

Reversible Inhibitors Arrest ClpP in a Defined Conformational State that Can Be Revoked by ClpX Association

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Abstract: Caseinolytic protease P (ClpP) is an important regulator of *Staphylococcus aureus* pathogenesis. A high-throughput screening for inhibitors of ClpP peptidase activity led to the identification of the first non-covalent binder for this enzyme class. Co-crystallization of the small molecule with *S. aureus* ClpP revealed a novel binding mode: Because of the rotation of the conserved residue proline 125, ClpP is locked in a defined conformational state, which results in distortion of the catalytic triad and inhibition of the peptidase activity. Based on these structural insights, the molecule was optimized by rational design and virtual screening, resulting in derivatives exceeding the potency of previous ClpP inhibitors. Strikingly, the conformational lock is overturned by binding of ClpX, an associated chaperone that enables proteolysis by substrate unfolding in the ClpXP complex. Thus, regulation of inhibitor binding by associated chaperones is an unexpected mechanism important for ClpP drug development.

Caseinolytic protease P (ClpP), a member of the serine hydrolase enzyme family, is a major regulator of bacterial cell homeostasis.^[1] The enzymatic complex consists of two adjacent heptameric rings that are connected by central α -helices E, forming a tetradecameric barrel (Figure 1a–c). The E helix is linked to strand β 9, which forms crucial hydrogen bonds across the heptamer interface. Different conformations of ClpP have been observed. Whereas an extended E helix (Figure 1a, PDB ID: 3V5E)^[2] is important for peptidolytic activity and tetradecamer stability, a kink in this helix leads to either compact (Figure 1b, PDB ID: 4EMM)^[3] or compressed (Figure 1c, PDB ID: 3QWD)^[4] states with misaligned catalytic triads. This distortion of

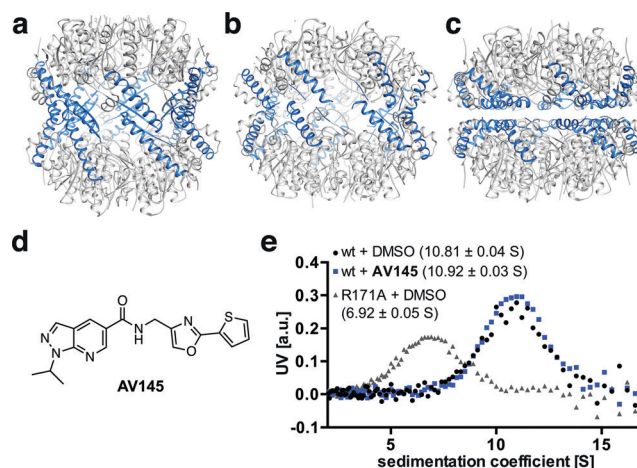


Figure 1. SaClpP in its a) extended (PDB ID: 3V5E), b) compact (PDB ID: 4EMM), and c) compressed (PDB ID: 3QWD) state. The E helices are shown in blue. d) Structure of AV145. e) Analytical ultracentrifugation of wild-type ClpP and the heptameric^[2] ClpP mutant R171A. DMSO served as a control. Sedimentation coefficient maxima are shown as the mean $\pm$ standard deviation.

catalytic residues induces a rotation of the conserved Pro125 (in strand β 9), which in turn pulls at helix E, resulting in its collapse. ClpP requires associated chaperones, such as hexameric ClpX, to unfold and digest larger protein substrates.^[1] Moreover, ClpX binding is believed to induce conformational selection of the active and extended state.^[5]

Thus far, only β -lactones and phenyl esters have been reported as specific ClpP inhibitors in whole proteome studies.^[6] Both compound classes covalently acylate the active-site Ser98.^[7] Covalent, irreversible binding is beneficial for proteome-labeling experiments, mechanistic studies, and for achieving a prolonged target residence time. The in vitro^[5,6] and in vivo^[8] application of irreversible ClpP inhibitors, however, has been limited by the low stability of their electrophilic motifs owing to hydrolysis.^[9] Thus far, all attempts to design non-covalent inhibitors have failed.^[7a] Surprisingly, given the importance of ClpP inactivation and the need for rational design, only one complex crystal structure of the protease with a nonspecific chloromethyl ketone (CMK) peptide ligand is available (PDB ID: 2FZS).^[10] Herein, we report the first co-crystal structure of a specific ClpP inhibitor with a novel, reversible mode of action, which was further exploited to synthetically optimize the ligand by rational design and in silico screening.

