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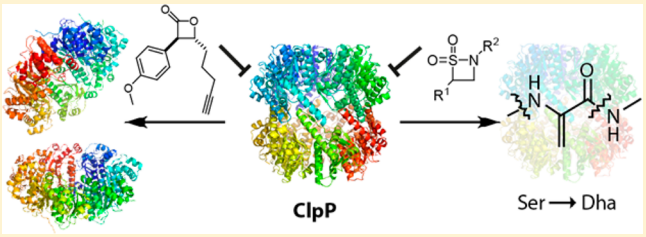
Disruption of Oligomerization and Dehydroalanine Formation as Mechanisms for ClpP Protease Inhibition

Malte Gersch,[†,§] Roman Kolb,[†,§] Ferdinand Alte,[‡] Michael Groll,[‡] and Stephan A. Sieber*[,†]

[†]Center for Integrated Protein Science at the Department of Chemistry, Institute of Advanced Studies IAS and [‡]Center for Integrated Protein Science at the Department of Chemistry, Technische Universität München, Lichtenbergstrasse 4, Garching D-85747, Germany

Supporting Information

ABSTRACT: Over 100 protease inhibitors are currently used in the clinics, and most of them use blockage of the active site for their mode of inhibition. Among the protease drug targets are several enzymes for which the correct multimeric assembly is crucial to their activity, such as the proteasome and the HIV protease. Here, we present a novel mechanism of protease inhibition that relies on active-site-directed small molecules that disassemble the protease complex. We show the applicability of this mechanism within the ClpP protease family, whose members are tetradecameric serine proteases and serve as regulators of several cellular processes, including homeostasis and virulence. Compound binding to ClpP in a substoichiometric fashion triggers the formation of completely inactive heptamers. Moreover, we report the selective β-sultam-induced dehydroalanine formation of the active site serine. This reaction proceeds through sulfonylation and subsequent elimination, thereby obliterating the catalytic charge relay system. The identity of the dehydroalanine was confirmed by mass spectrometry and crystallography. Activity-based protein profiling experiments suggest the formation of a dehydroalanine moiety in living *S. aureus* cells upon β-sultam treatment. Collectively, these findings extend our view on multicomponent protease inhibition that until now has mainly relied on blockage of the active site or occupation of a regulatory allosteric site.



INTRODUCTION

Over 100 different protease inhibitors are currently applied in the clinic as therapeutic agents,[1] and the most prevalent strategy for protease inhibition is represented by blockage of the active site with covalently or noncovalently acting compounds.[2,3] In addition, a limited number of allosteric and exosite inhibitors has been described.[4] Among those are small molecules that inhibit the formation of the physiologically active dimers of HIV protease[5] and of the human Kaposi's sarcoma associated herpes virus (KSHV) protease.[6] Covalent allosteric binders have been described for caspases trapping the enzyme in an inactive, dimeric form.[7] Although they are not proteases, HIV integrase and the C-reactive protein can be inhibited by shifting their oligomerization equilibria.[8,9]

Bacterial organisms possess several multicomponent proteolytic machineries such as ClpXP, FtsH, HslVU, and the Lon protease[10−13] to break down damaged and regulatory, short-lived proteins which are of pivotal importance to cell survival and pathogenicity.[14,15] Despite considerable sequence divergence, these systems all share a general topology in which proteolytic subunits and AAA+ ATPases as chaperones act together in barrel-shaped complexes. Chaperone and protease may form two domains in one polypeptide chain (as in FtsH and Lon) or may be encoded by different genes (as in ClpXP and HslVU) to allow further flexibility: for example, with different chaperones (ClpA, ClpC, and ClpX) being capable of binding the proteolytic subunit ClpP.[16−18] In the functional ClpXP complex, hexameric ClpX stacks on top of the tetradecameric serine protease ClpP that is built by two heptameric rings.[19−21] It has been demonstrated that the handle domain linking the two heptameric rings of the ClpP protease is highly dynamic[22,23] and that ClpP proteins adopt different conformations (Figure 1A).[24−26] In the compressed form, the catalytic residues are misaligned and the E helix in the handle domain is either kinked or structurally flexible. Upon adoption of the extended, active conformation, the catalytic triad aligns properly.

We recently described a hydrogen-bonding network that links the oligomeric state with the alignment of the catalytic triad in ClpPs from *Staphylococcus aureus* (SaClpP)[25] and *Listeria monocytogenes*.[27] In an active tetradecamer, Arg171 and Asp170 engage in interactions across the heptamer−heptamer interface that, in turn, cause a backbone motion of the catalytic Asp172 and enable alignment of the catalytic triad (Ser98, His123, Asp172). Similar to the case for ClpP from *Escherichia coli*,[28] mutation of any of these network residues as well as mutations destabilizing the extended E helix led to catalytically inactive, heptameric proteins.

