.* 2009; 5:469–478. [PubMed: 19465933]
37. Rupnik M, Pabst S, Rupnik M, von Eichel-Streiber C, Urlaub H, Soling HD. *Microbiology.* 2005; 151:199–208. [PubMed: 15632438]
38. Egerer M, Giesemann T, Herrmann C, Aktories K. *J. Biol. Chem.* 2009; 284:3389–3395. [PubMed: 19047051]
39. Raboy V. *Phytochemistry.* 2003; 64:1033–1043. [PubMed: 14568069]
40. Prochazkova K, Shuvalova LA, Minasov G, Voburka Z, Anderson WF, Satchell KJ. *J. Biol. Chem.* 2009; 284:26557–26568. [PubMed: 19620709]

41. Pruitt RN, Chagot B, Cover M, Chazin WJ, Spiller B, Lacy DB. *J. Biol. Chem.* 2009; 284:21934–21940. [PubMed: 19553670]
42. Barrett AJ, Rawlings ND. *Biol. Chem.* 2001; 382:727–733. [PubMed: 11517925]
43. Fuentes-Prior P, Salvesen GS. *Biochem. J.* 2004; 384:201–232. [PubMed: 15450003]
44. Boatright KM, Salvesen GS. *Curr. Opin. Cell Biol.* 2003; 15:725–731. [PubMed: 14644197]
45. Pop C, Salvesen GS. *J. Biol. Chem.* 2009; 284:21777–21781. [PubMed: 19473994]
46. Bergsbaken T, Fink SL, Cookson BT. *Nat. Rev. Microbiol.* 2009; 7:99–109. [PubMed: 19148178]
47. Lamkanfi M, Dixit VM. *Immunol. Rev.* 2009; 227:95–105. [PubMed: 19120479]
48. Feinstein-Rotkopf Y, Arama E. *Apoptosis.* 2009; 14:980–995. [PubMed: 19373560]
49. Yi CH, Yuan J. *Dev. Cell.* 2009; 16:21–34. [PubMed: 19154716]
50. Hardy JA, Lam J, Nguyen JT, O'Brien T, Wells JA. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:12461–12466. [PubMed: 15314233]
51. Scheer JM, Romanowski MJ, Wells JA. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:7595–7600. [PubMed: 16682620]
52. Erlanson DA, Wells JA, Braisted AC. *Annu. Rev. Biophys. Biomol. Struct.* 2004; 33:199–223. [PubMed: 15139811]
53. Chai J, Wu Q, Shiozaki E, Srinivasula SM, Alnemri ES, Shi Y. *Cell (Cambridge, Mass.)*. 2001; 107:399–407.
54. Romanowski MJ, Scheer JM, O'Brien T, McDowell RS. *Structure (London)*. 2004; 12:1361–1371.
55. Hardy JA, Wells JA. *J. Biol. Chem.* 2009; 284:26063–26069. [PubMed: 19581639]
56. Witkowski WA, Hardy JA. *Protein Sci.* 2009; 18:1459–1468. [PubMed: 19530232]
57. Datta D, Scheer JM, Romanowski MJ, Wells JA. *J. Mol. Biol.* 2008; 381:1157–1167. [PubMed: 18590738]
58. Elliott JM, Rouge L, Wiesmann C, Scheer JM. *J. Biol. Chem.* 2009; 284:6546–6553. [PubMed: 19117953]
59. Shahian T, Lee GM, Lazic A, Arnold LA, Velusamy P, Roels CM, Guy RK, Craik CS. *Nat. Chem. Biol.* 2009; 5:640–646. [PubMed: 19633659]
60. Waxman L, Darke PL. *Antiviral Chem. Chemother.* 2000; 11:1–22.
61. Kan CC. *Curr. Top. Med. Chem.* 2002; 2:247–269. [PubMed: 11944819]
62. Batra R, Khayat R, Tong L. *Nat. Struct. Biol.* 2001; 8:810–817. [PubMed: 11524687]
63. Pray TR, Reiling KK, Demirjian BG, Craik CS. *Biochemistry.* 2002; 41:1474–1482. [PubMed: 11814340]
64. Pray TR, Nomura AM, Pennington MW, Craik CS. *J. Mol. Biol.* 1999; 289:197–203. [PubMed: 10366498]
65. Marnett AB, Nomura AM, Shimba N, Ortiz de Montellano PR, Craik CS. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:6870–6875. [PubMed: 15118083]
66. Nomura AM, Marnett AB, Shimba N, Dotsch V, Craik CS. *Biochemistry.* 2006; 45:3572–3579. [PubMed: 16533039]
67. Shimba N, Nomura AM, Marnett AB, Craik CS. *J. Virol.* 2004; 78:6657–6665. [PubMed: 15163756]
68. Shelton CC, Zhu L, Chau D, Yang L, Wang R, Djaballah H, Zheng H, Li YM. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:20228–20233. [PubMed: 19906985]
69. Kopan R, Goate A. *Genes Dev.* 2000; 14:2799–2806. [PubMed: 11090127]
70. Selkoe DJ. *Neurol. Clin.* 2000; 18:903–922. [PubMed: 11072267]
71. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H. *Nature.* 2005; 435:959–963. [PubMed: 15959515]
72. Wolan DW, Zorn JA, Gray DC, Wells JA. *Science.* 2009; 326:853–858. [PubMed: 19892984]
73. Putt KS, Chen GW, Pearson JM, Sandhorst JS, Hoagland MS, Kwon JT, Hwang SK, Jin H, Churchwell MI, Cho MH, Doerge DR, Helfferich WG, Hergenrother PJ. *Nat. Chem. Biol.* 2006; 2:543–550. [PubMed: 16936720]

