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Supplementary Information for

Activation by sub-stoichiometric inhibition

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Supplementary Information Text

Materials and Methods

Bacteria and Plasmids

Strain KU98 (*degP::kan treA::spec ΔmalF3 ΔphoA* (*PvuII*) *phoR araD*139 *Δ(ara-leu)*7697 *ΔlacX*74 *galE galK thi rpsL* F' *lacI*^{q} *pro*) (1) was used for *degP* expression. Point mutants were constructed by oligonucleotide directed mutagenesis following standard procedures. All plasmids were derivatives of pCS20 expressing *wt degP* with a C-terminal His tag. MC4100 (F^{-} *araD139 Δ(argF-lac)U169 rpsL150* (Str^{R})*relA1 flbB5301 deoC1 ptsF25 rbsR*) (2) was used to establish *tsr-phoA* reporter system for DegP *in vivo* activity. *tsr-phoA* construct comprises of sequence coding for first 164 codons of *tsr*, followed by first 48 bp of IS50R and sequence coding for mature AP (aa 27-471, Uniprot numbering). *tsr-phoA* was cloned into modified pCS19 vector carrying chloramphenicol resistance gene using *NcoI*/*HindIII* restriction sites. Strain BW30270 (*F^{-}, lambda^{-}, ilvG, rfb-50, rph^{+}, fnr^{+}*) (*E. coli* Stock Center no. 7925) and derivates thereof containing single chromosomal knockouts of following proteases: *degP*, *ptrA*, *prc* and *degQ* were used to confirm that *in vivo* degradation of Tsr-PhoA is mediated by DegP. Rosetta2(DE3) (F^{-} *ompT hsdS*$_{B}$(r_{B}^{-} m_{B}^{-}) *gal dcm* (DE3) pRARE2) was obtained from Merck and used for the expression HTRA1 (residues 158-480) with a N-terminal StrepII-tag in pET21d vector. All constructs were verified by DNA sequencing.

Unless otherwise stated, cells were grown in LB-broth supplemented with appropriate antibiotics (200 μg/ml ampicillin, 30 μg/ml chloramphenicol, 50 μg/ml kanamycin) under aerobic conditions at 30°C.

Inhibitor and substrate synthesis

SPMFKGV-pNA and DPMFKLV-$B(OH)_2$ were synthesized as described (3, 4). Peptides were obtained from Intavis AG, Tuebingen, Germany.

Protein purification

Purification of DegP and HTRA1 was carried out as described (5, 6).

Size exclusion chromatography

100 μM DegP monomer-equivalent was preincubated with various concentrations of the DPMFKLV-$B(OH)_2$ inhibitor for 15 min at 37°C. 100 μl of the sample was loaded on Superose 6 10/300 column (GE Healthcare) pre-equilibrated with 50 mM NaPi, 150 mM NaCl, pH 8.0 buffer.

Chemical crosslinking

1 μM (monomer equivalent) of either purified wt DegP or active-site mutant DegP S210A was incubated with various DPMFKLV-$B(OH)_2$ inhibitor concentrations for 10 min at 37°C followed by addition of 0.1 % glutaraldehyde. After 2 min, reaction was stopped by addition of 30 μl 1 M Tris-HCl pH 8.0 and samples were further incubated for 10 min at RT before addition of 20 μl SDS-PAGE sample buffer. 2.5 μl aliquots (ca. 60 ng) were subjected to electrophoresis using 3-8 Tris-acetate gels (Invitrogen) and subsequently visualized with silver staining.

Western blots

Samples for Western blot analyses were prepared by harvesting cells by centrifugation (13,000 × *g* for 1 min). Pellets corresponding to an optical density at 600 nm (OD_{600}) = 5

were resuspended in SDS-PAGE sample buffer supplemented with 50 mM DTT. Samples were boiled for 10 min and subjected to electrophoresis through either 10% Bis-Tris SDS-PA gels (Invitrogen) with MOPS running buffer or 12% Tris-Glycine SDS-PA gels.

BW30270 and its derivates containing single chromosomal knockouts of following proteases: *degP*, *ptrA*, *prc* and *degQ* (7) and the *tsr-phoA* plasmid were grown at 30°C overnight. ON cultures were diluted in fresh LB to an OD_{600} of 0.05 and incubated further at 30°C. Aliquots of cells were removed at indicated time points, with time point zero corresponding to OD_{600} 0.5. Whole cell lysates were subjected to SDS-PAGE and immunoblotting. Immunoblotting was performed using mouse monoclonal antibody against AP (1:10,000 dilution, Caltag, South San Francisco, CA, USA). Secondary antibody was AP-conjugated anti-mouse (1:20,000 dilution).