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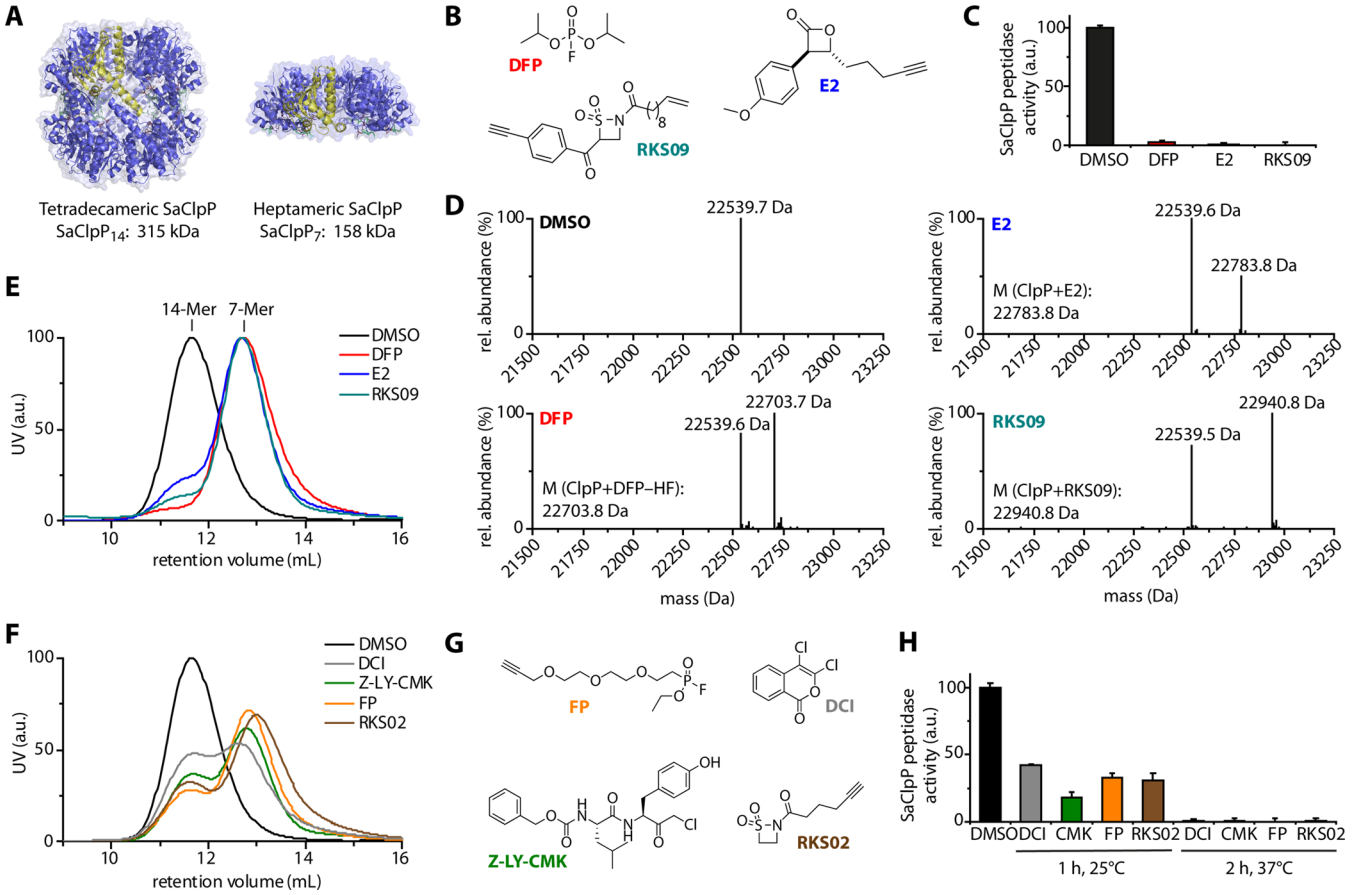

Figure 1. Small-molecule-induced disruption of SaClpP oligomerization: (A) cartoon representation of SaClpP tetradecamer (extended conformation) and SaClpP heptamer (compressed conformation), with one subunit highlighted in yellow; (B) structures of DFP, E2, and RKS09; (C) SaClpP peptidase activity after compound treatment (1 h, room temperature; incubation carried out with 50 μM SaClpP to allow for direct SEC analysis and then dilution of the sample to 1 μM for kinetic and MS experiments); (D) intact protein mass spectrometry after compound treatment showing incomplete modification. with measured masses indicated above the peaks and expected masses are given in the lower left corner; (E) size-exclusion chromatograms showing heptamer formation upon compound treatment; (F) size-exclusion chromatograms showing partial induction of heptamer formation (1 h, room temperature); (G) chemical formulas of further compounds tested; (H) compounds with partial heptamer formation showing remaining peptidase activity that decreased after longer incubation times and elevated temperatures.