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A previous high-throughput screen (HTS) with about 140 000 compounds from the COMAS library (MPI Dortmund) did not reveal a single non-covalently binding SaClpP inhibitor with an $IC_{50} < 2 \mu M$.^[6a] However, the low activity of ClpP with the standard Suc-Leu-Tyr-AMC substrate requires at least $1 \mu M$ ClpP to record significant turnover, so that the potencies of the best inhibitors are still in the micromolar concentration range. We therefore reinvestigated our previous HTS results by lowering the selection criteria to an $IC_{50} \leq 10 \mu M$.^[6a] Four compounds of the HTS fulfilled this prerequisite (Supporting Information, Figure S1). Mass-spectrometric analysis of the intact proteins revealed that **AV145** binds non-covalently (Figure 1d, Figure S1), and medicinal-chemistry considerations made this compound the most promising candidate for further analysis. Compound **AV145** lacks any reactive groups and consists of three characteristic heterocycles, a pyrazolopyridine as well as a 2-(thiophen-2-yl)oxazole moiety. Time-dependent incubation of SaClpP with **AV145** did not change the IC_{50} (Figure S2), supporting the fact that the compound is a reversible inhibitor. Furthermore, analytical ultracentrifugation demonstrated that **AV145**, unlike most phenyl esters and lactones, did not induce dissociation of ClpP into heptamers (Figure 1e).^[6a, 7b]

To gain insights into the mechanism of inhibition, we co-crystallized **AV145** with SaClpP and solved the complex structure by molecular replacement at 3 Å resolution ($R_{free} = 27.4\%$, Table S1, PDB ID: 5DL1). The asymmetric unit contains a ClpP tetradecamer with the compound bound to every subunit. Despite variations in occupancy, model building into the averaged $2F_o - F_c$ electron-density map allowed the unambiguous positioning of **AV145** near to the active site between α -helix E and strand $\beta 9$ (Figure 2a). Surprisingly,

