Heterooligomeric Complexes

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Vibralactone as a Tool to Study the Activity and Structure of the ClpP1P2 Complex from *Listeria monocytogenes***

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Nature provides a rich source of bioactive compounds comprising a diverse set of electrophilic core structures that are poised to react with corresponding nucleophilic residues such as serine and cysteine in enzyme active sites.[1–3] These residues are usually relevant for catalysis and therefore display fine-tuned reactivity towards their dedicated substrates.[4] We and others previously investigated the dedicated targets of monocyclic β-lactones which turned out to be potent and selective inhibitors of diverse disease-associated enzyme classes.[2,3,5–8] Covalent inhibition of the caseinolytic peptidase ClpP, for instance, resulted in a dramatic attenuation of bacterial virulence.[3] ClpP is an important, highly conserved heat shock protein with additional regulatory functions in many pathogens.[9–11] Some organisms such as *Listeria monocytogenes* genetically encode for two functionally and structurally uncharacterized ClpP isoforms (ClpP1 and ClpP2). So far, all β-lactones were reported to target solely ClpP2 and not ClpP1,[2] raising the question whether monocyclic lactones lack suitable reactivity to interact with the ClpP1 active-site nucleophile.

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Supporting information for this article (including details on the synthesis and characterization of compounds, bioassays, electron microscopy, image processing/3D reconstruction, structure prediction, homology modeling as well as proteome preparation and labeling) is available on the WWW under http://dx.doi.org/10.1002/anie.201104391.

We herein expand the scope of natural-product-derived β-lactones to strained bicyclic ring systems which may exhibit enhanced reactivity profiles. The natural products omuralide, salinosporamide, and vibralactone (VL) represent such desired scaffolds and have been reported to be potent proteasome or lipase inhibitors.[12–14] We utilized a chemical proteomic strategy termed "activity-based protein profiling (ABPP)"[15–17] to demonstrate that vibralactone (VL), contrary to monocyclic β-lactones, binds to both ClpP1 and ClpP2 in *L. monocytogenes*. Moreover, by combining transmission electron microscopy (TEM) and homology modeling/structure predictions, we were able to determine the quaternary structure of the hetero-oligomeric complex (Figure 1).

VL was synthesized as described by Zhou and Snider[18] (Scheme 1 in the Supporting Information) and modified with an alkyne handle in the final step for target discovery by ABPP (Figure 1).[19] Target analysis started by the incubation of this vibralactone probe (VLP) with intact living cells of *L. welshimeri* and its pathogenic counterpart *L. monocytogenes*. Upon cell lysis, the proteome was treated under click chemistry (CC)[20–22] conditions with rhodamine azide and the targets were visualized by fluorescent SDS-PAGE analysis (Figure 1 in the Supporting Information). Two strong fluorescent bands for approximately 20 kDa proteins were present at comparable intensities in *L. welshimeri* as well as in *L. monocytogenes* (Figure 2A) down to a VLP concentration of 3.4 μM (Figure 1C in the Supporting Information). Pre-incubation with various concentrations of unmodified VL gradually abolished the labeling of these bands, demonstrating that the natural product exhibits comparable target selectivity (Figure 2B). Mass spectrometric (MS) analysis revealed that the lower band corresponds to ClpP2 which has been labeled by monocyclic β-lactones before.[2] Interestingly, the upper band corresponds to ClpP1 which could not be addressed by any other β-lactone probe (Figure 2A, Table 1 in the Supporting Information). While ClpP2 orthologues from various organisms exhibit a high sequence homology (77% identity between *L. monocytogenes* and *S. aureus*) with a tetradecameric barrel-shaped assembly in crystal structures,[23–26] ClpP1 shares only 41% identity with ClpP2 (Figure 2 in the Supporting Information). This raises the question whether ClpP1 exhibits a different fold and function and assembles with ClpP2 in mixed complexes, as previously suggested for hetero-oligomeric complexes of different ClpP isoforms.[27]

To address these questions, we recombinantly overexpressed ClpP1 and ClpP2 independently as well as by means of a co-expression vector system in *Escherichia coli*. Co-expressed ClpP1P2 was isolated through a C-terminal strep