In vivo assays. Strain MC4100 carrying *tsr-phoA* plasmid was grown ON. Cultures were diluted in fresh LB to an OD_{600}=0.05 and inhibitor (dissolved as 100x stock in DMSO) was added at the concentrations indicated. Cells were grown further at 30°C for 18 hours.

Immunoblotting was performed using rabbit polyclonal antisera against MBP-DegP (1:20,000 dilution) and monoclonal mouse antibody against AP (1:10,000 dilution, Caltag, South San Francisco, CA, USA). Secondary antibodies were fluorescently labeled goat anti-rabbit AlexaFluor633 and goat anti-mouse AlexaFluor488 (Invitrogen). Immunoblots were visualized using Typhoon 9000 laser scanner (GE Healthcare) and signal intensity of Tsr-AP bands was analyzed using raw, unmodified scans with TotalLab Quant software (TotalLab). Results presented are representative of three independent experiments.

Protease assays

DegP activity assays with synthetic substrate SPMFKGV-pNA were performed in 100 μl of 50 mM NaPi pH 8.0 at 37°C by measuring the changes in OD_{405} (Molecular Devices Spectramax 5e reader) continuously for 2 h at 37°C. SPMFKGV-pNA concentration was 0.5 mM. Before addition of the substrate, 1 μM DegP was preincubated with either DMSO or inhibitor/activator for 5 min at 37°C. In cases where both inhibitor and activator were used, DegP was first incubated with inhibitor for 5 min, followed by the addition of an activator peptide and further incubation for 5 min before addition of substrate. DMSO concentration in all assays was 2%.

DegP assays with the periplasmic domain of RseA as a substrate were performed in similar manner, except that the buffer was 50 mM HEPES supplemented with 50 mM NaCl and 0.1% CHAPS, pH 8.0 and RseA concentration was 20 μM. Samples were removed at various time points and subjected to SDS-PAGE on 15% Bis-Tris gels in MES running buffer. Cleavage of RseA was visualized by Coomassie staining. Gels were subsequently scanned and the intensity of RseA bands were quantified with TotalLab Quant software (TotalLab) using raw, unmodified gel images. Intensity of RseA band in control sample containing DegP and DMSO was set as 1.

HTRA1 activity was determined using its native substrate tau in 50 mM HEPES, 75 mM NaCl pH 8.0 buffer at 37°C. 1 μM HTRA1 was pre-incubated with either DMSO or inhibitor for 5 min. Subsequently, either buffer or allosteric peptide SYAAWIDVEDL was added and samples were incubated for another 5 min before addition of tau. Aliquots were removed at various time points and subjected to SDS-PAGE using 10% Bis-Tris gels in MOPS running buffer. Cleavage of tau was visualized by Coomassie staining. Gels were subsequently scaned and the intensity of tau bands was quantified with TotalLab Quant software (TotalLab) using raw, unmodified gel images.

Isothermal Titration Calorimetry

All ITC experiments were performed at 37°C using a MicroCal ITC200 microcalorimeter. Purified proteins (25-40 μM) and ligands/inhibitor (0.25 - 4 mM) were dialyzed/dissolved in appropriate buffer and degassed for 15 min in a thermovac sample degasser before titration. ITC experiments were initiated with a 300 μl solution of protein in the temperature-controlled cell, a 40 μl solution of peptide in the titrator syringe and stirring at 700 rpm.

In experiments with DPMFKLV-$B(OH)_2$, 1 μl aliquots of the inhibitor (400 μM) were injected into the sample cell containing 40 μM DegP monomer equivalent with spacing between injections ranging from 340-450 in 100 mM NaH_2PO_4 pH 8.0 buffer. The area under each peak was integrated and plotted against the molar ratio inhibitor/DegP inside the sample cell using Origin-ITC software. After subtracting for the heat of peptide dilution, the different binding steps were dissected and fitted separately using "one set of sites" model yielding K_d of 6 μM for the first binding step and 33 nM for the second binding step.