Significant interest in ClpP inactivation started with the discovery of its crucial role in virulence of *S. aureus*, a pathogen that causes severe infections in the clinics and is difficult to treat through the occurrence of multiresistance.[29,30] For instance, a ClpP knockout strain was severely impaired in murine infection studies,[31] indicating a major role of ClpP in virulence regulation.[32] In line with these results, covalent small-molecule inhibitors of ClpP of the β-lactone class led to a marked decrease of virulence factor secretion.[33] While studying the binding site of the β-lactone inhibitors, we characterized mutants of SaClpP that were unexpectedly purely heptameric and catalytically inactive (e.g., L150A, N151Q, T169A).[34] Intrigued by the apparent sensitivity of the heptamer–tetradecamer transition switch, we were curious whether we could exploit this disassembly mechanism for protease inhibition triggered by small molecules. We therefore screened a compilation of both commercially available and custom-synthesized, covalent serine protease inhibitors and looked for major changes in SaClpP oligomerization upon ligand binding. We identified several active-site-directed compounds that induced inactive heptamers, and we thereby validated heptamer formation as an inhibition strategy. Moreover, we characterized the sultam-induced conversion of the active site serine into a dehydroalanine as a complementary inhibition mechanism.

RESULTS

In search for compounds that disassemble tetradecameric SaClpP into inactive heptamers, we selected molecules from various chemical classes for testing (Supplementary Figure 1, Supporting Information). Our focused library comprised β-lactones, a fluorophosphonate, a fluorophosphate, a dichloroisocoumarin, sulfonyl fluorides, β-sultams, and chloromethyl ketones as reactive moieties.[3,26,33,35−38] In order to obtain a comprehensive picture of inhibitor binding to SaClpP, we determined the size of the protein complexes using a calibrated size exclusion chromatography column (Supplementary Figure 2A), measured peptidase activity with a fluorogenic substrate assay, and used intact protein mass spectrometry (MS) as a readout for covalent protein modification. Since the effect of covalently acting compounds is time-dependent, we performed all experiments after 1 h of incubation at room temperature. The analysis revealed distinct inhibition profiles so that the compounds were classified into three categories: (a) inhibitors that modify the active sites partially and change the oligomerization to smaller species, (b) inhibitors that modify

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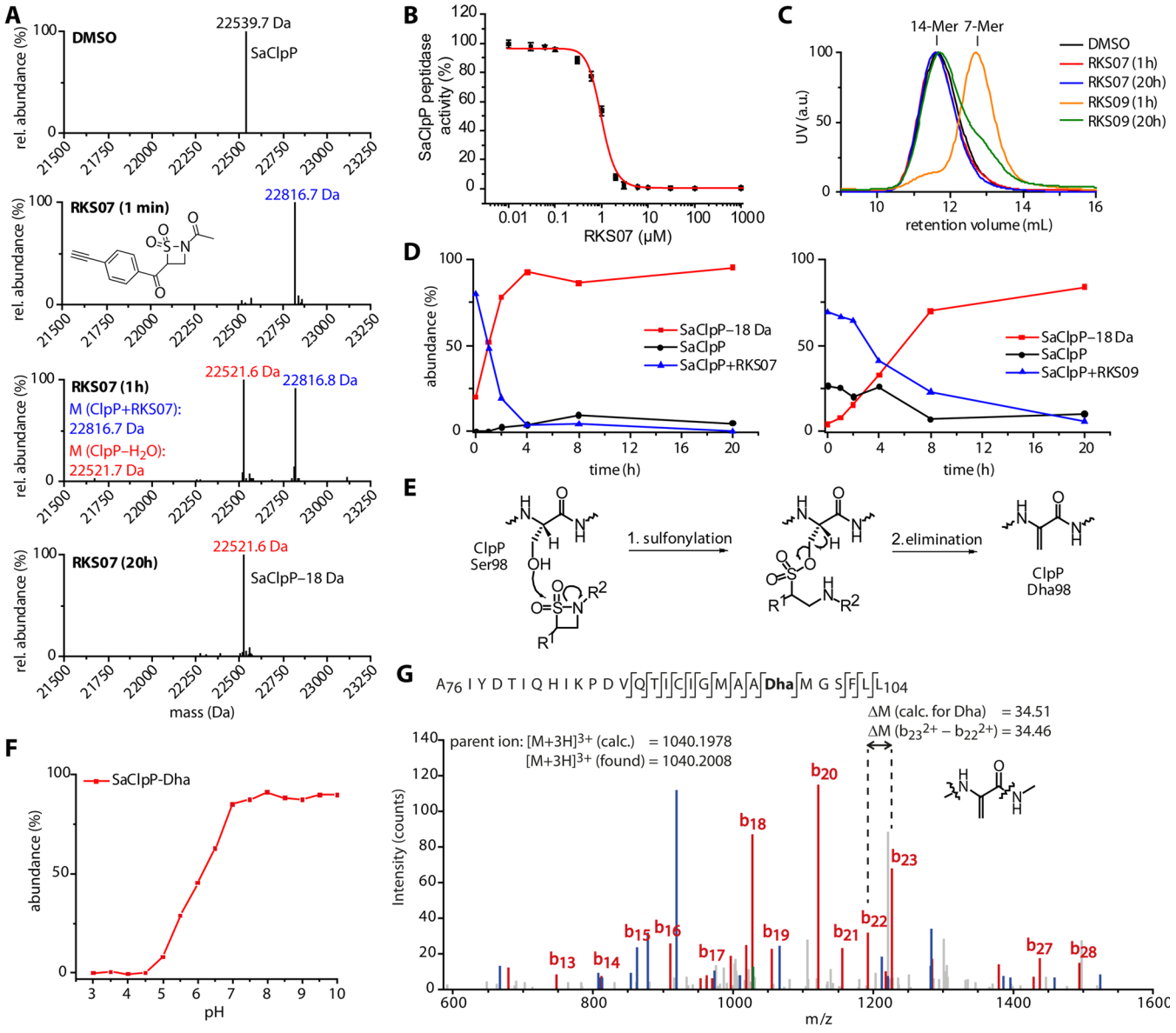

