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Allosteric Regulation of Proteases

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Allostery is a basic principle of control of enzymatic activities based on the interaction of a protein or small molecule at a site distinct from an enzyme's active center. Allosteric modulators represent an alternative approach to the design and synthesis of small-molecule activators or inhibitors of proteases and are therefore of wide interest for medicinal chemistry. The structural bases of some proteinaceous and small-molecule allosteric protease regulators have already been elucidated, indicating a general mechanism that might be exploitable for future rational design of small-molecule effectors.

Introduction

At any time and in any physiological state, hundreds of cellular proteins are being processed or degraded in a highly controlled fashion. Although usually several factors are involved in these processes, intrinsic hydrolytic cleavage is performed exclusively by proteases.

All proteases are strictly regulated, because their proteolytic activity is potentially harmful for non-substrate proteins and because they play critical roles in innumerable biological processes. Consequently, dysregulation often leads to severe pathophysiological states that in principle could be medicated by protease inhibitors or activators.

Proteases are therefore important drug targets in the pharmaceutical industry.[1,2] The vast majority of protease inhibitors on the market act at the active sites of proteases, and several of them have achieved blockbuster status. Besides these results, however, spectacular failures have also occurred in the development of protease inhibitors or activators, often caused by unexpected off-target effects, resulting in toxic or clinically inefficient drugs.[1] The development of protease-modulating drugs still remains a formidable challenge, demanding the advancement of current drug design strategies.

Allosteric protease regulation by small molecules might represent a promising alternative approach to active site inhibitors. In this review we therefore detail the structural basis of allosteric protease regulation in biologically relevant protein–protease interactions. Furthermore, we show that small-molecule allosteric effectors often act in a similar manner, illustrating the generality of allosteric protease regulation.

Proteases

Proteases catalyze the hydrolysis of peptide bonds. They are categorized by their active site residues—variously an aspartate (aspartylprotease), cysteine (cysteine protease), serine (serine protease), threonine (threonine protease), or metal cation such as Zn^{2+} (metalloprotease)—and by their substrate cleavage mode, leading to endo- or exopeptidases. A more sophisticated classification of proteases, internationally accepted in the field of protease research, is used in the MEROPS database. In MEROPS, all known proteases are grouped into clans and families on the basis of their common evolutionary origins, as well as their natural and synthetic inhibitors.[3]

Proteases are a highly diverse group of enzymes and occur as single- to multidomain proteins of diverse molecular architectures. In contrast, the three-dimensional folds of proteases of the same family are usually highly conserved, as exemplified by the S1A family of serine proteases, which contains, for example, the widely studied proteases trypsin, chymotrypsin, thrombin, and factor VIIa (Figure 1 A).[4] The catalytic hydrolysis

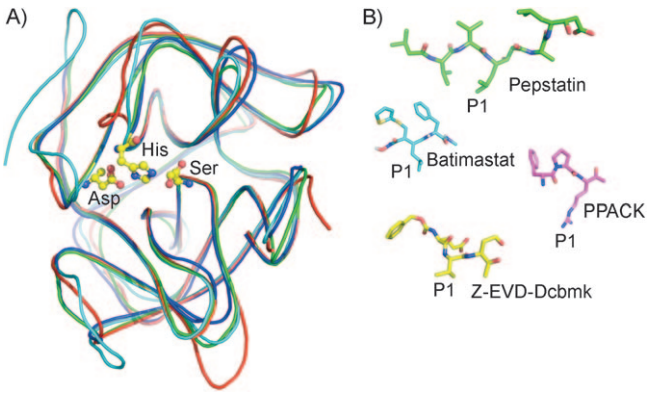

Figure 1. A) Overlay of the three-dimensional structures of the S1 serine proteases trypsin (green, PDB ID: 1TPO), chymotrypsin (blue, PDB ID: 1DLK), thrombin (red, PDB ID: 1AI8), and factor VIIa (cyan, PDB ID: 1KLI), highlighting their highly conserved fold. Active site residues are shown as balls and sticks. B) Examples of peptidic protease inhibitors from different protease classes, highlighting the conserved β-sheet-like binding interaction.

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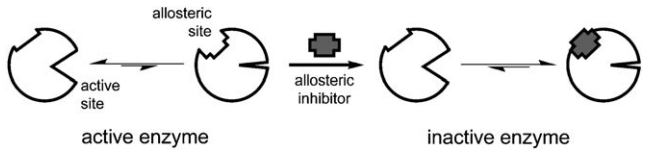
of substrates occurs at the active sites of the proteases, and among the different protease families these often differ in their amino acid configurations and/or topologies, whereas substrate binding takes place in specialized substrate binding pockets responsible for cleavage specificity. Importantly, all proteases recognize β-strands in their active sites, which further complicates the development of selective active-site-directed protease inhibitors (Figure 1 B).[5]

Allosteric Enzyme Regulation

Allosteric enzyme regulation is a modulation of the activity of an enzyme resulting from an interaction of an inhibitor at a site distinct from its active center. Although allostery was initially thought to be restricted to oligomeric proteins, culminating in the theoretical Monod–Wyman–Changeux (MWC) model and its further refinement in the Koshland–Némethy–Filmer (KNF) model,[6,7] recent findings indicate that allostery is an intrinsic property of all dynamic proteins.[8,9]