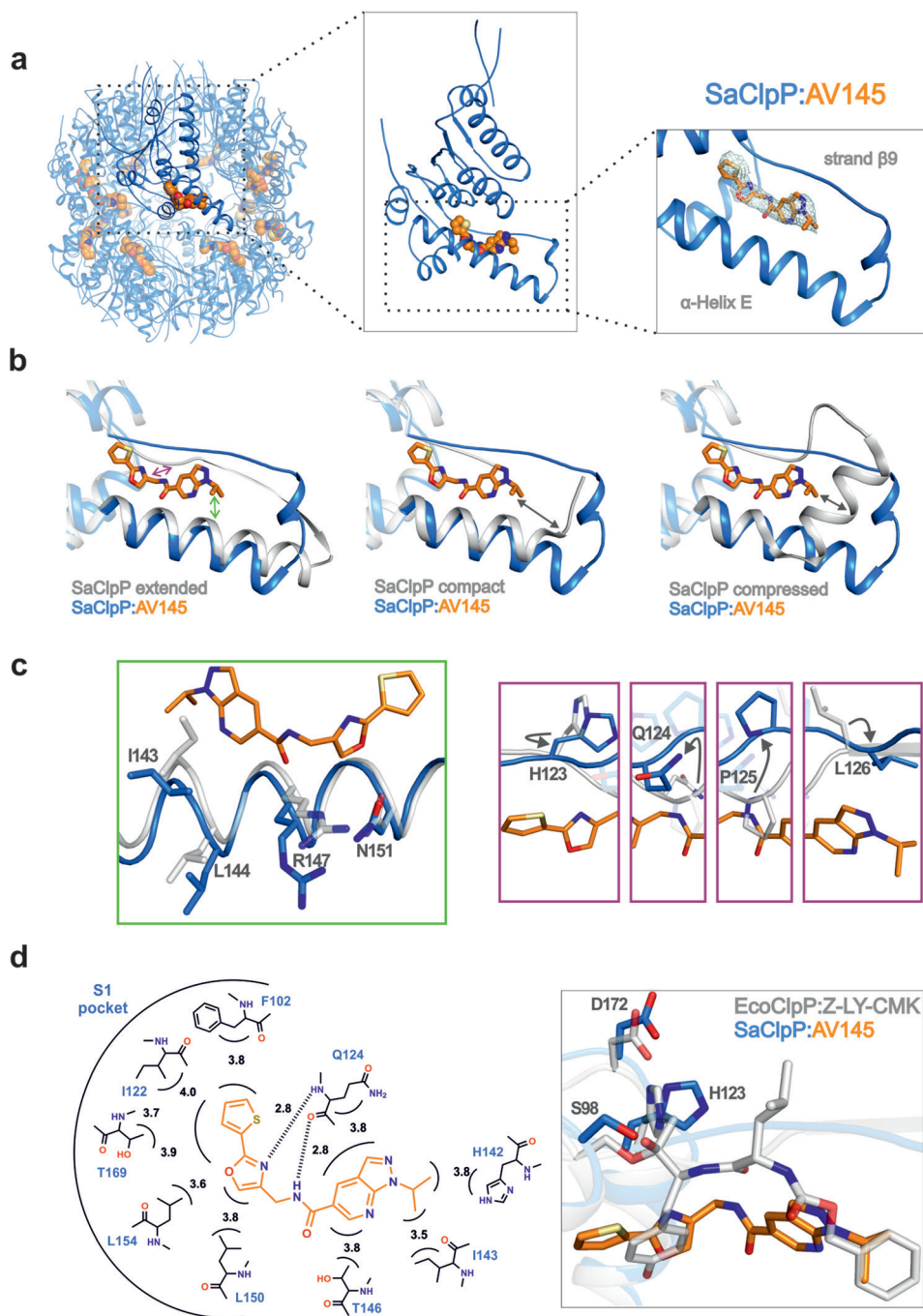


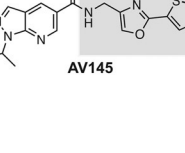
Figure 2. a) Binding of **AV145** to SaClpP (PDB ID: 5DL1). b) Structural superposition of SaClpP:**AV145** with the extended, compact, or compressed form of SaClpP. Structural changes between the extended and co-crystal structure are indicated by colored arrows. c) Detailed exploration of the structural changes of α -helix E and strand $\beta 9$ compared to the extended form. d) Analysis of the specific enzyme-inhibitor contacts and overlay with the EcClpP:Z-LY-CMK structure (PDB ID: 2FZS), illustrating the non-covalent inhibition mechanism of **AV145**. Color code: SaClpP:**AV145** marine, **AV145** orange, SaClpP:extended/compact/compressed and EcClpP:Z-LY-CMK gray; heteroatoms: O red, N blue, S yellow.

overlays with structures of the apo enzyme in its extended, compact, and compressed form revealed that the inhibitor binds in a non-substrate-like mode and induces an unprecedented conformational state (Figure 2b). Although helix E is almost completely aligned as in the extended, active enzyme, binding of **AV145** displaces Ile143 and Arg147 because of

der Waals contacts with Phe102, Ile122, His142, Ile143, Thr146, Leu150, and Leu154 as well as Thr169 stabilizes compound binding (Figure 2d). Overall, this novel mode of action explains why **AV145** is a non-covalent SaClpP inhibitor. Superposition of our structure with the Z-LY-CMK bound EcClpP structure (PDB ID: 2FZS)^[10] reveals differences (Figure 2d). Unlike CMK, **AV145** does not influence the catalytic center of SaClpP directly, but rather transmits its function through neighboring residues. Such a type of indirect binding has not been observed for this class of enzymes thus far.

