OPEN ACCESS

University of BRISTOL

Peer reviewed version

Link to published version (if available):
10.1073/pnas.1918721117

Link to publication record in Explore Bristol Research
PDF-document

University of Bristol - Explore Bristol Research
General rights

Activation by sub-stoichiometric inhibition

Melisa Merdanovic[a], Steven G. Burston[b], Anna Laura Schmitz[a], Steffen Köcher[a], Stefan Knapp[c], Tim Clausen[d], Markus Kaiser[a], Robert Huber[a,f,1], and Michael Ehrmann[a,1]

Affiliations

[a]Center of Medical Biotechnology, Faculty of Biology, University Duisburg-Essen, 45117 Essen, Germany

[b]School of Biochemistry, University of Bristol, Biomedical Sciences Building, Bristol BS8 1TD. U.K.

[c]Institute of Pharmaceutical Chemistry, Goethe-University Frankfurt, 60438 Frankfurt, Germany

[d]Research Institute of Molecular Pathology, 1030 Vienna, Austria

[e]School of Biosciences, Cardiff University, Cardiff CF10 3US, U.K.

[f]Max-Planck-Institute of Biochemistry, 82152 Martinsried, Germany

[1]To whom correspondence may be addressed. Email: michael.ehrmann@uni-due.de or huber@biochem.mpg.de.

Classification: BIOLOGICAL SCIENCES: Biochemistry

Keywords: protease, allostery, cooperativity, inhibitor, drugs, DegP, HtrA, HTRA1

Word count: 4645

Abstract

Startling reports described the paradoxical triggering of human mitogen activated protein kinase pathway when a small molecule inhibitor specifically inactivates the BRAF V600E protein kinase but not wt-BRAF. We performed a conceptual analysis of the general phenomenon "activation by inhibition" using bacterial and human HtrA proteases as models. Our data suggest a clear explanation that is based on the classic biochemical principles of allostery and cooperativity. Although sub-stoichiometric occupancy of inhibitor binding sites results in partial inhibition, this effect is overrun by a concomitant activation of unliganded binding sites. Therefore, when an inhibitor of a cooperative enzyme does not reach saturating levels, a common scenario during drug administration, it may cause the contrary of the desired effect. The implications for drug development are discussed.

Significance Statement

Small molecule inhibitors of enzymes are commonly derived from native ligands and represent invaluable and extensively applied tools in chemical biology. In addition to the well-known issue of selectivity, paradoxical curiosities were described over decades that have wide implications in drug discovery and treatment. Of particular interest are observations where low concentrations of inhibitors may activate their targets instead of causing partial inhibition. Our conceptual analysis explains the general phenomenon of "activation by inhibition" as a ligand-induced transition between the T state and the R state in the classic Monod-Wyman-Changeux model of activation, in which the inhibitory ligand acts as an activator.

Introduction

The activity of complex enzymes is precisely regulated by sophisticated molecular mechanisms that are described by the fundamental biochemical principles of allostery, cooperativity and oligomerization. Allostery is defined as the interaction of binding sites at a distance, allowing for the regulation of catalytic activity. Thus, a ligand bound to one site affects the affinity of another site for the same or a different ligand by inducing transitions between distinct conformational states. In 'classic' biochemistry, the couplings of binding sites are differentiated into functional interactions. Identical binding sites and homotropic ligands are the basis of cooperativity while a more general functional interaction of individual binding sites and heterotropic ligands is known as allostery. Allosteric effects may be observed between individual domains of monomeric proteins or between protomers of oligomeric protein complexes. Moreover, the allosteric switch between the resting and the active conformations can be accompanied by rather dramatic events such as changes in oligomeric states. While well-known studies have been performed on e.g. hemoglobin, the oxygen binding protein of red blood cells, or aspartate transcarbamoylase, a key enzyme in pyrimidine synthesis (1, 2), recent evidence suggests that prokaryotic DegP represents an exceptionally suitable model for addressing the underlying mechanisms of allostery, cooperativity and activation by oligomerization (3, 4).