Enzymes can adopt several conformations, some of them catalytically active, with others being inactive. Equilibria exist between these different states, and these can be shifted upon reversible ligand binding or irreversible trapping at the allosteric binding sites to result in redistributions of the conformation ensembles (Scheme 1).[10,11] Such redistributions can often



Scheme 1. General model of allosteric regulation. A dynamic protein exists in an equilibrium of several low-energy conformations, which are variously enzymatically active or inactive. Reversible binding to or irreversible trapping of a distinct conformation shifts this equilibrium, inducing an alteration of enzyme activity.

be visualized by structural analysis of proteins in the absence and in the presence of allosteric effectors, enabling the characterization of the allosteric binding site and of the structural basis of the functional coupling to the active site.[12,13] Although most allosteric interactions are associated with structural alterations, it is important to note that enzyme conformations can also differ only by a mean conformational change resulting from altered atomic fluctuations.[14,15]

Allostery is a very rapid, reversible, and energy-free principle of regulation of enzyme activities. A vast body of our current knowledge on allosteric regulation dates back to studies from the 1960s and '70s, when metabolic pathways of cells were systematically investigated for feedback regulatory mechanisms. Only recently has interest in allosteric regulation of enzymes revived, after the realization that all dynamic proteins are in principle amenable to it.[16,17] The vast majority of allosteric regulators were identified after study of the binding sites of known enzyme modulators or by extensive enzyme kinetic studies. In addition, high-throughput screening of compound libraries, often in conjunction with structural studies, has yielded various allosteric regulators. An alternative chemical biology approach, named tethering, has also been successfully applied to the identification of allosteric effectors and their corresponding binding sites.[18,19]

Besides the implications of allosteric regulation in many physiological processes, it also represents a highly interesting approach for drug discovery. To date, most enzyme inhibitors target the active sites of enzymes. However, active site topologies are often strongly conserved between distinct members of the same enzyme family and so are difficult to address in a selective manner by small molecules. Moreover, active site inhibitors often mimic the transition state during enzyme catalysis, resulting in polar, substrate-like compounds, often with unfavorable pharmacokinetic properties. This is even more evident for proteases, in which active site inhibitors frequently consist of peptidic structures with electrophilic warheads. Allosteric sites are usually less conserved and can be addressed by more drug-like compounds, offering opportunities to identify structurally unique low molecular weight protein modulators.[20]

Allosteric Regulation of Proteases

A continuously growing number of publications points towards the eminent role of allostery in protease regulation. However, most reports are concerned with allosteric protein–protein interactions occurring during protease assembly, while study of allosteric regulation of proteases by proteinaceous effectors and particularly by small molecules is still in its infancy. To keep this review informative and concise, the focus will be on representative examples from three families: the trypsin/chymotrypsin-like serine proteases (MEROPS S1A), the HtrA serine proteases (MEROPS S1B), and the caspases, a family of cysteine proteases (MEROPS C14A). These protease families are of medicinal interest, and structural studies both of natural proteinaceous allosteric effectors and of low molecular weight allosteric effectors are available.

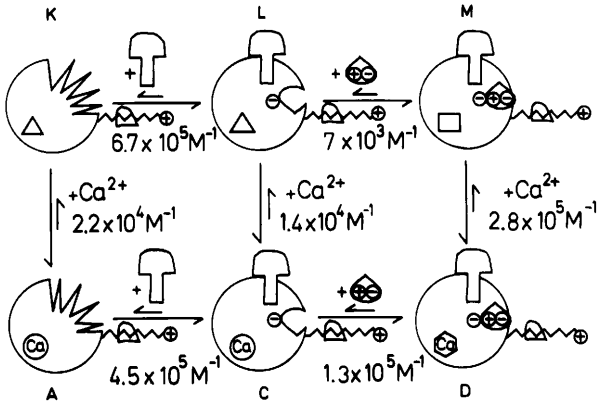
Trypsin/Chymotrypsin-like Serine Proteases (MEROPS S1A Family)

The S1A serine protease family is the largest of all protease families. All members are endopeptidases, featuring a highly conserved fold and a classical catalytic triad consisting of serine, histidine, and aspartate in the active site (Figure 1 A).[21,22] In general, they fall into three main categories: trypsin-like proteases that preferentially cleave proteins after basic amino acids, chymotrypsin-like proteases that cleave after hydrophobic amino acids, and elastase-like proteases that preferentially cleave after small, hydrophobic amino acids. All S1A proteases are synthesized as inactive zymogens and are activated upon a distinct proteolytic cleavage.[23]

The structural basis of the activation of S1A proteases was elucidated in seminal studies by Bode and Huber in the 1970s.[24] In the zymogen, four structural segments are deformed and flexible: the N terminus up to residue 19, as well

as residues 142–152, 184–193, and 216–223 (chymotrypsin numbering), which together form the activation domain. A proteolytic cleavage of the zymogen at a distinct bond results in an ordering of the activation domain as the newly formed N terminus inserts into a preformed binding pocket to form a salt bridge with Asp194 (chymotrypsin numbering). This rearrangement also brings about the correct formation of the S1 substrate binding site, oxyanion hole, and adjustment of the catalytic triad and is accompanied by a general stiffening of the overall structure. Consequently, the zymogen is commonly referred to as a disordered conformation, whereas the active protease adopts an ordered conformation.

