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PII: S0021-9258(22)00997-8
DOI: https://doi.org/10.1016/j.jbc.2022.102553
Reference: JBC 102553

To appear in: Journal of Biological Chemistry

Received Date: 2 July 2022
Revised Date: 23 September 2022
Accepted Date: 25 September 2022


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Structure of the drug target ClpC1 unfoldase in action provides insights on antibiotic mechanism of action

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Keywords: Mycobacterium tuberculosis, Antibiotics, Chaperone, protein degradation, cryo-electron microscopy
Abstract:

The unfoldase ClpC1 is one of the most exciting drug targets against tuberculosis. This AAA+ unfoldase works in cooperation with the ClpP1P2 protease and is the target of at least four natural product antibiotics: cyclomarin, ecumicin, lassomycin and rufomycin. Although these molecules are promising starting points for drug development, their mechanisms of action remain largely unknown. Taking advantage of a middle domain mutant, we determined the first structure of *Mycobacterium tuberculosis* ClpC1 in its apo, cyclomarin and ecumicin bound states via cryo-electron microscopy. The obtained structure displays features observed in other members of the AAA+ family and provides a map for further drug development. While the apo and cyclomarin bound structures are indistinguishable and have N-terminal domains (NTDs) that are invisible in their respective EM maps, around half of the ecumicin-bound ClpC1 particles display three of their six NTDs in an extended conformation. Our structural observations suggest a mechanism where ecumicin functions by mimicking substrate binding, leading to ATPase activation and changes in protein degradation profile.

Introduction:

Clp proteases are composed of two heptameric rings forming a cylinder with 14 proteolytic sites compartmentalized within its central chamber (1). While ClpP alone is able to rapidly hydrolyze peptides, the degradation of large proteins requires the presence of a hexameric AAA+ ATPase complex, such as ClpX or ClpC1. These ATPases activate the Clp proteases,
but also bind protein substrates, unfold them, and translocate them into the proteolytic compartment. Unlike most bacteria and mitochondria, *Mycobacterium tuberculosis* (*Mtb*) contains two clp genes, clpP1 and clpP2, that form an active complex containing one ClpP1 and one ClpP2 ring (unless otherwise stated, ClpP1P2, ClpC1 and ClpX refer to *Mtb* proteins) (2, 3). In addition to genetic evidence that ClpC1, ClpX, ClpP1 and ClpP2 proteins are essential for viability, the relevance of these targets has been reinforced by the discovery of multiple natural product antibiotics (NPAs) that kill *Mtb* by targeting this system. The specific potential of ClpC1 as a drug target in *Mtb* was proven by the discovery of 4 potent and chemically diverse NPAs acting on this protein (4–10). Indeed the cyclic peptides ecumicin, cyclomarin, rufomycin and lassomycin, all binding ClpC1, are among the most powerful anti-tuberculosis molecules to emerge recently. Ecumicin, for example, displays potent selective anti-tuberculosis activity with an MIC value 50 times lower than that of rifampicin or isoniazid, the first line drugs for the treatment of tuberculosis (TB)(4, 5).

ClpC1 is a member of the class II AAA+ family of proteins, which contains an N-terminal domain (NTD) and two distinct ATP-binding modules, D1 and D2. While no full-length structure of *Mtb*ClpC1 is currently available, considerable structural work has been performed on the easy-to-handle NTD domain (7, 11, 12). Curiously, despite representing only a small portion of the full protein, all the NPAs have been shown to bind to the ClpC1-NTD domain and high resolution X-ray structures of the binding sites are available for cyclomarin, ecumicin and rufomycin (7, 11, 12).

While this allows a proper mapping of the NPAs binding pockets, it is still not clear how binding to the NTD can translate into functional impairment of the remaining protein. The NTD lacks ATPase activity, which is present in the D1 and D2 domains, and is connected to these domains by a long disordered loop. Somehow, binding to the NTD must induce changes in the remaining domains. Curiously, despite binding to similar parts of the NTD, the different NPAs lead to distinct effects.