74. Peterson QP, Goode DR, West DC, Ramsey KN, Lee JJ, Hergenrother PJ. *J. Mol. Biol.* 2009; 388:144–158. [PubMed: 19281821]
75. Denault JB, Drag M, Salvesen GS, Alves J, Heidt AB, Deveraux Q, Harris JL. *Nat. Chem. Biol.* 2007; 3:519. author reply 520. [PubMed: 17710090]
76. Wu TD, Schiffer CA, Gonzales MJ, Taylor J, Kantor R, Chou S, Israelski D, Zolopa AR, Fessel WJ, Shafer RW. *J. Virol.* 2003; 77:4836–4847. [PubMed: 12663790]
77. Kato D, Boatright KM, Berger AB, Nazif T, Blum G, Ryan C, Chehade KA, Salvesen GS, Bogyo M. *Nat. Chem. Biol.* 2005; 1:33–38. [PubMed: 16407991]
78. Powers JC, Asgian JL, Ekici OD, James KE. *Chem. Rev.* 2002; 102:4639–4750. [PubMed: 12475205]
79. Ostermeier M. *Protein Eng., Des. Sel.* 2005; 18:359–364. [PubMed: 16043448]
80. Guntas G, Mansell TJ, Kim JR, Ostermeier M. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:11224–11229. [PubMed: 16061816]
81. Guntas G, Ostermeier M. *J. Mol. Biol.* 2004; 336:263–273. [PubMed: 14741221]
82. Buskirk AR, Liu DR. *Chem. Biol.* 2005; 12:151–161. [PubMed: 15734643]
83. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. *Cell (Cambridge, Mass.)*. 2006; 126:995–1004.
84. Shen A, Lupardus PJ, Morell M, Ponder EL, Sadaghiani AM, Garcia KC, Bogyo M. *PLoS One*. 2009; 4:e8119. [PubMed: 19956581]
85. Fischer U, Janssen K, Schulze-Osthoff K. *BioDrugs*. 2007; 21:273–297. [PubMed: 17896835]
86. Fischer U, Schulze-Osthoff K. *Pharmacol. Rev.* 2005; 57:187–215. [PubMed: 15914467]
87. Porter AG. *Nat. Chem. Biol.* 2006; 2:509–510. [PubMed: 16983381]
88. Yang S, Liu J, Thor AD, Yang X. *Oncol. Rep.* 2007; 17:1229–1235. [PubMed: 17390070]
89. Gao J, Sidhu SS, Wells JA. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:3071–3076. [PubMed: 19208804]
90. Dennis MS, Eigenbrot C, Skelton NJ, Ultsch MH, Santell L, Dwyer MA, O'Connell MP, Lazarus RA. *Nature*. 2000; 404:465–470. [PubMed: 10761907]
91. Sohn J, Grant RA, Sauer RT. *Cell (Cambridge, Mass.)*. 2007; 131:572–583.
92. Süel GM, Lockless SW, Wall MA, Ranganathan R. *Nat. Struct. Biol.* 2003; 10:59–69. [PubMed: 12483203]
93. Zorn JA, Wells JA. *Nat. Chem. Biol.* 2010; 6:179–188. [PubMed: 20154666]
94. Wrabl JO, Hilser VJ. *PLoS Comput. Biol.* 2010; 6:e1000722. [PubMed: 20361049]
95. Liu J, Nussinov R. *PLoS Comput. Biol.* 2009; 5:e1000527. [PubMed: 19798438]
96. Saldanha SA, Kaler G, Cottam HB, Abagyan R, Taylor SS. *Anal. Chem.* 2006; 78:8265–8272. [PubMed: 17165815]
97. Berger AB, Witte MD, Denault JB, Sadaghiani AM, Sexton KM, Salvesen GS, Bogyo M. *Mol. Cell*. 2006; 23:509–521. [PubMed: 16916639]
98. Eisenmesser EZ, Millet O, Labeikovsky W, Korzhnev DM, Wolf-Watz M, Bosco DA, Skalicky JJ, Kay LE, Kern D. *Nature*. 2005; 438:117–121. [PubMed: 16267559]
99. Henzler-Wildman K, Kern D. *Nature*. 2007; 450:964–972. [PubMed: 18075575]
100. Kern D, Eisenmesser EZ, Wolf-Watz M. *Methods Enzymol.* 2005; 394:507–524. [PubMed: 15808235]
101. Tzeng SR, Kalodimos CG. *Nature*. 2009; 462:368–372. [PubMed: 19924217]
102. Volkman BF, Lipson D, Wemmer DE, Kern D. *Science*. 2001; 291:2429–2433. [PubMed: 11264542]
103. Fraser JS, Clarkson MW, Degnan SC, Erion R, Kern D, Alber T. *Nature*. 2009; 462:669–673. [PubMed: 19956261]
104. Pfeifer G, Schirmer J, Leemhuis J, Busch C, Meyer DK, Aktories K, Barth H. *J. Biol. Chem.* 2003; 278:44535–44541. [PubMed: 12941936]
105. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. *Nat. Immunol.* 2009; 10:241–247. [PubMed: 19221555]