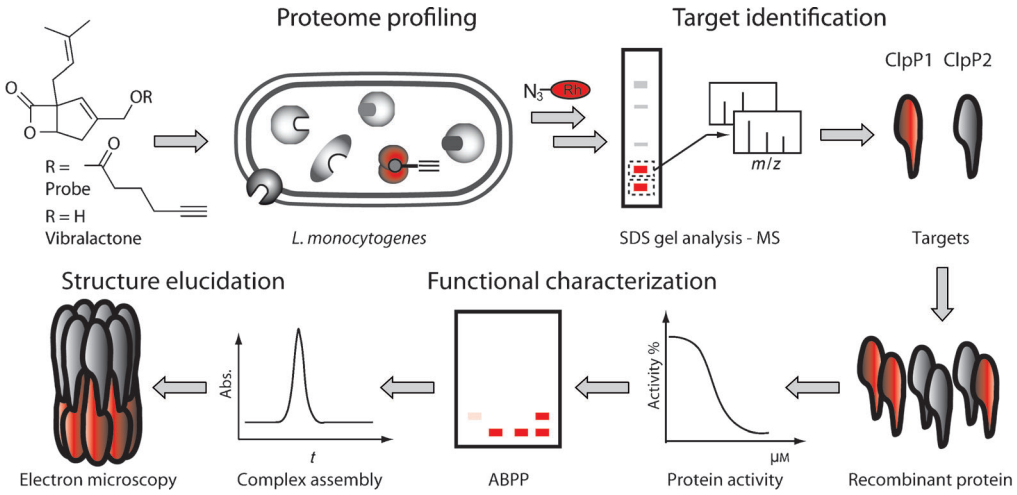

Figure 1. Discovery platform for vibralactone (VL) and the structural organization of ClpP1P2. The vibralactone probe (VLP) was used in living cells, and its dedicated targets were identified by SDS gel analysis and MS. Functional studies with the two prominent targets ClpP1 and ClpP2 reveal a hetero-oligomeric assembly that was further investigated by electron microscopy (EM).

tag at ClpP2 by affinity chromatography, whereby enrichment of ClpP1 is only possible if both enzymes form a stable hetero-oligomeric complex. Indeed, the existence of a hetero-oligomeric ClpP1P2 complex was confirmed by labeling with VLP (Figure 3 in the Supporting Information). Vice versa, equimolar mixing of purified ClpP1 with ClpP2 for different durations showed resulted in equal labeling of both enzymes between 24 h and three days (Figure 4 in the Supporting Information), indicating that ClpP2 triggered activation of ClpP1. While co-expressed ClpP1P2 revealed the same labeling pattern with VLP as that observed in the native *Listeria* proteomes, ClpP1 alone did not interact with VLP and selected monocyclic lactones (U1P, D3), in contrast ClpP2 was labeled by all lactones (Figure 3 in the Supporting Information). This suggests that the presence of ClpP2 is crucial for ClpP1 acylation and therefore likely for its activation at least in vitro.

In a peptidase activity assay with a fluorogenic model substrate VLP, VL and the monocyclic lactone U1P inhibited ClpP2 with EC_{50} values of 27 μM, 154 μM, and 4 μM (Figure 2C) as well as an equimolar ClpP1/ClpP2 mixture with EC_{50} values of 41 μM, 167 μM, and 3 μM, respectively (Figure 2D). In addition, both VL and VLP could be detected covalently attached to the active-site residue S98 of ClpP2 by LC–MS (Figure 5 in the Supporting Infor-