Using small-angle X-ray scattering (SAXS), we have recently shown that ClpC1 exists in an equilibrium between a resting state and the active hexameric state, but how this
equilibrium is modulated in *Mtb* is still difficult to understand (7). MecA, an adaptor that modulates ClpC in other species, does not exist in the *Mtb* genome, but a homologue of the ClpS adaptor protein has been shown to bind to ClpC1 (13). Another possibility is that substrate binding, as proposed by others, can shift the ClpC1 equilibrium towards a hexamer (14). It is assumed, based on the extensive AAA+ literature and obtained structural data, that the hexameric state is the ClpC1 functional form. Indeed, only the hexameric form permits the formation of a surface for the interaction with ClpP, as well as a pore linked with loops that can couple ATP hydrolysis to mechanical substrate pulling. It is rather unlikely that the structure observed for the resting state, despite the low resolution, can interact with ClpP.

Taking advantage of a single mutation, we were able to stabilise the hexameric state of this important drug target and obtain structures in the presence of cyclomarin and ecumicin. Interestingly, half of the ecumicin bound particles display three of their 6 NTDs in an extended conformation, suggesting a potential mechanism of how ecumicin modulates ClpC1 function.

Results:

Mutation of Phenylalanine 444 into Alanine results in fully functional *Mtb*ClpC1 with a stabilized hexameric state.

Although the ClpC1-NTD has been extensively studied by X-ray crystallography and solution NMR (7, 11, 12, 15), no structural information on the full-length ClpC1 has so far been reported. This can be rationalized by several key factors. Firstly, we have shown that ClpC1 exists in an equilibrium between a resting state and the active hexameric state (7). Second, the active hexamer is only formed in the presence of ATP which is rapidly converted into ADP. Finally, the ClpC1P1P2 *Mtb* system is, compared to homologues from *Staphylococcus aureus* (Sa) and *Bacillus subtilis* (Bs), more insoluble and therefore harder
to work with in vitro (7, 16). Indeed, these limitations and the fact that so far all the NPAs targeting ClpC1 bind to the ClpC1 NTD have led others to employ chimeras of ClpC1 and the D1D2 domains of Sa to study the function and mode of action of cyclomarin (16). While this is an interesting approach, it has the limitation of inferring results from non-physiological proteins and therefore not providing any direct and useful structural data for drug development. This is particularly evident, considering the existing mechanistic differences between the Sa and Mtb proteins. For example, whereas SaClpC depends on MecA to catalyse the degradation of GFPssra by ClpP, ClpC1 is fully capable to do so independently of the adaptor (2, 17–19). In fact, MecA does not exist in the Mtb genome. It is therefore fundamental to obtain structural information on ClpC1 in its functional state.

Given the presence of an equilibrium between a decameric resting state and the active hexameric ClpC1 states in solution, we rationalized that the stabilization of the ClpC1 hexamer could allow us to obtain structural information on this important drug target. Previously, stabilization was achieved by removing intrinsically disordered loops, allowing the elucidation of the only ClpC X-ray structure described to date (20), but at the expense of enzymatic activity and therefore mechanistic relevance. Carroni et al. have demonstrated that point mutations in the middle domain (MD) of SaClpC shift the equilibrium towards the hexameric state (14). We hypothesized that similar modifications could also stabilize the active hexameric state of ClpC1, allowing subsequent structural characterization. In particular, residue F436 in SaClpC (F444 in Mtb) has been shown to be important for the resting state-hexamer equilibrium (Fig 1A, 1B and S2 A).