For experiments involving allosteric ligands, 2 μl peptide aliquots (250 μM DYFGSALLRV; 2 mM DNRGDNVYFF) were dispensed into the cell containing either 25 μM DegP (monomer equivalent) or 25 μM DegP plus 8 μM, 25 μM or 125 μM inhibitor with a 120-180 s equilibration time between injections. Experiments were done in 50 mM NaH_2PO_4 pH 8.0. The heats of binding were corrected for the heat of peptide dilution and data were analyzed using Origin software with "one set of sites" model.

ITC experiments involving HTRA1 were performed in similar manner in a buffer containing 100 mM NaH_2PO_4, 150 mM NaCl pH 8.0. HTRA1 concentration in the cell was

25 μM (monomer equivalent). Where appropriate, HTRA1 was additionally co-incubated with 8 μM, 25μM or 125 μM DPMFKLV-B$(OH)_2$ inhibitor. Concentration of ligands in the syringe was 0.625 mM for DPMFKLV-B$(OH)_2$ and 1.5 mM for SYAAWIDVEDL.

Non-analytical Mechanistic Modelling

As the concentrations of substrate, inhibitor or activating peptides varied, the observed data of DegP activity were fitted to a mathematical model of the postulated DegP allosteric mechanism using the software package DynaFit v4 (BioKin Ltd.) (8). Since the complete mechanism is likely to be highly complex and therefore liable to suffer from over-parameterization two approaches were taken to avoid this problem. Firstly, the principle of Occam's razor (or the Principle of Parsimony) was applied and the simplest mechanistic model was used that could be justified by the data. This meant excluding the possibility that once a DegP trimer was activated, assembly into 6-mer, 12-mer or 24-mer complexes further enhanced the catalytic rate constant of each active site. This can be partially justified by the finding that the switch from hexamer to dodecamer only occurred when the inhibitor was above a 2-fold molar excess. However, it is clear from the steady-state proteolytic activity data at various concentrations of DegP (Fig. S1A) that the increased stability of higher order oligomers at higher DegP concentrations is likely to enhance the rate of conversion of DegP from its resting state to the activated state via thermodynamic coupling of the assembly steps to the dissociation of the initial hexameric conformation. Secondly, parameters that had been determined using simpler scenarios (e.g. substrate-only) or using other techniques (e.g. ITC) were constrained or fixed in more complex mechanistic models, thus avoiding the problem of over-fitting.

Allosteric Mechanism

The core allosteric mechanism used in the kinetic modelling (Fig. 1D). A concerted model of allostery was used based on the fact that in a sequential model, the binding of an inhibitor molecule at one site would not be expected to activate the whole complex. The resting state (T-state in MWC parlance) is the inactive hexamer in which the LA loops from subunits of one trimer ring protrude into the active sites of the opposite ring. This results in a relatively weak affinity for any substrate peptide. In the model it exists in equilibrium with an active conformation of the hexamer (R-state) in which binding to the active site is no longer inhibited by the inter-ring interaction with the LA loops and so can bind substrate much more tightly. This activated hexamer is also destabilized and readily dissociates into active trimeric rings that are then competent for assembly into higher order oligomers. In the absence of substrate $K_1 = [R]/[T]$ while K_2 is the equivalent conformational equilibrium where the two conformations have activating peptide bound to the PDZ1 domain of each subunit. K_{TA} and K_{RA} are the dissociation constants for the interaction between activator and the T-state conformation, and R-state conformation respectively. The active DegP conformation has a relatively high affinity for either peptide substrate or a peptide competitive inhibitor, and can bind a mixture of both within the trimeric ring. The substrate binds to each subunit of the activated trimer with affinity K_{RS} while the affinity for the inhibitor is K_{RI}.