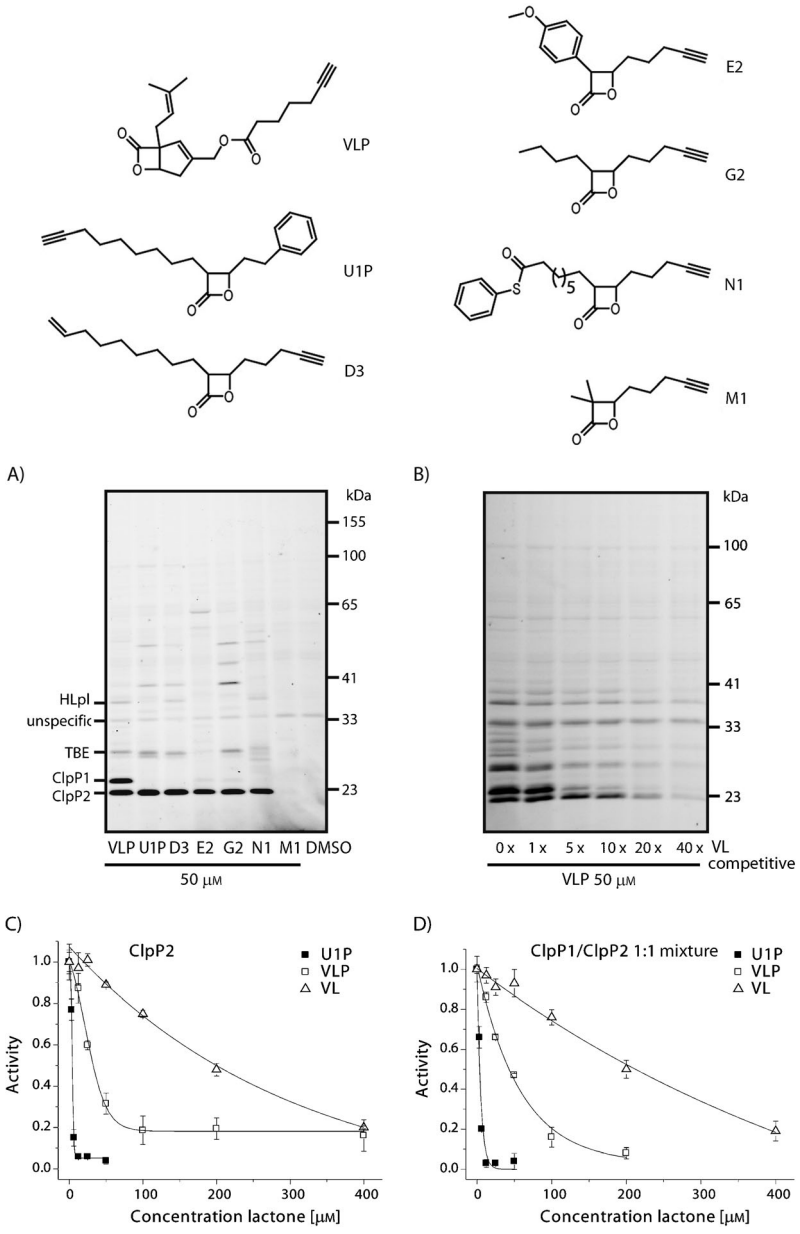

Figure 2. A) Fluorescent SDS-PAGE analysis of the *L. monocytogenes* proteome after incubation with VLP and different monocyclic β-lactones. B) Competitive ABPP experiment with VLP and different-fold excess of VL. Activity of recombinant C) ClpP2 and D) ClpP1/ClpP2 (1:1 mixture) after incubation with VLP, VL, and U1P at different concentrations.

mation). These results confirm together with heat denaturation studies (Figure 1D in the Supporting Information) that probe labeling is active-site directed and occurs only with the native, folded protein. No enzyme activity was observed for purified ClpP1 under any conditions tested. The optimum of peptidase activity was found at pH 7 (Figure 6A in the Supporting Information), and interestingly ClpP2 and ClpP1P2 activity increased by more than twofold after the addition of 5% glycerol (Figure 6B in the Supporting Information).[28]

To correlate enzyme activity with the quaternary organization of ClpP1, ClpP2, and ClpP1P2 we subjected all purified proteins to size-exclusion chromatography. While ClpP2 and co-expressed ClpP1P2 were found as tetradecameric complexes at 5% glycerol, lack of glycerol resulted to a large extent in disassembly into heptamers (Figure 7A in the Supporting Information). This suggests that glycerol, a stabilizing agent of the compact native form of flexible proteins, supports oligomerization into tetradecamers as reported previously.[29] Interestingly, ClpP1 eluted as a heptameric complex, independent of the glycerol content, emphasizing that ClpP1/ClpP2 activity depends on the oligomeric complex assembly with tetradecamers as active and heptamers as inactive forms. This is further supported by VLP incubation in the absence of glycerol with ClpP2 and co-expressed ClpP1P2 which shows less labeling for ClpP2 and no ClpP1 labeling in the co-expressed complex (Figure 7B in the Supporting Information).