By means of site directed mutagenesis, we mutated the homologous residue in ClpC1 to an alanine (F444A), to test if it could shift the ClpC1 equilibrium. Using a Superose 6 10/300 GL column we observed that, as previously shown (7), wild-Type ClpC1 (ClpC1WT) migrates as a species larger than the canonical hexamer. By contrast, in the presence of ATP, the F444A main species (ClpC1F444A) eluted in a volume consistent with the size expected for a ClpC1 hexamer (Fig1C). Furthermore, the presence of hexameric rings in the mutant was also confirmed by negative stain electron microscopy (EM) (Fig1D).
After successful stabilization of the hexameric state, we sought to verify that biological activity was maintained in mutant ClpC1F444A. ClpC1F444A ATPase activity was checked using an assay monitoring the fluorescence decrease at 340 nm, associated with NADH to NAD⁺ conversion as the ADP formed by ClpC1 ATPase activity is reconverted into ATP by pyruvate kinase and phosphoenolpyruvate dehydrogenase (18). As shown in Fig 2A, ClpC1F444A displayed an increase in the ATPase activity in comparison to ClpC1WT. An increase in ATPase activity was also observed for the SaClpC1F436A mutant, which was explained by a shift in the resting state–active hexamer equilibrium towards the latter, which is consistent with described size exclusion chromatography (SEC) experiments (14).

Although cyclomarin and ecumicin binding sites are located at the ClpC1 NTD and therefore distant from the F444A mutation, we tested the functional consequences of cyclomarin and ecumicin binding on the mutant. Using saturating concentrations (10 μM), ecumicin binding to the WT results in a strong increase in ClpC1 ATPase activity while cyclomarin does not. Curiously, a similar increase in ATPase activity is observed using the F444A mutant, showing that ecumicin effects do not exclusively result from the modulation of the ClpC1 resting state - hexamer equilibrium (Fig 2A). An important in vivo function of ClpC1, if not the most relevant, is to associate with ClpP1P2 and target proteins for degradation by the protease. The roles of ClpC1 in this process are multiple, as it is responsible for substrate recognition, unfolding and translocation but also for association with ClpP1P2 and its concomitant allosteric activation (2, 18). Because these processes are very hard to study independently, ClpC1 function is often evaluated indirectly by measuring protein target degradation, i.e. the rate at which a protein is degraded by ClpP1P2 in association with ClpC1 (2). Two substrates that are commonly used are FITC-casein (casein with a fluorescein fluorophore attached to lysine residues) and green fluorescent protein (GFP), representing 2 classes of proteins. FITC-Casein, which lacks a well-defined tertiary structure, is usually used as a model for unfolded substrates, while the stable, beta-barrel containing GFP is used as a model for a structured protein. While in the case of FITC-casein, protein degradation results in a net fluorescence increase as fluorescein quenching is reduced with degradation, in the case of GFPssra, degradation
of the protein fluorophore results in a decrease in fluorescence (18). As can be seen in Fig 2B and 2C, the F444A mutant is fully functional to catalyse the degradation of both FITC-casein and GFPssra by ClpP1P2, showing that this mutation does not impair ClpC1 enzymatic activity.

Reflecting different modes of action, the effects of cyclomarin and ecumicin in protein degradation by the ClpC1P1P2 complex are distinct. At the concentration used, cyclomarin is a moderate activator of FITC casein degradation and a weak inhibitor of GFPssra degradation while for ecumicin, we observe a mild activation of FITC casein degradation and a strong inhibition of GFPssra degradation (Fig 2B and 2C). The different effects of these 2 NPAs are also clear when they are used together. Indeed, cyclomarin is able to prevent both ATPase activation (Fig2D) as well as GFP degradation inhibition induced by ecumicin (FigS1), showing that they are competing for a similar pocket but they induce distinct biochemical effects. The distinct structural consequences resulting from cyclomarin and ecumicin binding are also clear from other biophysical data. Differential scanning fluorimetry (DSF) is a very useful method to monitor ligand binding to a given target. We have previously shown that Arginine phosphate (ArgP) is able to stabilize the NTD and we aimed to test the effects of cyclomarin and ecumicin on the NTD (7). Using sypro orange as a fluorescence reporter we obtained a Tm of 77.7 ± 0.3 and 83.6 ± 0.3 °C, for the apo and ArgP bound NTD. These values are higher than the ones we previously reported using intrinsic tryptophan fluorescence as a reporter that were 69 and 79 °C for the WT and ArgP bond NTD (7). Quite striking was the difference observed between cyclomarin and ecumicin. While cyclomarin binding resulted in very strong stabilization of NTD, with a calculated Tm of 91.5 ± 0.9 °C. Ecumicin, on the contrary, did not stabilize the domain and in fact lead to a decrease in the Tm (Fig S2B).