The heat shock factor DegP functions as a conformation-specific protease/chaperone complex that channels substrates into repair, assembly or degradation pathways (3, 5). DegP is a cage-forming protease, where the size of the cage is determined by the number of assembled trimeric subcomplexes. DegP protomers consist of a serine protease domain and two C-

terminal PDZ domains. PDZ domains are protein modules that bind the C-terminal 3–4 residues of target proteins (6). Recent studies revealed how ligands of PDZ1 domain serve as allosteric activators inducing positive cooperativity by triggering conformational changes and large structural rearrangements including switches between various oligomeric states where hexamers represent the resting state while higher oligomers represent the active state (7-10). DegP has two binding sites per protomer, the catalytic site and the peptide binding site of the PDZ1 domain that are linked by an allosteric circuit. In addition, activation domains that are shared between adjacent protomers mediate concerted activation of the catalytic sites within the trimeric subcomplexes. This architecture is the basis of positive cooperativity where ligand binding to one binding site of one protomer not only increases the affinity of ligands for the second site within the same protomer but in addition increases the affinity for ligand binding to the neighboring protomer.

Results

These mechanistic considerations suggested a hypothesis where sub-stoichiometric binding of a substrate-derived inhibitor would activate DegP instead of causing partial inhibition. This behavior can be explained because the ligand-bound active site is structurally connected to neighboring protomers and their unoccupied active sites and PDZ1 domains. Conceptually, these allosteric interactions should cause increased affinity of unoccupied sites for their ligands ultimately resulting in increased enzymatic activity. To initially test this model of regulation, we used the synthetic substrate SPMFKGV-pNA to measure DegP activity in combination with the peptidic boronic acid inhibitor DPMFKLV-B(OH)$_2$ that targets the active site Ser residue (4, 11). The steady-state rate of substrate hydrolysis varied sigmoidaly as a function of

the substrate concentration, characteristic of substrate-induced allostery (SI Appendix, Fig. S1A). Kinetically, each time-course showed a significant lag-phase before activation of the proteolytic activity while increases in DegP concentration reduced both the length of the lag-phase and both the steepness and extent of the activation (SI Appendix, Fig. S1A). This observation is consistent with a previously-established mechanism in which DegP undergoes large changes in oligomeric state i.e. from the resting state hexamer, which dissociates into trimers and reassembles to form active 12-mer and even 24-mer assemblies (12, 13). This drastic change in oligomeric states normally occurs in response to the binding of substrate to the active site or the binding of allosteric peptides to the PDZ1 domain. The presence of the lag-phase (SI Appendix, Fig. S1A) reflects the rather slow interconversion of DegP between the resting conformation and the allosterically-activated states due to the absence of a heterotropic allosteric activator binding to the PDZ1 domains and also the relatively weak binding of the peptide substrate (SPMFKGV-pNA) used here. Note that SPMFKGV-pNA does not bind to the PDZ domain, because this substrate does not contain a C-terminal carboxylate which is essential for binding (6).

Activation by sub-stoichiometric inhibition. Binding of DPMFKLV-B(OH)$_2$ was investigated using ITC. Measurements indicated a two-step binding mode of the inhibitor to DegP, expected for a cooperative enzyme. The weaker-binding phase (K_d = 6 μM) likely reflects the binding of the inhibitor to the resting state conformation, followed by a second, much tighter binding event (K_d = 33 nM) reflecting inhibitor binding to the fully activated proteolytic sites (Fig. 1A). Binding of the inhibitor to the active site of a cooperative enzyme is expected to promote the conformational switch to a tighter binding conformation, thereby

resulting in activation of the enzyme at sub-stoichiometric concentrations. To quantify

activation by sub-stoichiometric inhibition, DegP was treated with various concentrations of

DPMFKLV-B(OH)$_2$, ranging from 1 nM to 10 µM, before measuring DegP activity using

SPMFKGV-pNA as a substrate. In agreement with our hypothesis, DegP activity is increased

by up to 3-fold at sub-stoichiometric concentrations of the inhibitor (Fig. 1B). To show that the

observed activation is not substrate-specific, the proteolytic assays were repeated using the

periplasmic domain of the antisigma factor RseA from *E. coli* as a substrate (14), revealing

similar effects (SI Appendix, Fig. S1B). As activation of DegP is usually connected with a

change in oligomeric state, DPMFKLV-B(OH)$_2$ was titrated to DegP and the oligomeric state

was determined by size exclusion chromatography and chemical crosslinking. Interestingly, a

switch from hexamer to dodecamer was only observed at above 2-fold excess of inhibitor, i.e.