Figure 2. Conversion of the active site serine into dehydroalanine by β-sultams: (A) intact protein MS revealing the presence of protein species 18 Da lighter than wild-type SaClpP, with measured masses being indicated above the peaks and expected masses given in the lower left corner; (B) efficient inhibition of SaClpP (1 μM) by RKS07 after 1 h of incubation at room temperature (IC_{50} = 0.98 ± 0.01 μM); (C) size exclusion chromatograms of β-sultam-treated SaClpP samples (data on RKS09 from Figure 1D shown for clarity); (D) time-resolved formation of SaClpP species upon treatment with RKS07 and RKS09 via protein MS (1 μM SaClpP); (E) mechanism for the formation of Dha involving sulfonylation of the active site serine and subsequent elimination; (F) generation of the SaClpP-RKS07 adduct (1 min, 10 μM SaClpP), dilution into buffers with different pHs, and quantification of Dha-SaClpP formation after 2 h by MS (1 μM SaClpP); (G) CID fragmentation spectrum of the peptide A_{76}IYDTIQHIKPDVQTICIGMAADhaMGSFLL$_{104}$, showing the presence of a dehydroalanine moiety at position 98 (y ions, blue; b ions, red). For a complete list of identified fragments, please refer to Supplementary Table 6.

all active sites and do not change the oligomerization, and (c) nonbinders.

Inhibition of SaClpP by DFP, RKS09, and E2 through Heptamer Formation despite the Presence of Unmodified Active Sites (Category a). To our surprise, we identified a total of seven compounds that led to only partial modification of the active sites but efficiently altered the oligomerization to smaller species (category a). In an MS experiment, diisopropyl fluorophosphate (DFP), β-sultam RKS09, and β-lactone E2 showed modification of 57%, 63%, and 35% of the active sites, respectively, but caused complete inhibition of SaClpP (Figure 1B–D). Interestingly, size exclusion chromatography revealed an almost quantitative shift from the tetradecamer to the heptamer (Figure 1E). We further confirmed the size of DFP-treated SaClpP by static light scattering that yielded masses of 304 kDa for unmodified SaClpP and 150 kDa for DFP-treated SaClpP, showing clearly the presence of a heptameric species. Although up to 65% of the active sites were unmodified in these samples, enzyme activity was completely blocked (Figure 1C). In the presence of the tetradecamer-stabilizing agent glycerin, higher occupancies by E2 were obtained, while glycerin alone did not change SaClpP peptidase activity (Supplementary Figure 3). These results strongly support the view of tetradecamers as active and heptamers as inactive species.[25] Furthermore, the data demonstrate a mechanism in which inhibitor binding at some but not all active sites destabilizes the

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tetradecamer and is sufficient for the dissociation into heptamers, thereby causing complete inhibition.