Structural characterization of *Mtb*ClpC1 by cryo-electron microscopy
Having shown that the MD mutant (ClpC1:F444A) is active, we attempted unsuccessfully to crystallize the resulting hexamer. We therefore set out to obtain structural information on ClpC1 via cryo-EM. To further stabilize the sample, considering that ATP is being continuously degraded even at low temperatures and that some AAA+ proteins have been shown to hydrolyze ATP analogues, we introduced two additional mutations in the double walker B motif (E288A and E626A). We hypothesized that these mutations allow nucleotide binding, but prevent hydrolysis and therefore further stabilize the hexameric assembly.

After initial cryo-EM grid screening, a dataset was collected of the apo form of \textit{Mtb} ClpC1:E288A:F444A:E626A (see Materials and Methods). Processing of the data resulted in a structure for apo ClpC1 determined to a resolution of 3.6 Å (see Materials and Methods, Supplementary Figure 3 and 4 and Supplementary Table 1). As expected, the overall structure of apo \textit{Mtb} ClpC1:E288A:F444A:E626A consists of a hexamer composed of 6 ClpC1 subunits (A-F), with the D1 and D2 domains arranged in a ring that together forms a pore through which the substrate can be actively translocated. In line with recent structures, including the recent cryo-EM structure of \textit{B. subtilis} ClpC (21), the observed ClpC1 hexamer is not symmetric, as observed originally in a crystal structure of ClpC (20), and instead adopts an asymmetric spiral structure (Fig 3A). An important characteristic of our structure is the observation of a 23 residue long peptide visible in its central pore. The presence of substrates in the pore formed by D1 and D2 has been previously reported for other members of the family but as no substrate has been added to our cryo-EM sample preparation it was probably taken up and trapped inside of the inactive protein during purification (Fig 3A and B). This unexpected finding is nevertheless useful as it provides important details on the ClpC1 mechanism of action.

Indeed, the substrate is bound by each of the two pore loops from subunits A-E in both D1 and D2, with the exception of pore loop 2 in D1 subunit E, forming a spiral along the substrate. Subunit E is positioned at the highest point and subunit A at the lowest. Subunit F is detached from the substrate in both D1 and D2 and the pore loops are not visible. Furthermore, all nucleotide binding sites in D1 are occupied by ADP while for D2 4 sites
are occupied by ADP (subunits B, C, D, E), while two are in their apo state (subunits A and F) (Fig 3C). This organization is in line with the asymmetric disposition we referred to above and has been reported for other members of the family, particularly for structures obtained using cryo-EM (21–23).