concentrations where DegP is inhibited (Fig. 1C, SI Appendix, Fig. S1C), suggesting that

activation by sub-stoichiometric inhibition can occur in the hexamer. Control experiments

show that the catalytically inactive DegP S210A mutant does not bind DPMFKLV-B(OH)$_2$, as

determined by ITC (SI Appendix, Fig. S1D), and changes of oligomeric states do not occur

even at high inhibitor concentrations (SI Appendix, Fig. S1C). To rule out that the observed

activation results from unspecific biochemical effects such as aggregation (15), we applied a

non-binding inhibitor derivative carrying a (D)-configuration at the valine boronic acid residue

that had no or only minor effects on DegP activity (SI Appendix, Fig. S1E).

Activation by sub-stoichiometric inhibition in combination with allosteric activators.
DegP is allosterically activated by the C-termini of misfolded proteins which bind to its PDZ1

domain (Fig. 1D) (16). We therefore tested whether allosteric peptides would modulate

activation by sub-stoichiometric inhibition. Two allosteric peptides displaying a 100-fold difference in affinity towards DegP's PDZ1 domain were selected. The K_d values for the binding of DYFGSALLRV, corresponding to the C-terminus of *E. coli* EfeB (17) and the synthetic peptide DNRDGNVYFF that was characterized previously (9) were 1.2 μM and 135 μM, respectively (SI Appendix, Fig. S2A, S3A). Titration experiments indicated sequence specific differences in the potency of allosteric activation ranging between about 10 and 3-fold when using SPMFKGV-pNA as a substrate (Fig. 2A, SI Appendix, Fig. S2B, S3B). To subsequently test the combined effects of allosteric activators and inhibitor, the concentrations of the two allosteric peptides, DYFGSALLRV (2.5 μM) and DNRDGNVYFF (500 μM), were kept constant. Subsequent titration of inhibitor caused an up to 4-fold further activation of DegP at sub-stoichiometric inhibitor concentrations (Fig. 2A, SI Appendix, Fig. S2B, S3B). Similar results were obtained using RseA of *E. coli* as a substrate. DPMFKLV-B(OH)$_2$ was titrated to DegP in the presence of 2.5 and 50 μM of the allosteric activator DYFGSALLRV and degradation of RseA was followed by SDS PAGE at various time points. Again, activation was observed at sub-stoichiometric levels of inhibitor (Fig. 2B). Similar data were obtained using DNRDGNVYFF (SI Appendix, Fig. S3C). Furthermore, analysis of kinetic data indicated that the K_i of the inhibitor changed in the presence of the PDZ activator from 28 nM to 0.6 nM for DYFGSALLRV and to 20 nM for DNRDGNVYFF, respectively (compare Fig. 1B, Fig. 2A, SI Appendix, Fig. S3B). Moreover, to demonstrate that the detected activation coincided with increased affinity of allosteric peptides, ITC experiments were performed at various inhibitor concentrations. As expected, the K_ds of both peptides showed 6-fold higher affinities, i.e. for DYFGSALLRV from 1.2 μM in the absence of inhibitor to 0.2 μM in its

presence and for DNRDGNVYFF from 135 µM to 23 µM, respectively (SI Appendix, Fig. S2A, S3A).