Interestingly, most allosteric protease effectors seem to induce a switch between these two defined, naturally occurring conformations. In 1976, Bode and Huber showed for trypsinogen (the zymogen of trypsin) that the inactive trypsinogen conformation can be switched into an active but non-processed trypsin-like conformation by addition of H-Ile-Val-OH, a dipeptide resembling the activating N terminus of trypsin.[25,26] This was the first description of allosteric activation of a normally inactive protease conformation by a small molecule (Scheme 2).



Scheme 2. Huber and Bode's "classic" trypsinogen activation scheme of 1976, illustrating the equilibrium shifts occurring during allosteric zymogen activation (kindly provided by R. Huber, Max-Planck-Institut für Biochemie, Martinsried, Germany). The schematized species K and A represent proteolytically inactive trypsinogen in the absence or in the presence of Ca^{2+}, L and B represent trypsinogen bound to the trypsin inhibitor PTI in the absence and in the presence of Ca^{2+}, and M and C represent trypsinogen bound to the trypsin inhibitor PTI and the activating H-Ile-Val-OH dipeptide in the absence and in the presence of Ca^{2+}. Reproduced with permission from a private communication by W. Bode and R. Huber.

Studies of the biological function of streptokinase, a hemolytic protein secreted by *Streptococci* strains, demonstrated its capability to induce a non-proteolytic activation of plasminogen, the zymogen of the trypsin-like protease plasmin.[27] This finding led to the proposal that streptokinase might act in a similar fashion to the trypsin-activating dipeptide: that is, through an intrusion of the N-terminal end of streptokinase into the activation pocket of plasminogen, thereby inducing allosteric activation. This general allosteric zymogen activation mechanism was consequently termed "molecular sexuality" by Huber and Bode.[25] Although later biochemical studies with streptokinase site-directed mutants confirmed this concept,[28] it took more than twenty years for this allosteric activation mechanism to be finally corroborated by a structural study on a molecular level. The validation of the "molecular sexuality" concept was achieved in the form of a co-crystal structure of prethrombin-2, a zymogen of the trypsin-like protease thrombin, and staphylocoagulase, a non-proteolytically active protein acting as a zymogen activator.[29] Staphylocoagulase is secreted by the human pathogen *Staphylococcus aureus* to initiate blood clotting in its host by nonproteolytic activation of thrombin.[30] As predicted from the activation model, the co-crystal structure revealed an insertion of the N-terminal end of staphylocoagulase into the activation pocket of prethrombin-2, thereby triggering an allosteric rearrangement into the ordered and proteolytically active thrombin conformation (Figure 2).[29]

These findings highlight the inference that small molecules, mimicking the activating N-terminal end sequences, should also be able to represent S1A protease activators by inducing allosteric rearrangement of the disordered zymogen structure into the ordered protease conformation. Consequently, it seems reasonable that a thorough systematic search could yield such medicinally interesting compounds.

A special case of proteolytic activation is represented by the trypsin-like protease factor VIIa. In contrast with other S1A proteases, the proteolytic cleavage of the zymogen factor VII to factor VIIa generates a protease with only low proteolytic activity. Full enzymatic activity is achieved only after complexation with a second protein, named tissue factor (TF), in the presence of Ca^{2+} cations.[31] Because factor VIIa plays a critical role in blood coagulation, and due to its unusual activation mechanism, several studies to elucidate the allosteric activation mechanism of factor VIIa by TF have been carried out.

To this end, X-ray structures of, for example, the zymogen factor VII, factor VIIa, and factor VIIa in complexation with TF have been generated (Figure 3B).[32–34] Unfortunately, the structure of the zymogen factor VII has to date been obtained only in complexation with a small-molecule ligand named A-183 (vide infra). Nevertheless, this structure is generally accepted as a representative model structure of native factor VII and reveals the usual disordered zymogen conformation, while factor VIIa in complexation with TF assumes the ordered active protease conformation. Proteolytically processed factor VIIa takes on an intermediate structure, which, however, resembles a zymogen-like structure rather than a protease conformation. The transformation of this partly disordered zymogen-like structure into the ordered, proteolytically active conformation is allosterically achieved by interaction with TF (Figure 3C).