To elucidate the quaternary structure of the ClpP1P2 complex in more detail, the samples were visualized by negative-stain electron microscopy (Figure 8 in the Supporting Information) which, revealed side and top views of barrel-shaped oligomers of 11.5 nm in height and 11.0 nm in diameter. The three-dimensional (3D) model at 15 Å resolution, obtained by single-particle reconstruction (Figure 3) revealed a tetradecameric assembly with a central pore, resembling the structures of ClpP from other species.[26] Most strikingly, however, the two heptameric rings of the tetradecameric assembly turned out to be nonidentical as one of the rings contained an additional mass at one end of the barrel (Figure 3A). Upon homology modeling of the tetradecamer using the predicted structures of ClpP1 and ClpP2, and the structure of ClpP from *E. coli* (pdb ID 1YG6) as a template, we were able to correlate this mass to the flexible N-terminal loop region which contains six additional amino acids in the sequence of ClpP2 that are not present in ClpP1 (Figures 2 and 9 in the Supporting Information). Although the predicted secondary-structure elements as well as tertiary structures of ClpP1 and ClpP2 monomers correlated extremely well, except for the N-terminal loop region (Figure 9A in the Supporting Information), the corresponding homoheptamer models exhibited distinct differences (Figure 4). The diameter of the pore in ClpP2 (2 nm) appeared, as a result of the

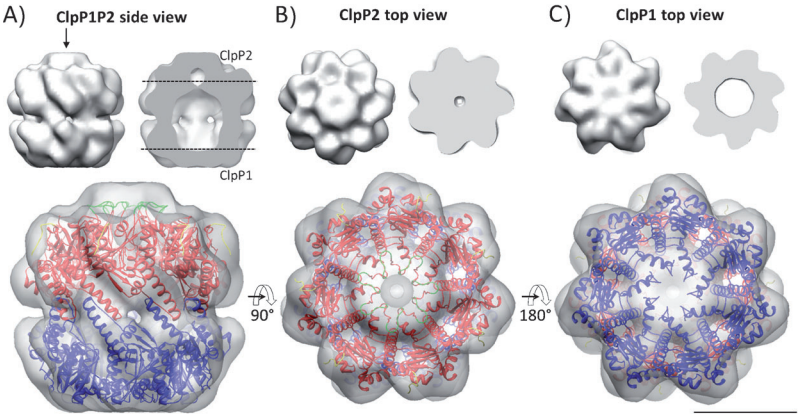

Figure 3. Three-dimensional model of the ClpP1P2 tetradecamer. Upper row: Surface representations and density cross sections of the ClpP1P2 tetradecamer as viewed from the side (A, central cross section) and along the sevenfold symmetry axis intercepting the ClpP2 (B) and ClpP1 rings (C). Positions of the cross sections in (B) and (C) are indicated by dashed lines in (A). Note the additional density on top of the ClpP2 ring (arrow). Lower row: Views of the oligomer in the same orientations as in the upper row with the docked homology model of the ClpP1P2 tetradecamer (ribbon representation) superimposed. The ribbon models of ClpP2 and ClpP1 are shown in red and blue, respectively. The N-terminal loop of ClpP2 is colored green, strep tag of ClpP2 yellow. Scale bar: 5 nm.