Unfortunately, several structural features are not visible in the ClpC1 apo structure, presumably due to flexibility or intrinsic disorder. One of these missing features are the LGF loops which, in line with other AAA+ ATPase structures, will likely only become structured upon interaction with the ClpP protease (22). Another unresolved feature is the middle domain, which is connecting D1 and D2 and carries the F444A mutation. The middle domain is presumably a key point for interactions with adaptors or binding partners and is usually only visible in the resting state, or when it is engaged with a binding partner (see MecA in ClpC1 or DnaK in the homologous ClpB) (14, 24). Although there is no high-resolution structure of the ClpC1 resting state, we presume that the overall organization of the resting state is similar to the one of SaClpC for two reasons: 1) our previously reported SAXS structure of the ClpC1 resting state overlays perfectly with the SaClpC cryo-EM structure of the resting state (PDB: 6EM9/6EM8) and 2) the same residues appear to be involved in the stabilization of the resting state as shown by our mutation experiments involving F444. It is thus likely that the MD of ClpC1 in the resting state is properly folded to provide contacts between the different MDs, thereby stabilizing the resting state, and only becomes unfolded upon formation of the hexamer. Finally, the NTD and its adjacent 26 residue linker are not visible in our apo ClpC1 structure. The NTD is thought to have an important role in substrate recognition and targeting to the ClpC1 pore, and it is the binding site of several recently identified NPAs against Mtb. Similar to the MD, the NTD can only be seen in the resting state or in structures where an adaptor or the substrate itself stabilize its position. The isolated NTD is a well folded globular domain and the fact that it is not visible in our apo ClpC1 structure presumably does not reflect on an intrinsic disorder of the domain but rather on the high flexibility of the linker and the resulting multiple orientations with respect to the D1/D2 domains.
Cryo-EM structures of *Mtb*ClpC1 bound to natural product antibiotics

Following successful structure determination of apo ClpC1, two additional datasets were collected on cryo-EM grids prepared after addition of 30 µM cyclomarin or ecumicin to purified *Mtb*ClpC1\(E_{288A/F444A/E626A}\) (see Materials and Methods). Processing of the two datasets resulted in a structure for cyclomarin-bound ClpC1 determined to a resolution of 3.3 Å, and two 3D classes of ecumicin-bound ClpC1 determined to resolutions of 4.3 Å and 8.6 Å respectively (see Materials and Methods, Supplementary Figure 3 and 4, and Supplementary Table 1).

The structure of cyclomarin-bound ClpC1 (Fig S2 C) is virtually identical to the apo ClpC1 structure, with a calculated root-mean-square deviation (r.m.s.d.) of 0.39 Å (over 3239 aligned \(C_α\)-atoms). No differences in nucleotide binding or substrate interaction are observed. Similar to the apo ClpC1 structure, a 23 residue substrate is trapped in the ClpC1 pore and the NTDs, MDs and LGF loops are invisible.

Processing of the *Mtb*ClpC1 dataset with added ecumicin resulted in 2D classes that included side-views revealing hints of the NTDs, present as a blurry sphere on top of the ClpC1 hexamer (Supplementary Figure 3, A). Ensuing hetero refinement using two 3D classes followed by final Non-Uniform refinements in cryoSPARC (25) resulted in two distinct maps at resolutions of 4.3 Å and 8.6 Å. The first map, populated by roughly 60 % of the particles, represents a structure identical to the ones observed for apo *Mtb*ClpC1 and cyclomarin bound *Mtb*ClpC1. The second map, containing 40 % of particles, has a significantly lower resolution, but reveals three globular domains positioned on top of the D1 domains of the hexameric ClpC1 ring in an organization reminiscent of an NTD-trimer observed in a hyperactive ClpB mutant bound to casein (26). Rigid-body fitting of the NTD-trimer taken from PDB 6OG3 (26) in the low resolution map results in a good fit (Figure 4, B), and provides a model where the antibiotic binding sites in helix 1 and 5 on the NTD and the ArgP/substrate binding sites are facing towards the centre of the ClpC1 hexameric ring (Figure 4A and B). The three remaining NTD domains, as well as all flexible linkers connecting the NTD domains with the adjacent D1 domain are not visible in the map, most
probably due to the inherent flexibility of the linker region. Nonetheless, the observed trimerization of the *Mtb*ClpC1 NTDs upon addition of ecumicin suggests that ecumicin can function by mimicking substrate binding.

**Discussion:**

It is well established that minor differences in protein structure may result in important functional differences. Therefore, even when structures of homologs are available, it is still important to obtain accurate structural information, particularly for drug development. For instance, while displaying high primary sequence homology to other ClpCs, *Mtb*ClpC1 displays unique mechanistic features. Indeed, contrary to its homologues, ClpC1, does not depend on activators as MecA and is fully competent to catalyse, together with ClpP1P2, the degradation of both unfolded (as casein) and folded (as GFPssra) substrates. In addition, *Mtb*ClpC1 is the sole target of the natural product antibiotics cyclomarin, lassomycin, ecumicin or rufomycin.