The elaborate nature of the TF-mediated regulation of factor VIIa suggests that low molecular weight ligands might also affect factor VIIa function. Two peptides—E-76 and A-183 (Figure 3A)—were discovered as potential allosteric inhibitors by phage display of naïve peptide libraries constrained with a single intramolecular cysteine bond.[35–37] The two compounds bind tightly to two distinct exosites of the protease domain,

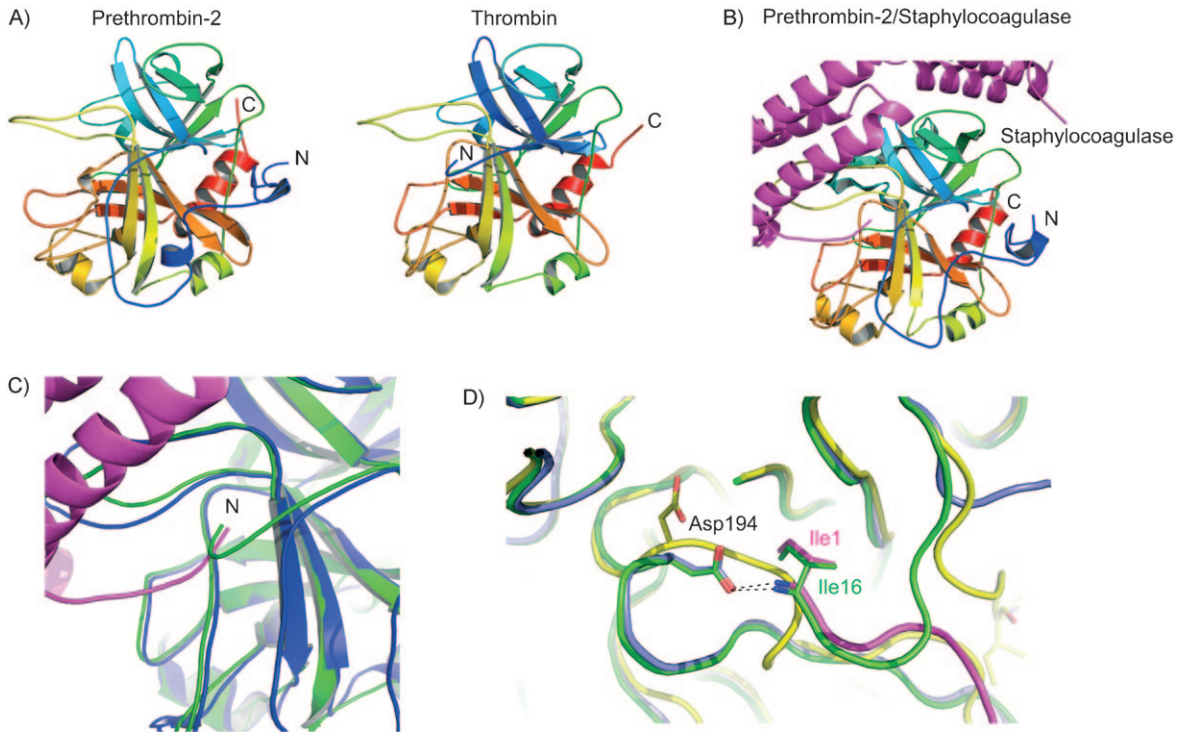


Figure 2. Activation mode of thrombin. A) X-ray structures of prethrombin-2 (PDB ID: 1HAG) and proteolytically active thrombin (PDB ID: 1PPB). B) Co-crystal structure of prethrombin-2 and staphylocoagulase (PDB ID: 1NU9). C) Overlay of activated thrombin (green) and the prethrombin/staphylocoagulase complex (thrombin shown in blue, staphylocoagulase shown in magenta). The N termini of processed thrombin and staphylocoagulase occupy the same position in the activation region of thrombin. D) Close-up of the activation region of thrombin. Overlay of prethrombin (yellow), thrombin (green), and the prethrombin/staphylocoagulase complex (prethrombin and staphylocoagulase shown in blue and magenta, respectively).

resulting in a significant reduction of the proteolytic activity of factor VIIa in complexation with TF. A structural study with E-76 and factor VIIa revealed that E-76 induced significant disorder in the protease conformation, illustrated by the loss of a conformation-stabilizing hydrogen bond and an increased flexibility in the corresponding loop.[35] It thus seems that E-76 acts as a compound that induces conformational switching from an ordered active protease conformation into a disordered, more flexible zymogen—or at least zymogen-like—structure, thereby causing the observed inhibition (Figure 3C and 3D).

Surprisingly, A-183 operates through a different mechanism. Unfortunately, the only X-ray analysis available to date is that in complexation with the zymogen factor VII, which demonstrated that A-183 binds to a factor VII exosite close to the active site.[37] Further characterization of the structural basis of the observed inhibition is difficult, but A-183-mediated disruption of the substrate binding site has been suggested.

In summary, although the regulation of factor VIIa is far more complex than in most S1A proteases, it has been possible to find peptidic allosteric regulators that induce a switch from the ordered active protease conformation to the disordered zymogen-like conformation, thereby following a mechanism similar to that of the allosteric zymogen activators.

HtrA Proteases (MEROPS S1B Family)

An alternative allosteric regulatory mechanism was found in the HtrA serine proteases after analysis of their activation in a cellular signaling cascade. Initially, HtrA proteases were identified for the first time in *E. coli* through the use of null mutants that either prevented growth at elevated temperatures (HtrA, for High temperature requirement) or lost the capability to digest misfolded proteins in the periplasm (DegP).[38,39] Consequently, this protease family was attributed to protein quality control, and their direct involvement in diverse biological processes such as unfolded stress response, various protein-folding diseases, and cancer has so far been demonstrated.