Fig. 1.

Simplified view of allostery in proteases. Top panel: binding of an activator to an allosteric site induces a shift in the conformational equilibrium around the active site, resulting in substrate binding and cleavage. Bottom panel: binding of an inhibitor to an allosteric site causes a shift in the conformational equilibrium around the active site that no longer permits substrate binding. In both cases, there are *multiple* pathways (wavy lines) through which the allosteric signal is propagated. The thicker line denotes a major pathway; thinner lines represent minor pathways. It should be noted that a conformational change is not a requirement for allosteric effectors to alter protein activity.⁷

Fig. 2.

Autoproteolytic activation of bacterial toxins by inositol hexakisphosphate (InsP₆). (A) Structure of inositol hexakisphosphate (InsP₆). Stick model and chemical structure are shown. (B) Schematic illustrating CPD-mediated autoproteolytic activation of the multidomain *Clostridium* sp. LGT and *V. cholerae* MARTX toxins. Left panel: the C-terminal end of LGTs (black squiggly line) binds to unknown cell surface receptors potentially through carbohydrate interactions. Binding results in receptor-mediated endocytosis of LGT toxins. Acidification of the endosome (H⁺ protons) induces a conformational change in the toxin that allows the putative translocation region (orange line) to insert into the endosomal membrane and form a pore that translocates the glucosyltransferase domain (blue triangle). The cysteine protease domain (pink circle) is also presumably translocated across the endosomal membrane. Exposure of the CPD to InsP₆ (yellow jagged oval) in the eukaryotic cytosol results in protease activation. Activated CPD cleaves after a conserved Leu residue at the glucosyltransferase-CPD junction, releasing the glucosyltransferase domain into the cell, where it has improved access to Rho GTPase substrates at the plasma membrane. Right panel: the multidomain *V. cholerae* MARTX toxin binds to cells and is believed to autotranslocate the central effector region across the plasma membrane through conserved repeat regions (red rectangles). Exposure of the CPD to InsP₆ in the eukaryotic cytosol activates the protease, which cleaves the toxin at three sites to separate effector domains. Proteolytic cleavage is necessary for optimal actin crosslinking domain activity, which induces cell rounding.

Fig. 3.

Structure of bacterial cysteine protease domains (CPDs) bound to InsP₆. (A) *V. cholerae* MARTX CPD (PDG ID 3EEB) and (B) *C. difficile* TcdA CPD (PDG ID 3HO6) are shown as ribbon structures; InsP₆ is shown as a stick model; the catalytic dyad Cys and His are labeled, and the side chains are shown as sticks. The N-terminus of the protein wraps around the CPD core and extends towards the active site (magenta ribbon). The β -flap structure, which bridges the InsP₆ binding site and substrate binding site, is colored in pink and shown in the inset. The conserved Trp residue is shown; this residue is critical for *V. cholerae* CPD to communicate InsP₆ binding to the active site.¹⁸ Residues that appear to stabilize the β -flap structure upon InsP₆ binding are labeled, and the side chains are shown as green sticks. InsP₆ interacting residues that may be stabilized by formation of the β -flap are indicated, and the side chains are shown as blue sticks.