Next, we characterized the fluorosphosphonate probe FP, the dichloroisocoumarin DCI, the β-sultam RKS02, and the chloromethyl ketone Z-LY-CMK (Figure 1G) and recorded partial active site modification by MS (Supplementary Figure 2C). In these cases, we detected two peaks in the size exclusion chromatogram corresponding to a tetradecameric and a heptameric species, with the latter being more prominent (Figure 1F). We then analyzed the degree of active site modification in the peaks of Z-LY-CMK-treated SaClpP by MS and found that the degree of modification was larger in the heptameric peak (33%) than in the tetradecameric fraction (17%), fitting well to an overall modification of 29% (Supplementary Figure 2C). As expected from the presence of unmodified active sites in the tetradecameric species, minor enzymatic activity could be detected by a fluorogenic substrate assay (Figure 1H). Longer incubation times (2 h) and higher temperatures (37 °C) led to full inhibition (Figure 1H) but not to complete modification by Z-LY-CMK (Supplementary Figure 2C). These results suggest a dynamic equilibrium between tetradecamers and heptamers. In this equilibrium, reassociation of heptamers into tetradecamers would be accompanied by a conformational change by which inhibitor binding at the remaining unmodified active sites can occur. The resultant higher occupancy would shift the equilibrium toward the heptameric population. This mechanism explains the increased modification in the presence of the stabilizing agent glycerin, the delayed complete modification by some compounds, and the increased degree of modification in the heptameric fraction.

Occupation of Each Active Site by D3, RM448, and RKS07 without a Change in the Oligomeric State (Category b). As described previously,[34] β-lactones D3 and RM448 result in an almost instant covalent modification of all 14 active sites and complete inhibition of peptidase activity. These small molecules retain the tetradecameric state (Supplementary Figure 2B), thus belonging to category b. Another compound of this category, the β-sultam RKS07, also abolished enzyme activity completely (Figure 2B) without changing the oligomerization (Figure 2C). Since we found the IC_{50} value of RKS07 to depend on the enzyme concentration (Supplementary Figure 4), we concluded an irreversible mechanism of inhibition.[39] We thus measured $k_{obs}/[I]$ values as quantitative measures of potency suitable for the characterization of covalent inhibitors. While RKS07 reacted with velocity comparable to that of β-lactones D3 and E2 (140 ± 9 M^{-1} s^{-1} (RKS07); 78 ± 6 M^{-1} s^{-1} (D3); 64 ± 3 M^{-1} s^{-1} (E2)), RKS09 reacted much faster with a $k_{obs}/[I]$ value of 1320 ± 82 M^{-1} s^{-1} (Supplementary Figure 6), thus representing the fastest ClpP inhibitor known to date.

Both 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) and phenylmethylsulfonyl fluoride (PMSF) as well as Z-L-CMK and *N*-p-tosylphenylalanyl chloromethyl ketone (TPCK) did not inhibit SaClpP (category c), as judged from peptidase activity measurements (Supplementary Figure 5).

Dehydroalanine Formation at the Active Site Serine Caused by β-Sultams. Unexpectedly, we found by MS two peaks in the RKS07-treated sample (Figure 2A): the larger peak corresponded to the joint masses of protein and inhibitor that can be rationalized by a simple addition reaction upon attack at the sulfur by the active site serine, fission of the S−N bond, and ring opening. The second, smaller species, however, was 18 Da lighter than the unmodified protein (Figure 2A). To resolve the identity of the 18 Da lighter SaClpP species formed upon treatment with RKS07, we studied the reaction of SaClpP with β-sultams in more detail. Time-dependent measurements showed that both RKS07 and RKS09 induced the formation of the 18 Da lighter SaClpP after 20 h of incubation as the sole species (Figure 2A,D). Formation occurred at different velocities and seemed to proceed through the sulfonylated enzyme, which decreased to the same extent as the 18 Da lighter species increased. Notably, partially modified SaClpP by RKS09 was heptameric, while incubation for 20 h led to the 18 Da lighter, inactive species and a change of the oligomerization back to the tetradecamer (Figure 2C). This is in accordance with the tetradecameric size of the RKS07-treated SaClpP (Figure 2C).

A mass decrease of 18 Da is consistent with the loss of a water molecule in the course of an elimination reaction of the sulfonylated active site serine to furnish a dehydroalanine moiety (Figure 2E). Remarkably, the addition reaction of RKS07 and SaClpP reached completion (>95%) within 1 min of incubation at room temperature (Figure 2A). We diluted this adduct into different buffers, showing a clear pH dependence of the second reaction step that proceeded to completion only at neutral or basic pH (Figure 2F and Supplementary Figure 7A).