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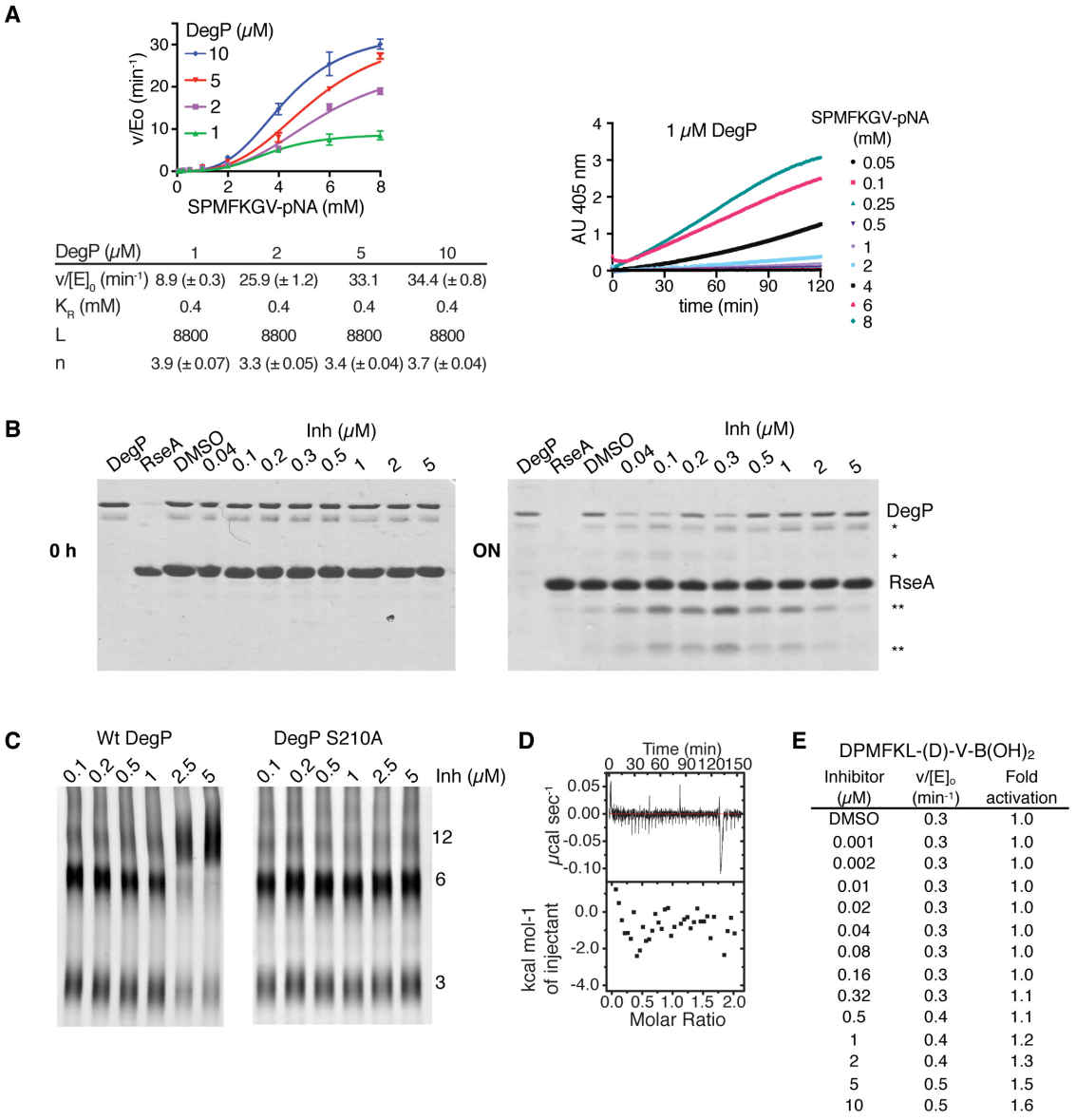
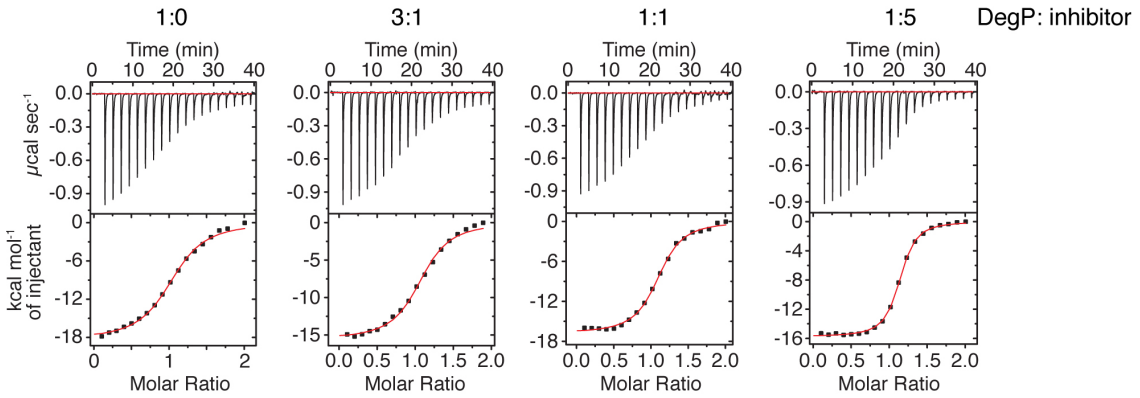