Activation by sub-stoichiometric inhibition *in vivo*. To further substantiate our results, we tested whether activation at sub-stoichiometric concentrations of inhibitor occurred in living cells. For these assays, we used the experimental system that led to the discovery of the *degP* gene (Fig. 2C) (18). When a *tsr-phoA* fusion is expressed in *E. coli*, the Tsr-AP hybrid protein is cleaved near the fusion joint mainly by DegP but only marginally by other cell envelope proteases (SI Appendix, Fig. S4). Therefore, these cells represent a suitable reporter system to measure DegP activity *in vivo*. Growing cells expressing the plasmid-derived *tsr-phoA* fusion and native chromosomal *degP* were treated with DPMFKLV-B(OH)$_2$ at various concentrations ranging between 50 nM and 100 µM. Subsequently, proteolytic processing of the Tsr-AP hybrid protein by DegP was determined by Western bloting (Fig. 2D). The pattern of Tsr-AP bands indicated that at high inhibitor concentrations (25 - 100 µM), proteolytic processing of Tsr-AP was reduced because DegP is inhibited. At intermediate inhibitor concentrations (0.5 - 5 µM), processing of Tsr-AP was increased, while at lower concentrations of inhibitor (0.13 - 0.05 µM) processing of Tsr-AP was comparable to the DMSO control. Densitometry of the signal intensity of the Tsr-AP band relative to the DMSO control indicated an up to 2.5-fold increase in DegP activity at 2.5 and 5 µM inhibitor concentrations, respectively (note that the reduced amount of DegP at low inhibitor concentrations is likely the result of autoproteolysis caused by its activation). Therefore, the *in vivo* reporter system supported the results obtained by biochemical assays using purified proteins. These data also showed that even moderate activation of DegP can significantly affect processing of target proteins *in vivo*.

Activation by inhibition in human HTRA1. To demonstrate that activation by inhibition is not a phenomenon that is specific to the bacterial enzyme DegP and its unique structural properties, we tested the established model using human HTRA1 for which X-ray crystallography has shown that DPMFKLV-B(OH)$_2$ binds to the active site in a canonical manner (4). While HTRA1 shares structural and functional features with DegP, such as a trimeric arrangement of protomers and the conserved activation domain, it differs in several aspects, such as surface accessible active sites and a mode of activation that is not strictly coupled to changes in the oligomeric state.

ITC measurements of DPMFKLV-B(OH)$_2$ binding to HTRA1 indicated a K$_d$ of 0.8 μM (SI Appendix, Fig. S5A). To test whether HTRA1 can be activated by DPMFKLV-B(OH)$_2$, we performed proteolytic digests using its native substrate tau and various concentrations of inhibitor. An up to 15-fold increase in HTRA1 activity was observed in the presence of the inhibitor (Fig. 3). However, in contrast to DegP, the highest activation of HTRA1 was not observed at sub-stoichiometric concentrations of the inhibitor, but at 20-fold excess. This effect might be best explained by the 24-fold lower affinity of the inhibitor for HTRA1. To overcome this limitation, HTRA1 was pre-incubated with an allosteric ligand of the PDZ domain, SYAAWIDVEDL (K$_d$=32 μM, SI Appendix, Fig. S5B) at concentrations 3-fold below (10 μM) and 5-fold above (150 μM) its K$_d$. Subsequently, DPMFKLV-B(OH)$_2$ was titrated to determine the effects of the inhibitor on HTRA1's activity. At both concentrations of the allosteric PDZ domain ligand SYAAWIDVEDL, HTRA1 was activated by the inhibitor up to 7-fold and 10-fold, respectively (Fig. 3, SI Appendix, Fig. S5C). As expected, in the presence of 150 μM SYAAWIDVEDL, the inhibitor concentrations where highest activation

could be observed shifted from 20 µM to 1-2 µM. In agreement with these considerations, the presence of the allosteric activator caused stronger inhibition at high inhibitor concentrations. In addition, ITC measurements revealed, in a concentration-dependent manner, an up to 4-fold increase of the affinity of the allosteric peptide in the presence of the inhibitor, i.e. from 32 to 8 µM (SI Appendix, Fig. S5B).

Discussion

Ligand-derived synthetic inhibitors of enzymes are widely used in basic research and as drugs. For inhibitors interacting with single binding sites of monomeric enzymes, dose-response correlations are hyperbolic. The response of the enzyme system to inhibition changes fundamentally when an enzyme has multiple binding sites that are allosterically connected. Here, regulation of activity is more complex and can therefore cause effects that may seem paradoxical at first sight. HtrA proteases such as DegP belong to the S1 family of serine proteases, prominent members of which include e.g. trypsin, chymotrypsin, thrombin and elastase. The latter are typically activated by zymogen conversion. This process involves proteolytic processing of N-terminal segments stabilizing conformations via as disorder-order transition of the activation domain comprising loops L1, L2 and LD. As the formation of the active site is conformationally linked with binding of the newly formed N-terminus into a preformed pocket, allostery is an integral part of the activation mechanism of these classic proteases (19). Even though activation of DegP does not include zymogen processing, the principles described above are conserved. However, the molecular mechanisms regulating DegP are more complex. Its loop LD reaches over from an adjacent protomer and the activation domain is extended by loop L3 and PDZ domain 1 to sense allosteric ligands of the