a

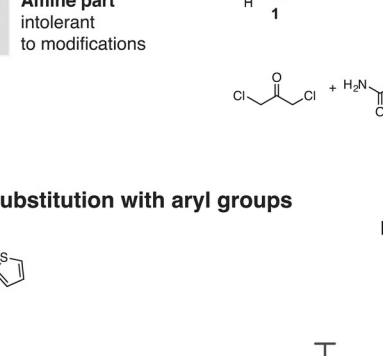
Acid part variable



AV145

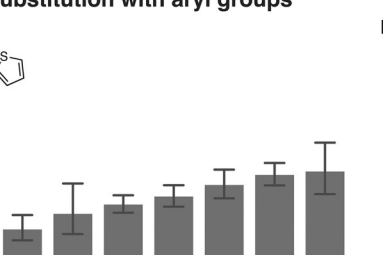
Amine part intolerant to modifications

b



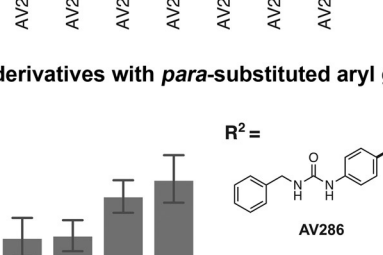
c

Derivatives by rational N-substitution with aryl groups



d

Virtual screening inspired derivatives with *para*-substituted aryl groups



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rational considerations or resulted from a virtual screen (see the Supporting Information). Alterations within the amine part, for example, by introduction of thiophene substituents and the replacement of the thiophene by a phenyl or oxazole group, resulted in inactive compounds (Figures S3, S4). However, modifications within the acid part were much more tolerated and led to improved inhibitors. Based on this, we explored the possibility of incorporating aromatic groups for π -stacking with His142 (part of the strand β 9) through the design of eleven derivatives with *N*-aryl substituents (Figure 3c).

The synthesis of these derivatives, exemplified for **AV280** in Figure 3b, involved N-alkylation of pyrazolopyridine **1** with 3,4-dimethylbenzyl bromide, followed by saponification of the ester with lithium hydroxide. Preparation of the oxazole amine part **3** started with the condensation of the appropriate aryl amide with dichloroacetone at 120°C. The resulting chloromethyl-substituted oxazole **2** was converted into amine **3** in two steps. Standard amide coupling of the acid with the amine resulted in the final products.

AV191 and **AV280**, which bear a benzyl and a 3,4-dimethylbenzyl substituent, respectively, showed a sixfold increase in ClpP inhibition compared to **AV145**, with IC_{50} values of 1.7 and 1.5 μ M, respectively (Figure 3c). Based on results from the virtual screening, we next replaced the pyrazolopyridine moiety by aryl rings with a *para* urea or a *para* carbamate substituent, resulting in **AV286** with an IC_{50} value of 0.9 μ M (Figure 3d). Notably, this value shows that a stoichiometric amount of **AV286** (relative to the monomer concentration of the protease) is sufficient to inhibit 50% of SaClpP. An increase in substrate concentration did not influence inhibitor binding (Figure S5).

With potent and reversible ClpP inhibitors at hand, we investigated whether the best compound of this series exhibits cell permeability and target selectivity in situ. Therefore, an **AV286**-related activity-based protein profiling^[11] photoprobe (**AV321**) equipped with a diazirine photocrosslinker and an alkyne tag was prepared (Figure 4a).^[12] Pleasingly, the probe retained a low IC_{50} value (2 μ M) for SaClpP peptidase inhibition (Figure S6). Living *S. aureus* cells were incubated with **AV321**, irradiated with UV light to form a covalent link between target protein and diazirine, lysed, and clicked to a functionalized azide tag (fluorescent dye or biotin) via the alkyne; the labeled proteome was then analyzed by fluorescence SDS-PAGE analysis (Figure 4b) or mass spectrometry (MS; Figure 4c). A dominant fluorescent protein band at the molecular weight of ClpP appeared on SDS-PAGE (Figure 4b). To confirm ClpP binding in situ we performed quantitative gel-free MS by isotope labeling.^[13] MS-based target enrichment was visualized by volcano plots (Figure 4c, Figure S7). In all runs, ClpP was highly enriched, and only a few putative off-targets (significance level: $p \leq 0.05$), such as the 50S ribosomal protein L1 (Protein ID ID: Q2G0P0), were detected. We next examined whether cell-permeable inhibitors reduced the production of α -hemolysin (hla), a predominant *S. aureus* toxin regulated by ClpP. Surprisingly, the general level of hla was high, suggesting that inhibition of intracellular proteolysis was not as efficient as the reduction of in vitro peptidase activity.