HtrA proteases belong to the S1B family and are multidomain proteins consisting of at least one chymotrypsin-like protease domain and one or two PDZ domains that assemble into complex and highly dynamic multimers.[40] Among them, DegS functions as a stress sensor in bacterial periplasmatic protein quality control.[41] Upon binding of hydrophobic peptides derived from the C-terminal ends of misfolded outer membrane proteins, an allosteric activation of DegS occurs. This leads to the initiation of a proteolytic signaling cascade, resulting in the expression of various stress genes. X-ray studies of DegS have confirmed this allosteric activation mechanism and provide insight into the structural basis of its activation (Figure 4B).[42,43] Intriguingly, the activating peptides bind to the PDZ domain with the penultimate amino residue extending into the chymotrypsin-like protease domain, thereby inducing a structural re-

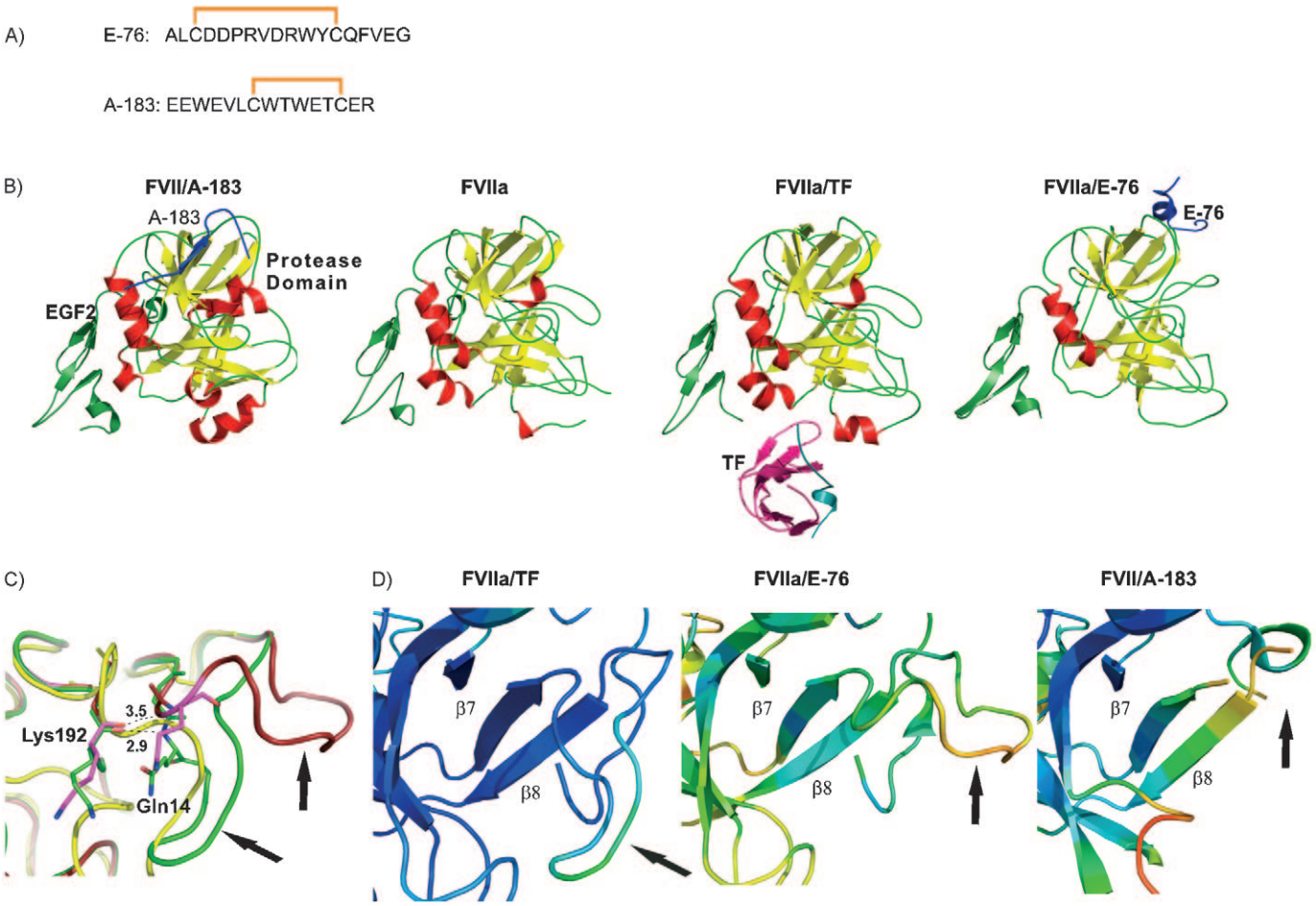


Figure 3. A) Sequences of E-76 and A-183. B) Structures of the zymogen factor VII/A-183 complex (PDB ID: 1JBU), factor VIIa (PDB ID: 1QFK), the factor VIIa/TF (PDB ID: 1DAN) complex, and factor VIIa in complexation with E-76 (PDB ID: 1DVA). C) Overlay of the activation region of the factor VIIa/TF complex (green), zymogen factor VII/A-183 (yellow), and factor VIIa in complexation with E-76 (red). Arrows indicate the relocation of the activation loop. D) Comparison of the positions of this flexible loop in the FVIIa/TF, FVIIa/E-76, and FVII/A-183 complexes, colored according to crystallographic B-factors representing structural flexibility (from blue to red; blue represents rigid regions, red highly flexible areas). Arrows illustrate the increasing disorder in the activation loop. While proteolytically active FVIIa/TF is still well defined and ordered, FVIIa/E76 shows already greatly increased flexibility. The activation loop in the zymogen FVII/A183, however, is highly disordered, resulting in a lack of electron density in the corresponding crystal.