PDZ domain. Moreover, activation by PDZ ligands is coupled to oligomer conversion, i.e. from the resting hexamer to active larger oligomers (9). The events triggered by a strong active site ligand such as DPMFKLV-B(OH)$_2$ involve the same elements, the interaction of which proceeds in reverse order, i.e. initial interaction of the ligand with the substrate specificity pockets and loop L2 triggers the rearrangement of loops L1 and LD* as well as of loop L3 leading to a structural rearrangement within the PDZ domain and a concomitant increase of affinity for its ligands. Therefore, high concentrations of the inhibitor have the same effect as allosteric ligands of the PDZ domain, i.e. triggering the conversion of hexamers into larger oligomers. Together, these allosteric communications across protomers result in the structural rearrangement of substrate-binding pockets, proper positioning of the catalytic triads and formation of the oxyanion holes in structurally connected active sites and ultimately in positive cooperativity. These processes can be considered as a ligand-induced transition between the T state and the R state in the classic Monod-Wyman-Changeux model of activation, in which the inhibitory ligand acts as an activator (20) (Fig. 1D). Therefore, activation does not require occupation of all allosteric sites.

Our model of activation of an enzyme by sub-stoichiometric occupancy of an inhibitor is reminiscent of other inhibitor activated systems. One prominent example is the activation of the mitogen activated protein kinase pathway when wild type BRAF is targeted by inhibitors such as PLX4032 (21). The paradoxical result has been readily explained by inhibitor-driven dimerization with unliganded CRAF causing allosteric disruption of autoinhibition and transactivation of CRAF leading to increased pathway activity (22). Therefore, the BRAF PLX4032 - unliganded CRAF complex represents another example of activation by sub-stoichiometric inhibition. Consistently, similar effects are observed with kinase-dead mutants

of BRAF (23). Moreover, additional allosteric events involving RAS-RAF interactions that are not RAS allele specific may come into play (22, 24), promoting additional activation and are thus comparable to those observed with allosteric peptides and HTRA1 and DegP. A similar example involving small molecule modulators of mammalian kinases describes activation by sub-stoichiometric inhibitor concentrations of PERK, a kinase of the unfolded protein response of the ER (25). It seems that this mode of regulation is widespread. For example, low ratios of the inhibitors maleate and *N*-phosphonacetyl-(L)-aspartate to aspartate transcarbamoylase cause an increase in enzymatic activity (20, 26). In addition, an engineered *E. coli* lipoprotein containing a hydrophobic C-terminus displayed similar concentration-dependent pattern of activation and inhibition of DegP as DPMFKLV-B(OH)$_2$, however the binding site of the lipoprotein-derived inhibitor and thus the underlying molecular mechanism remain to be elucidated (27).

The molecular mechanisms described here have wide implications for drug development. If an inhibitor that targets a cooperative enzyme is not equally distributed across all tissues, reflecting the well-known problem of bioavailability, the inhibitor will be efficient in tissues where distribution is good but it will activate the target protein in tissues where concentrations are low causing the opposite of the desired effect. Thus, allosteric effects are not only important for basic research but they have also considerable importance for clinical applications. In general, our work supports the notion that a careful consideration of classic biochemical principles is likely to significantly reduce side effects and failed efforts in drug discovery (28).

Materials and Methods

The synthetic substrate SPMFKGV-pNA of DegP and the peptidic boronic acid inhibitor DPMFKLV-B(OH)$_2$ were prepared and used as described (4, 11). The cell based assays of DegP activity employing a Tsr-AP hybrid protein were done as described (18). Methods for protein purification and ITC measurements followed previously described protocols. They are described in detail in the SI Appendix, which includes materials and methods and figures.

Data Availability

All data are included in the manuscript and supporting information.