DegP (μM)	1	2	5	10
v/[E]$_0$ (min^{-1})	8.9 (± 0.3)	25.9 (± 1.2)	33.1	34.4 (± 0.8)
K_R (mM)	0.4	0.4	0.4	0.4
L	8800	8800	8800	8800
n	3.9 (± 0.07)	3.3 (± 0.05)	3.4 (± 0.04)	3.7 (± 0.04)

Inhibitor (μM)	v/[E]$_o$ (min^{-1})	Fold activation
DMSO	0.3	1.0
0.001	0.3	1.0
0.002	0.3	1.0
0.01	0.3	1.0
0.02	0.3	1.0
0.04	0.3	1.0
0.08	0.3	1.0
0.16	0.3	1.0
0.32	0.3	1.1
0.5	0.4	1.1
1	0.4	1.2
2	0.4	1.3
5	0.5	1.5
10	0.5	1.6

Fig. S1. A) Cleavage rates at various concentrations of DegP (1, 2, 5 and 10 μM) plotted vs concentrations of SPMFKGV-pNA (0.05, 0.1, 0.25, 0.5, 1, 2, 4, 6 and 8 mM). Error bars indicate standard deviation of the experimental data (N=4). Data were fitted to the MWC model for DegP. L, equilibrium constant T/R in the absence of ligand; K_R, dissociation constant for substrate binding to activated DegP; v/[E]$_0$, maximum turnover rate for activated DegP; parenthesis, 95% confidence limit of the fit (Left). Progress curves of DegP activity (1 μM) in presence of various concentrations of SPMFKGV-pNA substrate. Changes in absorbance at 405 nm, indicating pNA release, were recorded in 1 minute intervals over 2 h. Standard deviation was < 20% (N=4) (right). B) Representative images of RseA digestion by DegP in the presence of the inhibitor (Inh). *, DegP fragments due to autoproteolysis; **, RseA cleavage products. C) Oligomeric state of wt DegP and DegP S210A in the presence of various concentrations of DPMFKLV-B(OH)$_2$ at 37°C revealed by crosslinking with of 0.1% glutaraldehyde. Oligomeric states are indicated. D) ITC measurement of the DPMFKLV-B(OH)$_2$ binding to the DegP S210A active site mutant. 2 μl aliquots of the inhibitor (250 μM) were injected into the sample cell containing 25 μM DegP S210A. E) Effect of DPMFKL-(D)-V-B(OH)$_2$ on DegP (1 μM) activity using SPMFKGV-pNA (500 μM) as substrate. Standard deviation was < 20% (N=3).

A



DegP : inhibitor	1:0	3:1	1:1	1:5
K_d (μM)	1.2	0.9	0.6	0.2
ΔH (cal mol^{-1})	-1.8e^4	-1.6e^4	-1.7e^4	-1.6e^4
ΔS (cal mol^{-1} K^{-1})	-32.0	-22.5	-25.7	-20.5
n	1.0	1.1	1.1	1.1