arrangement in the protease domain from a disordered zymogen-like structure into an ordered chymotrypsin-like conformation (Figure 4C).

Interestingly, an allosteric activation mechanism was also elucidated for DegP, a structurally and also functionally more complex HtrA protease, indicating a general regulatory mechanism for all HtrA proteases.[44–46] At low temperatures, DegP acts as a molecular chaperone, whereas at higher temperatures it functions as a protease, selectively digesting proteins that are misfolded or without any apparent tertiary structure.[47] Binding of suitable hydrophobic peptides to the allosteric binding site leads either to activation of the protease at low temperatures or to amplification of the protease activity of DegP at higher temperatures. The allosteric "chemical" activation thus overpowers even the temperature regulation of DegP.[44,48]

HtrA proteases therefore represent anomalous proteases. While allostery in most proteases represents an additional regulatory element acting equipollent to other regulatory mechanisms, allosteric control of the proteolytic activity of HtrA proteases is superordinated over other regulatory mechanisms. Consequently, these proteases are prime targets for the development of small-molecule allosteric effectors through the synthesis of, for example, peptidomimetic analogues of the activating peptides.

Caspases (MEROPS Family C14A)

Caspases are the central elements of apoptosis, or programmed cell death.[49] Their name is a composition of cysteine protease and aspartate, the residue after which caspases preferentially cleave protein substrates. Caspases belong to the C14A family and are each characterized by a catalytic dyad consisting of a cysteine and histidine residue. They are biosynthesized as inactive single-chain proenzymes, which are cleaved upon proteolytic activation into monomers consisting of large α- and small β-subunits. Two monomer units subsequently arrange into a highly conserved proteolytically active homodimer