We next digested the modified enzyme with trypsin and chymotrypsin and analyzed the resulting peptides by nano-LC-MS/MS (sequence coverage of SaClpP 100%; see Supplementary Tables 4−6). We identified peptides that upon collision-induced dissociation (CID) fragmentation showed the mass corresponding to dehydroalanine (Dha) instead of the catalytic serine 98 (Figure 2G). A dehydroalanine on a protein surface was recently used to install posttranslational modifications on purified proteins by addition of thiols.[40] Despite extensive attempts, we were unable to observe complete covalent addition of several sulfur nucleophiles to Dha-SaClpP. Heat denaturation in the presence of thiols yielded only 30% modified protein (Supplementary Figure 8). This indicates that the Dha in the ClpP active site is either sterically or electronically distinct from Dha moieties in loops on protein surfaces. To confirm the existence of the Dha residue and to investigate putative cross links between the electrophilic Michael system and a nearby nucleophile, we solved the crystal structure of the modified enzyme to 2.3 Å resolution (R_{free} = 0.221). Consistent with MS data, a Dha could be refined at the position of all 14 active site serine residues (Figure 3,

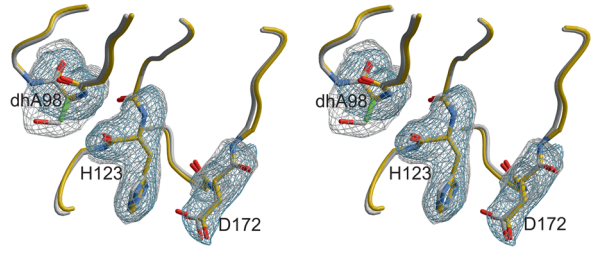

Figure 3. Crystal structure of Dha-SaClpP: stereoview on the active site of Dha-containing SaClpP in the crystal structure with 2.3 Å resolution. The electron density (blue mesh) is consistent with a dehydroalanine moiety (for comparison, the gray mesh denotes Ser98 in the wild type structure). The $2F_o - F_c$ electron density maps are contoured at 1.0 σ; amino acids have been excluded prior to phase calculations.

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Supplementary Table 3). The electron density clearly depicted the lack of the hydroxyl group and provided no evidence for an intramolecular Michael-type attack. No other structural changes were observed by comparison of Dha-SaClpP with the wild type protein. Collectively, these data further confirm the formation of a dehydroalanine residue through elimination of the sulfonylated active site serine upon treatment with β-sultams.

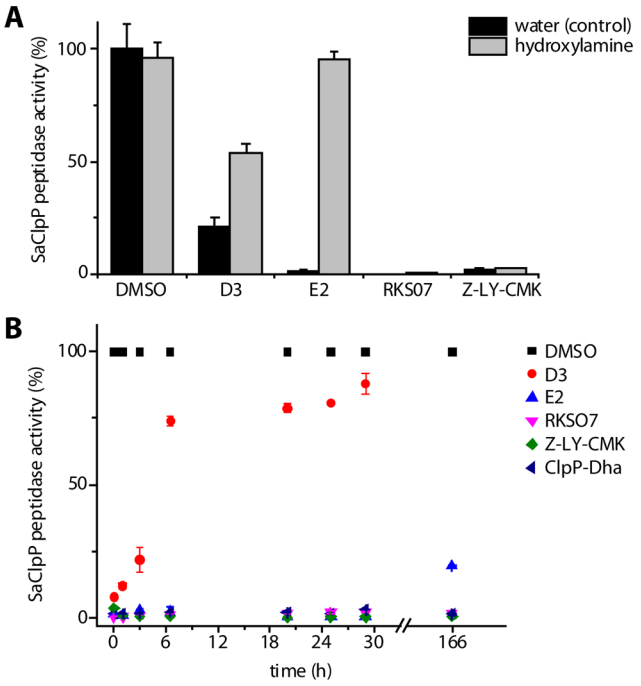

Figure 4. (A) Reversal of inhibition of SaClpP by β-lactones (D3, E2) by hydroxylamine treatment. (B) Allowance of prolonged inhibition by both dehydroalanine formation and disruption of oligomerization (incubation at 37 °C; see Supplementary Figure 7B for DMSO controls). In both experiments, incubation was carried out with 10 μM SaClpP and 10× excess of inhibitor and aliquots were diluted to 1 μM SaClpP for activity measurements.