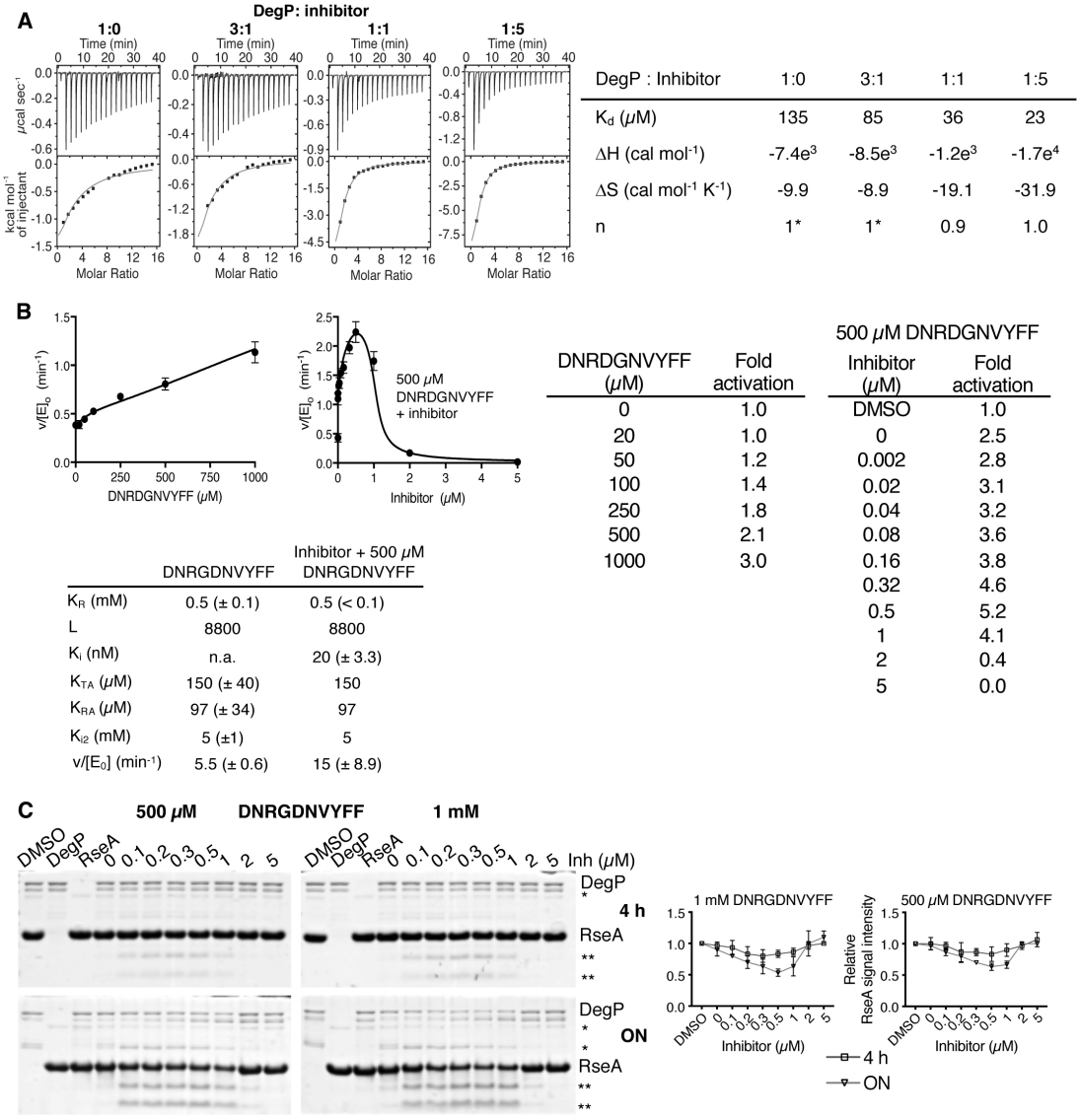
B

DYFGSALLRV (μM)	Fold activation
DMSO	1.0
0.25	1.1
0.5	1.2
1	1.5
2.5	3.0
5	5.4
10	7.0
25	8.2
50	9.5
100	10.3
250	10.5
500	9.4

2.5 μM DYFGSALLRV

Inhibitor (μM)	Fold activation
DMSO	1.0
0	2.2
0.002	2.0
0.02	2.5
0.04	2.9
0.08	3.5
0.16	4.1
0.32	4.3
0.5	3.5
1	0.5
2	0.1
5	0.0

Fig. S2. A) ITC thermograms of binding of DYFGSALLRV to 25 μM wt DegP in the absence or presence of DPMFKLV-B(OH)$_2$. DegP:inhibitor ratio in the ITC cell is indicated above the thermograms. Thermodynamic parameters were obtained using "one set of sites" model. B) Fold activation of DegP activity (relative to the DMSO control) corresponding to the data in Fig. 2B. Standard deviation was < 20% (N=3).



DegP : Inhibitor	1:0	3:1	1:1	1:5
K_d (μM)	135	85	36	23
ΔH (cal mol^{-1})	-7.4e^3	-8.5e^3	-1.2e^3	-1.7e^4
ΔS (cal mol^{-1} K^{-1})	-9.9	-8.9	-19.1	-31.9
n	1*	1*	0.9	1.0

DNRDGNVYFF (μM)	Fold activation
0	1.0
20	1.0
50	1.2
100	1.4
250	1.8
500	2.1
1000	3.0

500 μM DNRDGNVYFF Inhibitor (μM)	Fold activation
DMSO	1.0
0	2.5
0.002	2.8
0.02	3.1
0.04	3.2
0.08	3.6
0.16	3.8
0.32	4.6
0.5	5.2
1	4.1
2	0.4
5	0.0