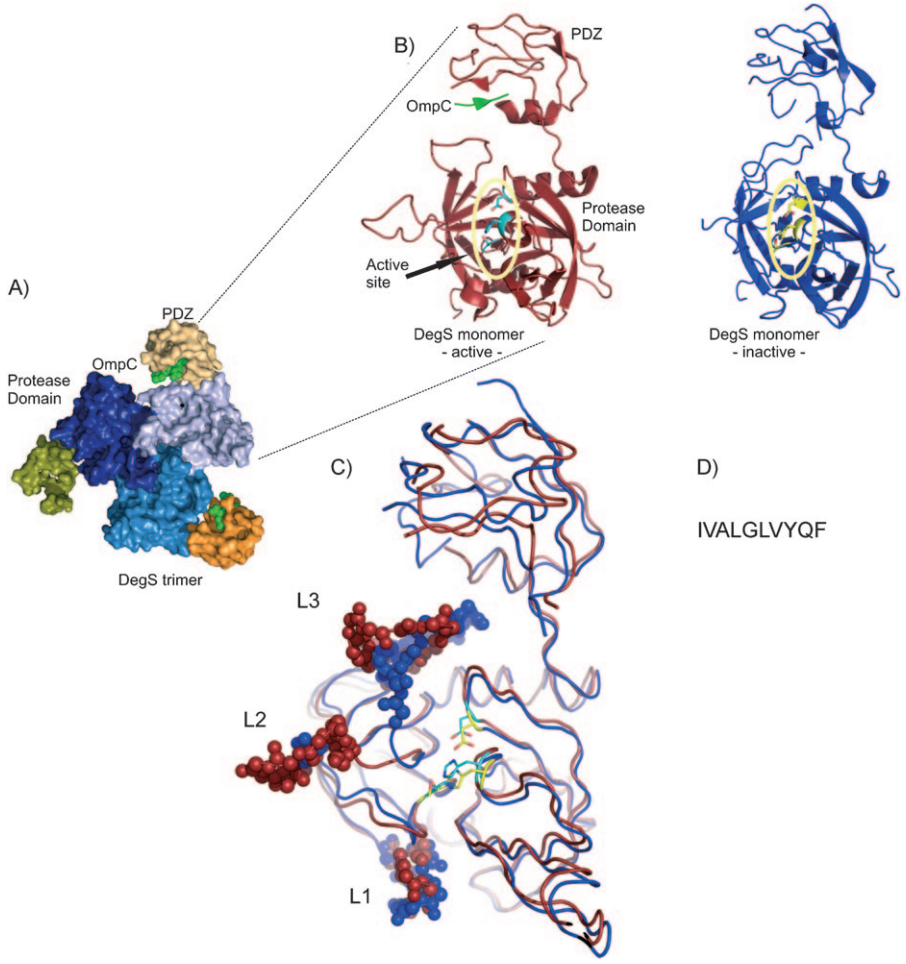


Figure 4. A) Structure of the DegS trimer in surface representation. B) DegS monomers in the presence (PDB ID: 1SOZ) and the absence (PDB ID: 1SOT) of the hydrophobic OmpC peptide. C) Overlay of DegS in the absence (blue) and in the presence (red) of the OmpC-derived peptide. Main chain atoms of the diverging loops L1, L2, and L3 are shown as spheres, the active site residues as sticks. D) Sequence of the OmpC model peptide.

(Figure 5A).[50] To date, several examples of allosteric regulation of caspases by either proteinaceous or small molecules are known.

Caspases-3, -7, and -9 are inhibited with high specificity by the multidomain endogenous inhibitor XIAP. However, while caspases-3 and -7 are inhibited by prevention of substrate access to the active site, caspase-9 is inhibited allosterically by the BIR3 domain of XIAP.[51] In vivo, caspase-9 exists predominantly as an inactive monomer. BIR3 binds selectively to the caspase-9 monomer at its dimerization side, thereby preventing the formation of the catalytically active caspase-9 homodimer. As a consequence, the active site loops remain in the inactive conformation of the caspase-9 monomer (Figure 5B). BIR3 thus traps caspase-9 in its inactive conformation, highlighting the preferred stabilization of natural state conformations by allosteric effectors.

In a second example, allosteric inhibition of caspase-2 was achieved with a designed ankyrin repeat protein (DARPin).[52] A structural study of its binding mode revealed that DARPin induces only relative small structural rearrangements of the loops constituting the active site. DARPin appears to fix caspase-2 in an inactive conformation unrelated to the zymogen structure, therefore representing an alternative allosteric binding mode (Figure 5C).

Wells's group developed a technique named tethering that can be employed for allocating allosteric sites in proteins. It is based on the reversible formation of disulfide bonds between cysteines on protein surfaces and a library of thiol-containing small molecules. Either native or artificially introduced cysteines can be addressed. The function of the five surface-exposed cysteines in caspase-3 was probed by the tethering technique.[18] Two compounds named DICA and FICA (Figure 5F) were identified as caspase-3 inhibitors, binding selectively to Cys264, located in a central cavity at the dimerization interface. The highly homologous caspase-7, featuring a 100% identical central cavity, was also inhibited by DICA and FICA upon cysteine binding. A co-crystal structure of these two small molecules with caspase-7 demonstrated different binding modes of DICA and FICA in the allosteric site, but an identical conformational rearrangement in the protease domain. Both compounds induce a switch of the active dimerized caspase-7 conformation into a procaspase-7-like conformation by irreversibly trapping the zymogen-like conformation (Figure 5D).

Caspase-1, which has only 20–30% amino acid sequence identity to caspase-3 and caspase-7, features a cysteine binding site not within but close to the cavity at the dimerization interface.[19] This Cys residue could also be targeted by tethering, and a suitable small molecule named compound 34 (Figure 5F) was identified as a caspase-1 inhibitor. Structure analysis of their co-crystal structure revealed an analogous allosteric inhibition mode, characterized by conformational switching into a zymogen-like structure (Figure 5E). Interestingly, the functional coupling of the allosteric and the active site requires only a small subset of the hydrogen bonding networks within the protease.[53]

These examples demonstrate that allosteric regulation of caspases is employed in nature but can also be achieved by small molecules. Also for this protease family, it seems that most allosteric effectors act through preferential binding to