Improved Inhibition by both Induction of Heptamers and Dehydroalanine Formation. The high propensity to hydrolysis of the acyl ester intermediate is the major drawback of β-lactone-based inhibitors. This feature is commonly observed with hydrolases inhibited by β-lactones, since the hydrolysis of an ester is part of their catalytic cycle and reactivation times of as low as 2 min are possible.[41,42] Hydroxylamine cleaves thioesters and, to a lesser extent, esters in aqueous solutions. As expected, β-lactone-treated SaClpP was found susceptible to hydroxylamine treatment, restoring the catalytic activity, while RKS07 and Z-LY-CMK treated samples were unaffected, proving the different inhibition mechanisms (Figure 4A). To test for sustained inhibition, we incubated SaClpP with several inhibitors (D3, E2, RKS07, Z-LY-CMK) at different temperatures (4, 25, 37 °C) and measured peptidase activity at several time points (Figure 4B, Supplementary Figure 7B–D). While the D3-treated enzyme regained its activity almost completely within 20 h at 37 °C, the E2-treated sample still showed 90% inhibition after 1 week at 37 °C normalized to the untreated control (Supplementary Figure 7B). This result was found to be in accordance with mass spectrometry results. It supports the view of E2-induced inactive heptamers in which also the acyl ester hydrolysis is impaired. ClpP incubated with RKS07 and Z-LY-CMK displayed no enzyme activity even after 1 week (see Supplementary Figure 7C,D for data at 25 and 4 °C). Accordingly, purified Dha-SaClpP was inactive at all time points.

The generality of the approach was investigated by screening ClpP proteins from *Listeria monocytogenes* (LmClpP2) and *Escherichia coli* (EcClpP) for compound-induced changes in their oligomerization state. E2 and RKS09 were able to induce heptamer formation in LmClpP2, as observed with SaClpP (Supplementary Figure 9A). In contrast, EcClpP only showed a slight shift after E2 and Z-LY-CMK treatment, while D3 induced partial formation of heptamers (Supplementary Figure 9B). Moreover, we observed the respective dehydroalanine proteins upon RKS07 treatment by mass spectrometry (Supplementary Figure 10).

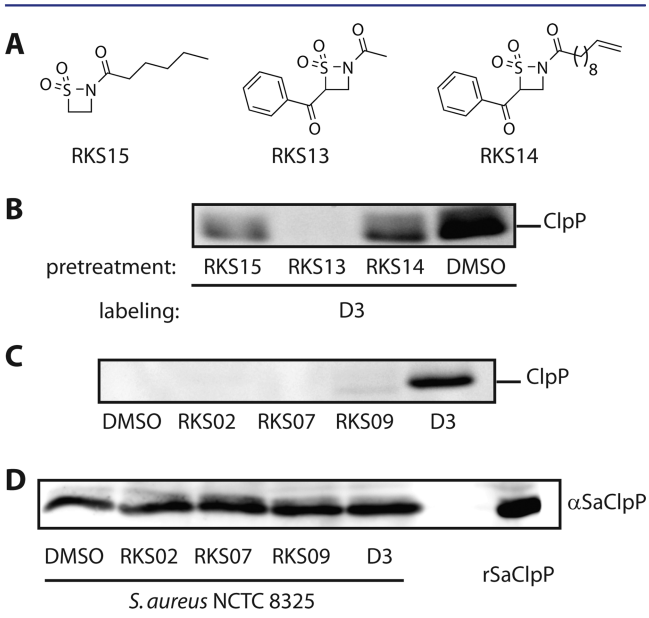

Figure 5. (A) Structures of the synthesized alkyne-free β-sultams. (B) Competitive labeling experiment in which living *S. aureus* cells were pretreated with DMSO or the compounds shown in (A) (1000 μM) and then labeled with ClpP-specific probe D3 (100 μM). (C) ABPP-labeling experiment in which living *S. aureus* cells were incubated with the probes indicated at 100 μM and, following cell lysis and click chemistry mediated rhodamine attachment to the probe, the proteome was separated by SDS-PAGE and imaged for fluorescence. See Supplementary Figure 11 for coomassie loading controls and images of the entire gels. (D) Western blot against SaClpPs after treatment of *S. aureus* cells with the indicated compounds for 2 h (see Supporting Figure 13 for an uncropped image).

Dehydroalanine Formation in Living Bacteria. To test if this reaction occurs in vivo, we synthesized alkyne-free analogues of the previous β-sultams termed RKS13, RKS14, and RKS15 (Figure 5A). We employed a competitive activity-based protein profiling (ABPP)[43−46] experiment in which we preincubated living *S. aureus* cells with alkyne-free β-sultams followed by addition of alkyne-functionalized D3 as an SaClpP-selective probe.[33] After cell lysis, a rhodamine fluorophore was attached to the probe via click chemistry,[47−49] the proteome was separated by SDS-PAGE, and the gel was imaged for fluorescence (Figure 5B, Supplementary Figure 11). In agreement with our in vitro data, no band in the case of

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RKS13 and only weak bands in the case of RKS14 and RKS15 were observed in the pretreated samples, indicating either the complete formation of dehydroalanine or the complete conversion of SaClpP into inactive heptamers owing to partial modification with inhibitor. To rule out the latter possibility, we treated living *S. aureus* cells with alkyne-functionalized probes, where we could not detect a fluorescent band in the molecular weight range of ClpP (Figure 5C; see Supplementary Figure 12 for the result of a time-dependent labeling experiment). Moreover, we showed by Western blot that the compounds do not alter the cellular concentration of ClpP (Figure 5D). These data suggest the formation of dehydroalanine-modified SaClpP in living cells.