	DNRGDNVYFF	Inhibitor + 500 μM DNRGDNVYFF
K_R (mM)	0.5 (± 0.1)	0.5 (< 0.1)
L	8800	8800
K_i (nM)	n.a.	20 (± 3.3)
K_{TA} (μM)	150 (± 40)	150
K_{RA} (μM)	97 (± 34)	97
K_{i2} (mM)	5 (±1)	5
v/[E_0] (min^{-1})	5.5 (± 0.6)	15 (± 8.9)

Fig. S3. Activation by sub-stoichiometric inhibition in combination with allosteric activators. A) ITC thermograms of binding of DNRDGNVYFF to 25 μM wt DegP in absence or presence of DPMFKLV-$B(OH)_2$. DegP:inhibitor ratio in the ITC cell is indicated above the thermograms. Thermodynamic parameters were calculated using "one set of sites" model (1* denotes that n was fixed to 1 during fitting). B) Activity of DegP (1 μM) with 500 μM chromogenic SPMFKGV-pNA substrate. (Left) In the presence of various concentrations of DNRDGNVYFF. (Right) In the presence of fixed concentration of DNRDGNVYFF (500 μM, corresponding to 4-fold of K_d) and various inhibitor concentrations. Error bars indicate standard deviation of experimental data (N=3). Fold activation of DegP relative to DMSO control is shown in tables. Data were fitted to MWC model for DegP (N=3). L, equilibrium constant T/R 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}, 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/$[E]_0$, maximum turnover rate for activated DegP; parenthesis, 95% confidence limit of the fit. C) Representative image of RseA (20 μM) degradation by DegP (1 μM) with fixed concentrations of the DNRDGNVYFF peptide (500 μM and 1 mM) and various inhibitor (Ihn) concentrations. *, DegP fragments due to autoproteolysis; **, 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 of experimental data (N=3).

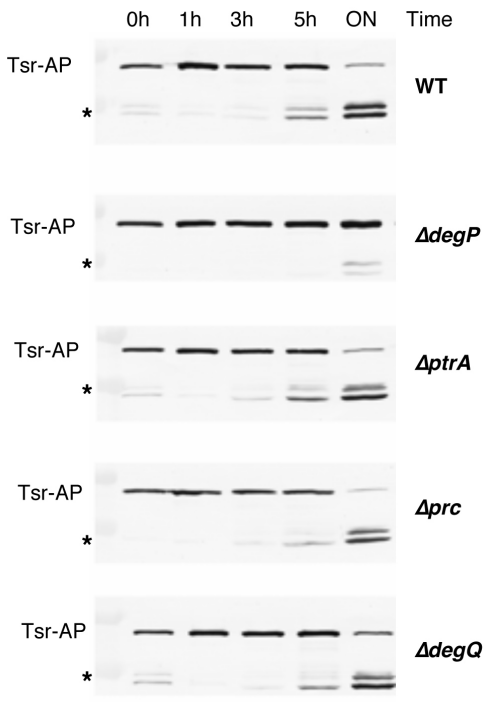
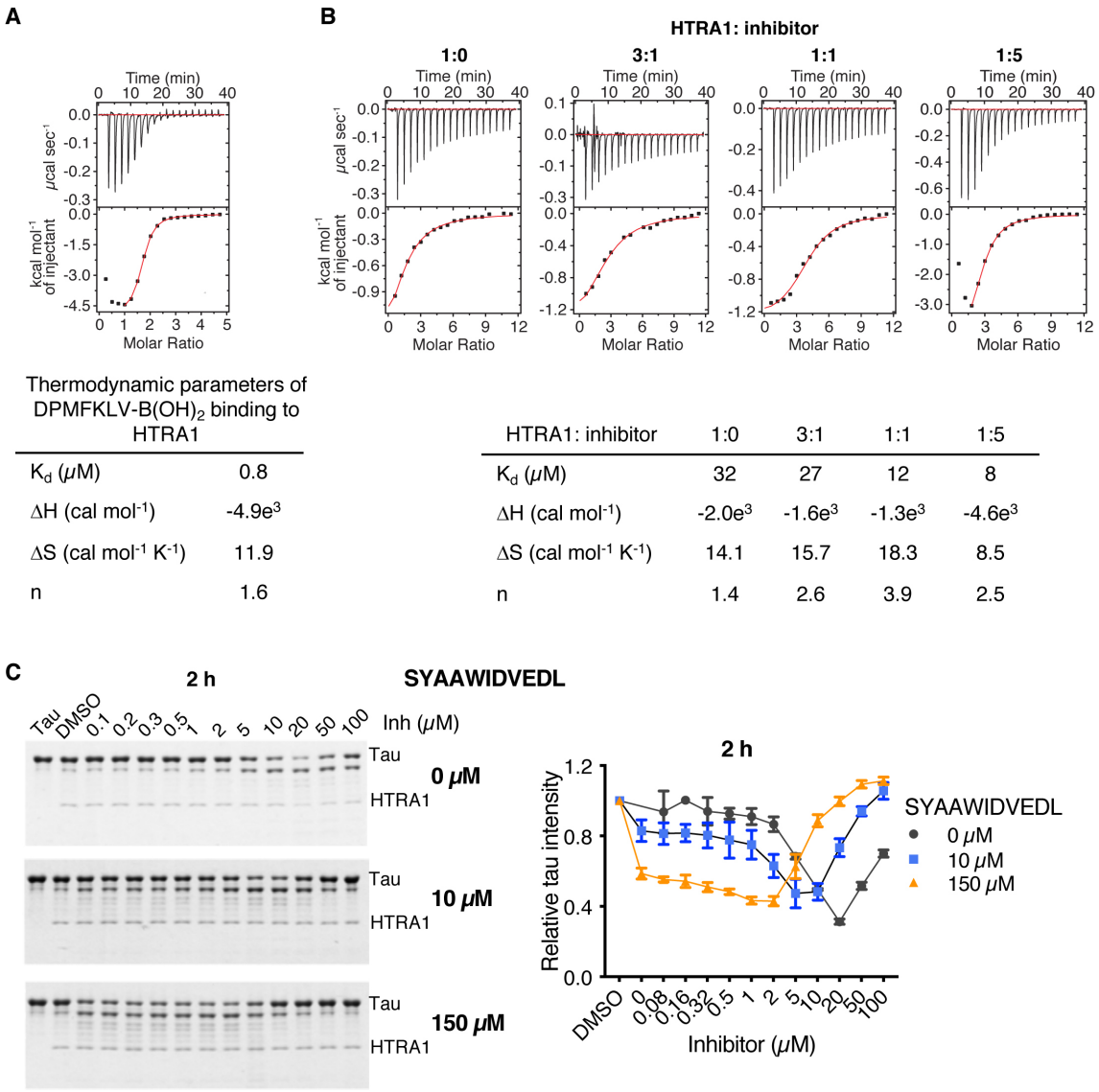