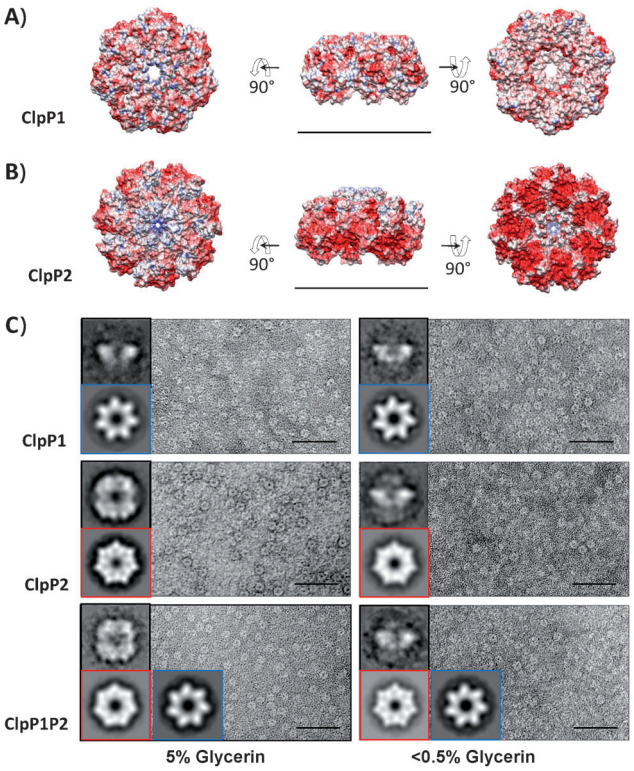

Figure 4. Distribution of charged residues on the surface of homology-modeled ClpP1 (A) and ClpP2 (B) homoheptamers. Residue color coding: negatively charged, red; neutral, white; positively charged, blue. Scale bars: 10 nm. C) Transmission electron micrographs of negatively stained ClpP1, ClpP2, and ClpP1P2 complexes (0.2 mg mL^{-1} protein, 1.5% (w/v) uranyl acetate) with either 5% glycerol (left) or diluted to a very low (<0.5%) glycerol concentration (right). Insets: Characteristic class averages. Top: Side views, bottom: top views after sevenfold symmetrization. For each data set, five class averages were calculated and compared from approximately 1500 top views along the sevenfold axis. Color coding of frames: ClpP1, blue; ClpP2, red. Scale bars: 100 nm, box sizes of class averages: 19 nm.

localization of the N-terminal loops at the entrance, narrower than in ClpP1 (3 nm; Figure 4). Moreover, whereas the distribution of the charged residues on the surface of ClpP1 seemed to be random (Figure 4 A and Figure 10 in the Supporting Information), the ClpP2 surface exhibited significant patches of high negative charge (Figure 4 B and Figure 10 in the Supporting Information). Indeed, these different characteristics were reflected by distinct appearances of the ClpP1 and ClpP2 homoheptamers in negative-stain microscopy. In line with the surface charge distribution, ClpP2 heptamers showed strong stain accumulation, masking the slight depressions present in the (predicted) structure and giving rise to a less-contoured class average (Figure 4 C). In contrast ClpP1 heptamers were only faintly stained and resembled a symmetric seven-pointed star. Also in agreement with the predicted structure, the central pore of ClpP2 appeared narrower in the negative stain image. In line with the results from size-exclusion chromatography (Figure 7 A in the Supporting Information), in co-expressed ClpP1P2 preparations containing 5% glycerol mainly tetradecameric oligomers were observed whose top views strongly resembled ClpP1 and ClpP2 homoheptameric rings (Figure 4 C). Upon dilution of glycerol to concentrations below 0.5%, we again found only class averages correlating to either ClpP1 or ClpP2 homoheptamers. As these homoheptameric rings were derived from the dissociation of the tetradecameric species, we conclude that the ClpP1P2 tetradecamers are mainly composed of homoheptameric ClpP1 and ClpP2 rings.

Surprisingly, a different structural assembly was reported and proposed for distantly related cyanobacterial ClpP isoforms composed of a tetradecamer of two heteroheptameric rings.[27,30] This indicates, consistent with our results, a functional specialization of ClpP isoforms with yet unexplored, presumably regulatory functions.

In conclusion, we have reported on the bicyclic β-lactone vibralactone as an unprecedented probe for labeling two isoforms of the important ClpP protease from *L. monocytogenes*. We expanded the scope of the probe as a tool to study the activity and assembly of ClpP1 and ClpP2 subunits in a hetero-oligomeric composition. Our results suggest that ClpP1 is activated by hetero-oligomerization with ClpP2, and the tetradecameric assembly enhances the catalytic activity. Finally, electron microscopic images indicate that the tetradecameric assembly is constituted by two homoheptameric ClpP1 and ClpP2 rings that are stacked on top of each other. These results provide the first insight into a novel complex assembly of an important class of bacterial enzymes.

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