References

1. C. Bohr, K. A. Hasselbach, A. Krogh, Ueber einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensäurespannung des Blutes auf dessen Sauerstoffbindung übt. *Skand. Arch. Physiol.* **16**, 401–412 (1904).

2. J. Gerhart, From feedback inhibition to allostery: the enduring example of aspartate transcarbamoylase. *FEBS J.* **281**, 612-620 (2014).

3. T. Clausen, M. Kaiser, R. Huber, M. Ehrmann, HTRA proteases: regulated proteolysis in protein quality control. *Nat. Rev. Mol. Cell Biol.* **12**, 152-162 (2011).

4. L. Truebestein *et al.*, Substrate-induced remodeling of the active site regulates human HTRA1 activity. *Nat. Struct. Mol. Biol.* **18**, 386-388 (2011).

5.   C. Spiess, A. Beil, M. Ehrmann, A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* **97**, 339-347 (1999).

6.   A. Ernst *et al.*, A structural portrait of the PDZ domain family. *J. Mol. Biol.* **426**, 3509-3519 (2014).

7.   T. Krojer, M. Garrido-Franco, R. Huber, M. Ehrmann, T. Clausen, Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. *Nature* **416**, 455-459 (2002).

8.   T. Krojer *et al.*, Structural basis for the regulated protease and chaperone function of DegP. *Nature* **453**, 885-890 (2008).

9.   M. Merdanovic *et al.*, Determinants of structural and functional plasticity of a widely conserved protease chaperone complex. *Nat. Struct. Mol. Biol.* **17**, 837-843 (2010).

10.   T. Krojer, J. Sawa, R. Huber, T. Clausen, HtrA proteases have a conserved activation mechanism that can be triggered by distinct molecular cues. *Nat. Struct. Mol. Biol.* **17**, 844-852 (2010).

11.   P. Hauske *et al.*, Selectivity profiling of DegP substrates and inhibitors. *Bioorg. Med. Chem.* **17**, 2920-2924 (2009).

12.   T. Krojer *et al.*, Interplay of PDZ and protease domain of DegP ensures efficient elimination of misfolded proteins. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 7702-7707 (2008).

13.   N. J. Thompson, M. Merdanovic, M. Ehrmann, E. van Duijn, A. J. Heck, Substrate occupancy at the onset of oligomeric transitions of DegP. *Structure* **22**, 281-290 (2014).

14.   S. Hasenbein *et al.*, Conversion of a regulatory into a degradative protease. *J. Mol. Biol.* **397**, 957-966 (2010).

15.   B. K. Shoichet, Screening in a spirit haunted world. *Drug Discov. Today* **11**, 607-615 (2006).

16.   M. Meltzer *et al.*, Allosteric activation of HtrA protease DegP by stress signals during bacterial protein quality control. *Angew. Chem. Int. Ed. Engl.* **47**, 1332-1334 (2008).

17.   X. Liu *et al.*, Crystal structure and biochemical features of EfeB/YcdB from Escherichia coli O157: ASP235 plays divergent roles in different enzyme-catalyzed processes. *J. Biol. Chem.* **286**, 14922-14931 (2011).

18.   K. L. Strauch, J. Beckwith, An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1576-1580 (1988).

19.   P. Hauske, C. Ottmann, M. Meltzer, M. Ehrmann, M. Kaiser, Allosteric regulation of proteases. *Chembiochem.* **9**, 2920-2928 (2008).

20.   J. Monod, J. Wyman, J. P. Changeux, On the Nature of Allosteric Transitions: A Plausible Model. *J. Mol. Biol.* **12**, 88-118 (1965).

21.   A. T. Weeraratna, RAF around the edges--the paradox of BRAF inhibitors. *N. Engl. J. Med.* **366**, 271-273 (2012).

22.   T. Jin *et al.*, RAF inhibitors promote RAS-RAF interaction by allosterically disrupting RAF autoinhibition. *Nat. Commun.* **8**, 1211 (2017).

23.   S. J. Heidorn *et al.*, Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* **140**, 209-221 (2010).

24.   F. Su *et al.*, RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N. Engl. J. Med.* **366**, 207-215 (2012).

25.   A. S. Mendez *et al.*, Endoplasmic reticulum stress-independent activation of unfolded protein response kinases by a small molecule ATP-mimic. *Elife* **4**, e05434 (2015).