Fig. S4. Proteolytic processing of Tsr-AP hybrid proteins by various proteases. Tsr-AP processing in wt *E. coli* cells (BW30270) and derivatives containing chromosomal knockouts of genes encoding the proteases indicated. Whole cell lysates were prepared from cells growing in rich media. Samples were taken at time points indicated, subjected to SDS-PAGE and immunoblotting. Anti-AP antibody was used to detect Tsr-AP. *, Tsr-AP degradation products.



Thermodynamic parameters of DPMFKLV-B(OH)$_2$ binding to HTRA1

K_d (μM)	0.8
ΔH (cal mol^{-1})	-4.9e^3
ΔS (cal mol^{-1} K^{-1})	11.9
n	1.6

HTRA1: inhibitor	1:0	3:1	1:1	1:5
K_d (μM)	32	27	12	8
ΔH (cal mol^{-1})	-2.0e^3	-1.6e^3	-1.3e^3	-4.6e^3
ΔS (cal mol^{-1} K^{-1})	14.1	15.7	18.3	8.5
n	1.4	2.6	3.9	2.5

Fig. S5. A) ITC thermogram of the DPMFKLV-B(OH)$_2$ binding to 25 μM wt HTRA1 and thermodynamic parameters obtained using "one set of sites" model. B) ITC thermograms of binding of SYAAWEDVIDL in the absence or presence of the DPMFKLV-B(OH)$_2$ to wt HTRA1 and respective thermodynamic parameters. HTRA1:inhibitor ratio in the ITC cell is indicated above the thermograms. C) Proteolysis of tau (10 μM) by HTRA1 (1 μM) in the absence or presence of fixed concentrations of SYAAWIDVEDL and various concentrations of DPMFKLV-B(OH)$_2$ after 2 h at 37°C (left side). Tau signal intensity was quantified using densitometry relative to the DMSO control (right side); error bars indicate standard deviation (N=3).