Fig. 4.

Comparison of bacterial CPDs (Family C80) to other clan CD family members. Side and end views of (A) *V. cholerae* MARTX CPD (PDB ID: 3EEB), (B) *C. difficile* TcdA CPD (PDB ID: 3HO6), (C) *Porphyromonas gingivalis* gingipain R (PDB ID: ICVR), and (D) human caspase-7 (PDB ID: ISHJ) shown as ribbon structures. Proteins are rainbow colored starting with the N-terminus in blue and ending with the C-terminus in red. The catalytic Cys and His residues are labeled for orientation, and the side chains are shown as sticks.

Fig. 5.

A conserved mechanism for allosterically inhibiting caspases using synthetic small molecules. (A) Structure of known allosteric inhibitors. FICA and DICA inhibit the executioners caspase-3 and caspase-7, while Compound 34 inhibits the initiator caspase, caspase-1. The sulfhydryl group used to tether the compounds to Cys residues at the dimer interfaces is highlighted. (B) Comparison of three ribbon structures of mature caspase-7. Left, active site bound by Ac-DEVD-CHO inhibitor (PDB ID: 1FIJ); middle, ligand-free apoenzyme (PDB ID: 1K86); right, allosteric site-bound with DICA inhibitor (PDB ID: 1SHJ). The side chains of residues that undergo conformational rearrangements upon allosteric inhibitor binding are shown as sticks. R187, Y223, and Gly188 are shown as purple sticks; L2' loop residues (K212-I213-P214-V215) are shown as green sticks. In the "on" state (active site-bound) structure, the allosteric dyad (R187 and Y223) are coupled through a π -cation interaction, and the L2' loops assume an "up" position, maximizing the distance between L2' loops. A large central cavity at the dimer interface is also apparent in the active site-bound structure. In the "off"-state structures (ligand-free and allosteric site-bound), the R187–Y223 interaction is disrupted, and the L2' loops move in closer proximity. In the allosterically inhibited structure, the L2' loops occlude the central cavity. (C) Surface representation of three structures of mature caspase-1 (adapted from Scheer *et al.*).⁵¹ Left, active site covalently modified by Z-VAD-FMK (PDB ID: 2HBQ); middle, ligand-free apoenzyme (PDB ID: 1SC1); right, allosteric site modified by Compound 34. The allosteric cavity at the dimer interface is indicated in red. (D) Schematic of caspase regulation following proteolytic cleavage. Once the zymogen procaspases are proteolytically activated, the caspases are in dynamic equilibrium between "zymogen-like" and "on"-state conformations in the absence of ligand. Binding of substrate (orange diamond) or

modification of the active site by inhibitors shifts the equilibrium such that the protease is effectively locked in an “on-state” conformation; conversely, modification of caspases by the allosteric inhibitor (red circle) at the dimer interface traps the protease in the “off”-state similar to the zymogen. Model adapted from Scheer *et al.*⁵¹

Fig. 6.

Proposed model for DD2-mediated inhibition of KSHV protease by monomer trapping. Inhibitor binding to a KSHV monomer prevents protease dimerization, effectively inhibiting enzyme activation. DD2 binds to dimerization interface residues, shifting the equilibrium towards a pre-existing folding intermediate, trapping the protease in the inactive, monomeric state. Model adapted from Shahian *et al.*⁵⁹

Fig. 7.

Proposed model for 1541-stimulated executioner procaspase activation. (A) Structures of 1541 and procaspase-3-specific 1541B. (B) Wolan *et al.* propose that procaspases are in dynamic equilibrium between “off” and “on” states, with the “off”-state being the most favored. Binding of the small molecule activator 1541 close to the active site of procaspase-3 shifts the equilibrium to an “on”-state conformation such that procaspase-3 undergoes autoproteolytic activation (black arrows). 1541 slowly stimulates removal of the prodomain and inter-subunit cleavage; the latter event is least favored. As mature caspase is produced, the autoproteolytic activation of procaspase-3 accelerates (orange arrows) due to a positive feedback loop. At high concentrations of 1541 ($>30\ \mu\text{M}$), 1541 saturates the active sites and leads to inhibition. Model adapted from Wolan *et al.*⁷²