■ DISCUSSION

ClpP is a crucial pathogenesis-associated enzyme in several bacterial pathogens such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*,[33,50,51] which demands novel ways for sustained inhibition. Here, we report the discovery of several covalently acting, active-site-directed compounds that lead to partial or complete dissociation of the proteolytic complex into catalytically inactive heptamers. Three compounds (DFP, RKS09, and E2) caused complete heptamerization, while other compounds shifted the oligomerization equilibrium toward the heptameric population. We have shown that this mechanism is not limited to SaClpP but could also be observed with other ClpP proteins. These results likely explain observations in the literature that DFP induces structural heterogeneity,[52] that DFP shows incomplete inhibition,[53] and that DFP displays nonstandard inhibition kinetics.[54] This is, to our knowledge, the first report in which a multimeric protease is inhibited by a ligand-induced disassembly triggered by an active-site-directed molecule.

It was previously speculated that transient fluctuations in the ClpP handle region might result in the formation of equatorial pores contributing to product release. This view is based on NMR data as well as on the observation of a functional ClpP enzyme where both axial pores are blocked by two ClpX hexamers.[17,22] These fluctuations would most likely be caused by a transition from the extended state into the compressed state, though the mechanism triggering this conformational switch remains to be elucidated. However, our results reveal that certain steric bulk at the active site, e.g. introduced by the peptidic inhibitor Z-LY-CMK, destabilizes the extended conformation and causes the formation of heptamers. In analogy, native peptide fragments with spacious side chains might serve as triggers for the conformational switch generating equatorial pores for product release next to the active site. Examination of the crystal structures of *E. coli* ClpP with bound Z-LY-CMK[36] as well as *Bacillus subtilis* ClpP with DFP[26] has not provided a clear explanation as to why ClpP proteins dissociate in solution. Our results suggest that steric bulk at the active site might shift the catalytic His123, thereby moving the catalytic Asp172 that would disrupt the Arg171−Asp170 interaction across the ring interface. This view is supported by the observation that small changes in this region (e.g., T169A) also led to heptamerization and by results that show a coupling of the catalytic triad with oligomerization.[25,27] Further experiments will have to clarify the molecular basis for this mechanism of ligand-induced oligomerization defects.

A mechanistically different inhibition principle for ClpP is represented by the selective conversion of the active site serine into a dehydroalanine moiety by β-sultams, as previously reported for β-lactamases.[55] This reaction proceeds rapidly through the sulfonylated serine.[35] Subsequent spontaneous elimination of this metastable intermediate results in an increase of the dehydroalanine species. The RKS09 compound displayed a unique behavior, since binding to ClpP caused disassembly of the holo-enzyme into heptamers that upon dehydration reassociate back into tetradecamers. The identity of the dehydroalanine was unequivocally confirmed by mass spectrometry and protein crystallography. Furthermore, activity-based protein profiling experiments suggest the formation of a dehydroalanine moiety in living *S. aureus* cells upon β-sultam treatment. These results are a significant advance over the previously reported dehydroalanine formation in isolated enzymes.[55,56]

Incubation of ClpP with β-sultams and subsequent dehydroalanine formation represents an elegant strategy to introduce a site-directed mutation into a protease active site without touching any other serine residue of the protein. Alternative methods to install a dehydroalanine have either been restricted to cysteine residues[57] or have been impaired by tedious optimization of reaction conditions[37] and, possibly, incomplete turnover.[58] With SaClpP having failed to react with PMSF, this reactivity is no longer a prerequisite for dehydroalanine formation in serine proteases. Moreover, inhibition by dehydroalanine formation is irreversible and thus long lasting in comparison to acyl ester intermediates formed by β-lactones and β-lactams that can be hydrolyzed. Collectively, we report two mechanisms for improved inhibition of multicomponent proteases. These findings extend our view on protease inhibition that until now has mainly relied on blockage of the active site or occupation of a regulatory allosteric site.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Text, figures, and tables giving experimental procedures, additional biochemical and kinetic data, proteomic data, compound characterization data, and ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

E-mail: stephan.sieber@tum.de.

Author Contributions

[§]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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