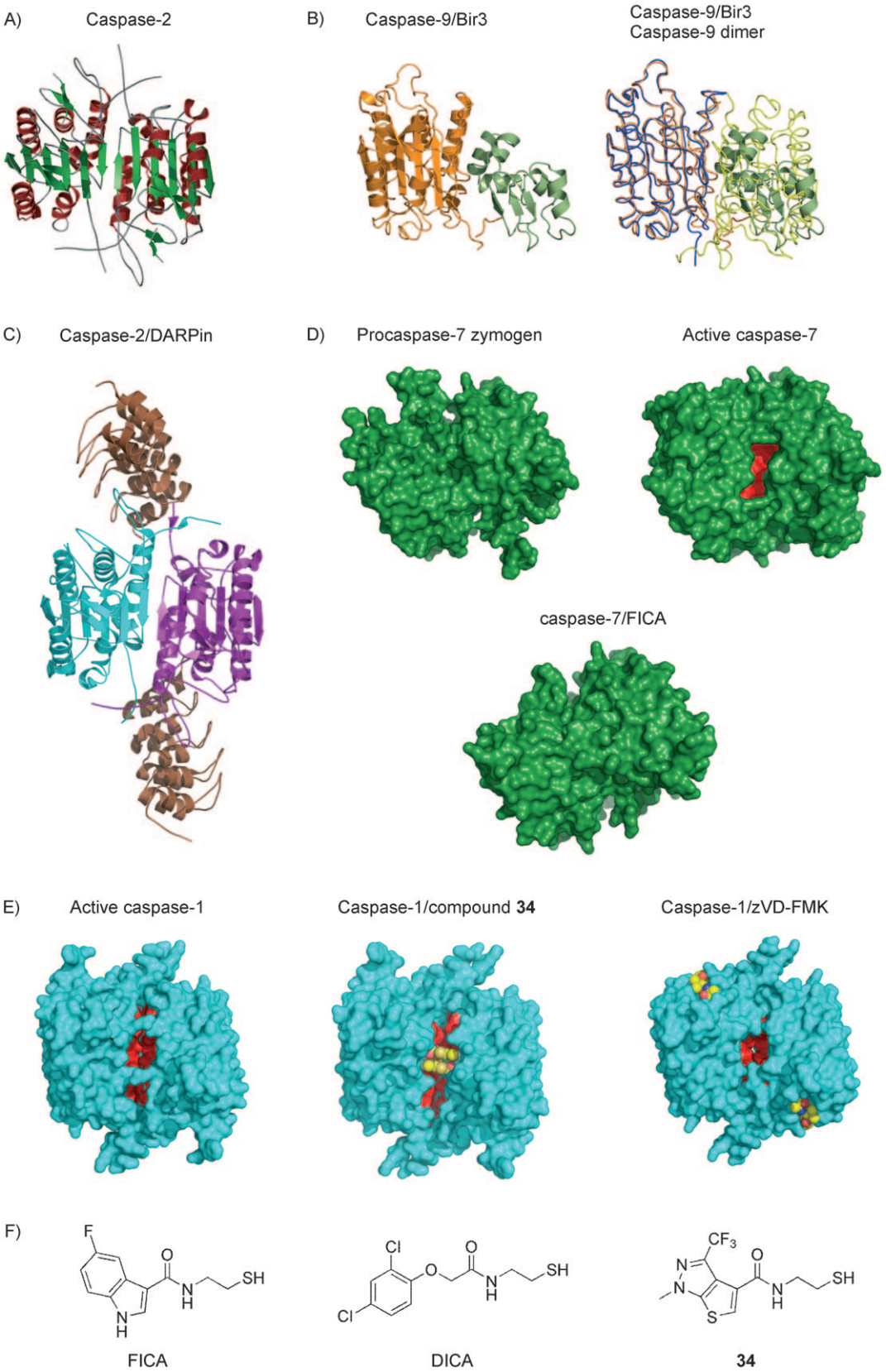


Figure 5. A) X-ray structure of proteolytically active dimeric caspase-2 (PDB ID: 1PYO). B) Complex structure of caspase-9 and BIR-3 (PDB ID: 1NW9) and overlay of the structure of active dimeric caspase-9 (PDB ID: 1JXQ) and caspase-9 in complexation with BIR3. C) Structure of caspase-2 and complex structure with DARPin (PDB ID: 2P2C). D) Surface representation of the dimeric structure of procaspase-7, active caspase-7, and FICA-inhibited caspase-7. The allosteric cavity is shown in red. E) Surface representation of the dimeric structure of active and compound 34- or zVAD-FMK-inhibited caspase-1. F) Chemical structures of FICA, DICA, and compound 34.

native protein conformations, thereby inducing the observed allosteric effect. However, the binding mode of DARPin demonstrates that previously unobserved conformations can also be addressed. Unfortunately, although suitable allosteric binding sites have been identified, no general design principles for reversible and small-molecule-based allosteric regulators of caspases can yet be given, and further research into this direction is required.

Conclusions and Outlook

Allostery is commonly used in biological systems to regulate the proteolytic activities of proteases. Initial studies of small-molecule allosteric effectors indicate that they act in a similar manner to proteinaceous effectors, either by mimicking their interaction or by targeting completely new sites. Consequently, this approach offers good prospects for medicinal chemistry as it circumvents the well known problems of active site inhibitors.

Most allosteric effectors appear preferentially to target native low-energy conformations such as those found in the zymogen or active protease structure. The preference for those conformations might be explained by the stabilities of those conformations. It is easier to shift the protein conformation equilibrium into a low-energy conformation, such as zymogen or active protease conformations, than into high-energy structures.

Although the principles of allosteric regulation are well understood, a fundamental question still remains. How can further small-molecule allosteric regulators be discovered? This question is not easy to answer, and it seems that several approaches can be employed to reach this goal. On the one hand, the available structural data on the interactions of proteinaceous allosteric effectors and their target proteases might be used for rational design of small-molecule mimics. On the other hand, screening of compound libraries represents a promising approach for finding new allosteric modulators. This calls for suitable compound libraries from, for example, diversity-oriented (DOS) or biology-oriented synthesis (BIOS).[54–58] Finally, tethering has demonstrated its potential to identify new allosteric sites and regulators.[18,19] However, the search for allosteric small-molecule regulators of proteases has just begun, and future research efforts will still be required to provide guidelines for a more rational design of allosteric effectors.

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Keywords: allosteric regulation · drug discovery · enzymes · proteases · small molecules

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