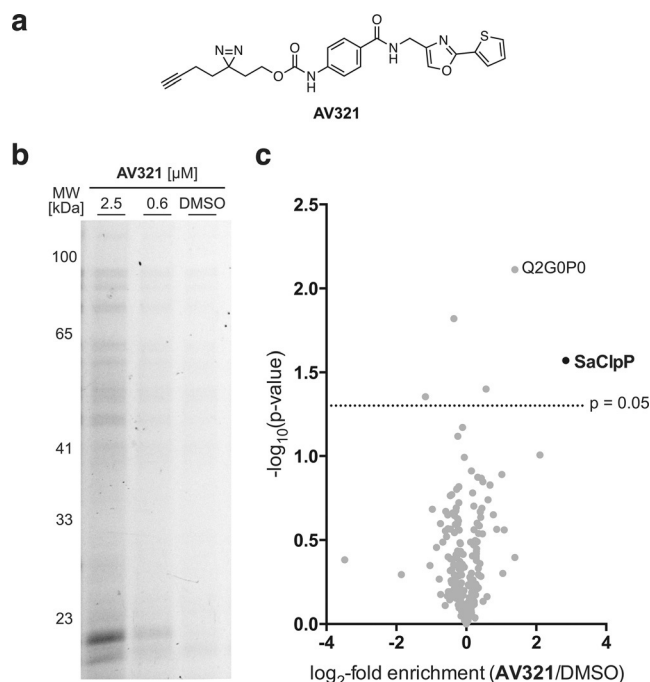


Figure 4. In situ target validation in living *S. aureus* cells with **AV321**. a) Chemical structure of the activity-based photoprobe **AV321**. b) Fluorescence SDS-PAGE analysis of the soluble fraction. c) Volcano plot representation of gel-free quantitative ABPP experiments measured on the Orbitrap XL. Data are derived from three biological replicates for DMSO and **AV321** (5 μ M), respectively. The $\log_2$ -fold enrichment values were z-score-normalized, and $\log_{10}$ (p values) were calculated using a two-sided one-sample Student's t-test. Protein enriched in addition to SaClpP: Protein ID Q2G0P0: 50S ribosomal protein L1. See the Supporting Information for further details and additional analysis.

To explore this finding in more detail, we tested the most potent peptidase inhibitors in a ClpXP protease assay with fluorescent GFP-SsrA (tagged for ClpXP degradation) as a substrate. Indeed, all inhibitors were largely inactive in this assay (Figure S8), demonstrating that ClpX overrides the inhibitor-induced conformational lock. To investigate the mechanistic basis of this unexpected behavior, we utilized a previously introduced activator of ClpP.^[14] This compound displaces ClpX in protease assays and was thus used as a surrogate for the chaperone (Figure S9). The molecule abolished **AV286** inhibition of ClpP in casein and peptidase assays in a concentration-dependent manner, which validates a conformational selection of the active enzyme state by ClpX or its surrogates (Figure S9).^[15] Our observation is in line with a recent study that showed a conformational switch to the active and extended state upon binding of acyldepsipeptides, which mimic ClpX binding.^[15] Thus, regulation by ClpX and other proteolytic activators represents an important and thus far neglected parameter for future ClpP inhibitor development.

In conclusion, **AV145** bound to ClpP arrested the enzyme in an unprecedented inactive conformational state by a significant rotation of Pro125 and an associated misalignment of the catalytic triad architecture. This binding mode was explored for the design of new compounds, which resulted in **AV286**, the first non-covalent inhibitor of ClpP with an

IC₅₀ value of <1 μM. Importantly, ClpX revoked non-covalent binding of the examined inhibitors to ClpP, a counter-intuitive finding when compared to related complex proteolytic systems, such as the proteasome, where stable proteolytic inhibitors for individual subunits have been described.^[16] Our results therefore strongly imply that regulators such as the ATP-dependent ClpX orchestrate and modulate various distinct conformational stages in the hydrolytic chamber of ClpP. For the identification of non-covalent ClpP inhibitors that are also active in situ, it is thus important not to solely focus on the peptidase activity of the isolated ClpP complex, as this, although easy to monitor, does not accurately reflect the situation in live cells. We hypothesize that sustained ClpP inhibition may either be achieved by increasing the small-molecule-induced conformational lock by, for example, the incorporation of large ligands that block the proteolytic channel, or by inhibitor discovery with the intricate ClpXP proteolytic complex. Both would certainly represent attractive starting points for the future optimization of anti-virulence compounds against *S. aureus* and its antibiotic-resistant strains.

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Keywords: caseinolytic protease · conformational selection · non-covalent inhibition · protein crystallography · structural rearrangement

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