26. J. Foote, H. K. Schachman, Homotropic effects in aspartate transcarbamoylase. What happens when the enzyme binds a single molecule of the bisubstrate analog N-phosphonacetyl-L-aspartate? *J. Mol. Biol.* **186**, 175-184 (1985).

27. H. Park, Y. T. Kim, C. Choi, S. Kim, Tripodal Lipoprotein Variants with C-Terminal Hydrophobic Residues Allosterically Modulate Activity of the DegP Protease. *J. Mol. Biol.* **429**, 3090-3101 (2017).

28. D. C. Swinney, Biochemical mechanisms of drug action: what does it take for success? *Nat. Rev. Drug Discov.* **3**, 801-808 (2004).

Figure legends

Fig. 1. Inhibition of DegP by DPMFKLV-B(OH)$_2$ A) ITC thermogram of binding of DPMFKLV-B(OH)$_2$ inhibitor to wt DegP. 400 µM inhibitor was titrated into the sample cell containing 40 µM DegP. K_ds of the two distinct binding steps are indicated. B) Kinetic parameters of DegP (1 µM) activity in the presence of the DPMFKLV-B(OH)$_2$ concentration indicated using the chromogenic substrate SPMFKGV-pNA (500 µM). Error bars = standard deviation of experimental data (n=3). Data were fitted to MWC model as described in supplementary information. L, equilibrium constant T/R in the absence of ligand; K_i, affinity of the inhibitor to activated DegP; K_R, dissociation constant for substrate binding to activated DegP; $v/[E]_0$, maximum turnover rate for the activated DegP species; parenthesis, 95% confidence limit of the fit. C) SEC analysis of DegP (100 µM) preincubated with ratios of DPMFKLV-B(OH)$_2$ indicated. Retention volumes and size (kDa) of calibration proteins thyroglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa) are indicated (left). Activity of eluted DegP fractions was determined using 500 µM SPMFKGV-pNA as substrate (right). Error bars indicate standard deviation (N=3). D) Cartoon of core allosteric mechanism. The T-state is the inactive hexamer. It exists in equilibrium with the dissociated trimers (R-state) which is competent to bind either substrate (S) or inhibitor (I) to the active site. Although the equilibrium in the absence of S or I lies towards the T-state (i.e. K_1 is small) the binding of S or I pulls the equilibrium towards the R-state by thermodynamic coupling. Alternatively, the inactive T-state binds activator to the PDZ1 domains moderately but the R-state PDZ1 domains bind activator more tightly (i.e. $K_{RA} \gg K_{TA}$) leading to activation, as once in the R-state it can bind S or I.

Fig. 2. Activation of DegP by substoichiometric inhibition A) Activity of DegP (1 µM) using chromogenic SPMFKGV-pNA (500 µM) as a substrate in the presence of various (left) or fixed

(right) concentrations of DYFGSALLRV (2.5 μM, corresponding to 2-fold of K_d) and various concentrations of the inhibitor DPMFKLV-B(OH)$_2$ (Inh). Error bars indicate standard deviation of experimental data (N=3). Data were fitted to MWC model as described in supplementary information. L, equilibrium constant between T and R conformations in the absence of ligand; K_R, dissociation constant for substrate binding to activated DegP; K_i, affinity of the inhibitor to activated DegP; K_{TA} and K_{RA} are the dissociation constants for the interaction between activator and the T-state conformation, and R-state conformation, respectively; K_{i2}, affinity of the activator for the active site; $v/[E]_0$ is maximum turnover rate for the activated DegP species; parenthesis, 95% confidence limit of the fit. B) Representative images of the digestion of the periplasmic domain of RseA (20 μM) by DegP (1 μM) in the presence of fixed concentrations of DYFGSALLRV (2.5 and 50 μM) and concentrations of DPMFKLV-B(OH)$_2$ indicated (upper panel). **, RseA cleavage products. Quantification of DegP activity using the signal intensity of RseA relative to the DMSO control (lower panel). Error bars indicate standard deviation (N=3). C) DegP activity *in vivo*. Cartoon of cell-based DegP reporter assay. Alkaline phosphatase (AP) is tethered to the N-terminal 164 amino acids of Tsr protein. DegP cleaves the Tsr-AP hybrid protein near the fusion joint. D) Inhibitor mediated activation of DegP activity *in vivo*. *E. coli* cells expressing the *tsr-phoA* fusion were grown ON at 30°C in rich medium with either DMSO (2%) or various concentrations of DPMFKLV-B(OH)$_2$. Whole cell extracts of equivalent number of cells were subjected to SDS-PAGE and Western blotting using antibodies against AP. *, Tsr-AP degradation products. Additional Western blots of the same samples using MBP-DegP antibodies (lower panel). Quantification of DegP activity using the signal intensity of Tsr-AP band relative to the DMSO control (upper panel). Error bars indicate standard deviation (N=3).
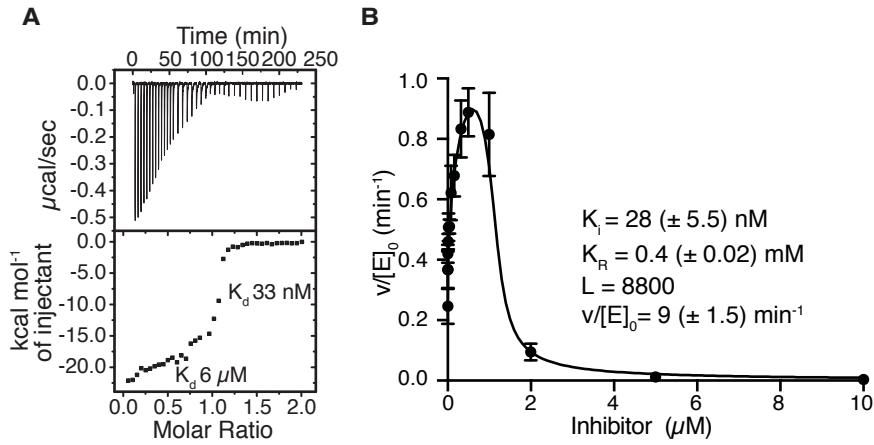
Fig. 3. Activation of HTRA1 by substoichiometric inhibition Proteolysis of Tau (10 µM) by HTRA1 (1 µM) in the absence or presence of the fixed concentrations of the allosteric peptide SYAAWIDVEDL (10 µM and 150 µM) and various concentrations of the inhibitor DPMFKLV-B(OH)$_2$ (Inh) after 5 h incubation at 37°C (left panel). Quantification of Tau signal intensity using densitometry (N=3) relative to the DMSO control, error bars indicate standard deviation (right panel). An additional 2 h time point of tau digests is shown in Fig. S5C.

AUTHOR CONTRIBUTIONS

MM performed experiments, SGB kinetic calculations, ALS, SK and MK chemical syntheses, MM, RH, SK, TC, MK and ME designed the study and analyzed data. The paper was written by ME with contributions by all authors.

Declaration of interests

The authors declare no competing interests.

A

	Lower	Upper
K_d (nM)	5650	33
ΔH (cal mol^{-1})	-2.4e^4	-1.6e^4
ΔS (cal mol^{-1} K^{-1})	-53.6	-15.7
n	1.3	1.1

B

K_i = 28 (± 5.5) nM
K_R = 0.4 (± 0.02) mM
L = 8800
$v/[E]_0$= 9 (± 1.5) min^{-1}

C

D

A

	DYFGSALLRV	Inhibitor + 2.5 μM DYFGSALLRV
K_R (mM)	0.4 (< 0.1)	0.3 (< 0.1)
L	8800	8800
K_I (nM)	n.a.	0.6 (< 0.1)
K_{TA} (μM)	660 (± 9.4)	607 (± 143)
K_{RA} (μM)	2.4 (± 0.1)	0.8
K_{I2} (mM)	6.8 (± 0.4)	2.0 (± 0.1)
$v/[E]_0$ (min^{-1})	5.5 (± 0.4)	9.6 (± 2.1)

B

DYFGSALLRV

C

D

SYAAWIDVEDL