In this study, we took advantage of a middle domain mutation (F444A) to stabilize the ClpC1 hexameric state. This mutation was chosen based on previous data obtained for *Sa*ClpC and our own results using *Mtb*ClpC1, that proved the existence of an equilibrium between a resting state and a hexameric form (7, 14). Indeed, the change of an aromatic phenylalanine to an alanine resulted in a large shift in the size exclusion profile towards the size expected for a hexameric complex.

While the introduction of the F444A mutation shifts the equilibrium towards the hexameric state, it may potentially modify *Mtb*ClpC1’s mechanism of action. For example, the first structure of a ClpC complex, the only one obtained using X-ray crystallography, was from an inactive mutant, unable to catalyse ATP hydrolysis and therefore protein degradation (20). We therefore biochemically characterized the F444A mutant with
respect to ATPase activity and ClpP1P2 mediated protein degradation of unfolded and folded substrates, and show that *Mtb*ClpC1_{F444A} is fully active, with increased specific activities versus the WT protein. This activation was not unexpected, considering that the equilibrium is shifted towards the active state, but it is worth mentioning that it is more moderate than the activation described for ClpC1 homologues with similar modifications in the MD (14). This fact likely reflects the functional differences between these homologues, but nonetheless shows that the F444A mutant is fully able to catalyze unfolded and folded protein degradation in association with ClpP1P2.

One of our goals was to understand the mechanism of action of antibiotics targeting ClpC1. Therefore, an important question was whether the F444A mutation might influence the effects of cyclomarin and ecumicin. It is known that even though cyclomarin and ecumicin largely share their binding surfaces in the NTD, their binding results in different mechanistic consequences. In fact, as we have shown here and has been reported elsewhere (4, 5), ecumicin results in a marked increase in ClpC1 ATPase activity, whereas cyclomarin fails to do so. Two other striking differences concern FITC-casein and GFPssra degradation in association with ClpP1P2. Although both cyclomarin and ecumicin lead to a mild activation of FITC-casein degradation, ecumicin is a much better inhibitor of GFPssra degradation.

Introduction of the F444A mutation and the shift towards a hexameric state does not abolish cyclomarin and ecumicin effects and a similar profile of activation and inhibition is observed for all enzymatic activities tested. This does not support the recent proposition, based on protein chimeras, that cyclomarin mechanism of action depends only on a shift of the resting state – hexamer equilibrium (16). If that was the case the effect of cyclomarin should be abolished with the introduction of the mutant. Indeed, the same conclusion could be simply derived from the fact that the two antibiotic effects are dissimilar. If ecumicin and cyclomarin effects were exclusively dependent on resting state - hexamer equilibrium, we would expect their action to be the same in all activities measured – which is not the case.
The cryo-EM structures we determined further corroborate our assumption that the F444A mutant is a valid model for an active ClpC1 hexamer. Supporting the validity of our approach, multiple structural features demonstrate that we have in fact a functional enzyme. First, the asymmetric hexameric structure we observe here has been observed for other members of the AAA+ family, including a very recent structure of SaClpC as well as ClpA (21, 22). This shows that the mutation we introduced does not impair hexamer formation, domain architecture or nucleotide binding. Even more important, the unexpected presence of a peptide bound in the pore formed by the D1 and D2 domains and in close contact with the canonical tyrosine loops demonstrate that F444A ClpC1 is fully competent to bind and translocate protein substrates.

In addition to the apo MtbClpC1 cryo-EM structure, we determined additional structures after addition of either cyclomarin or ecumicin antibiotics to purified MtbClpC1. Both cyclomarin-bound MtbClpC1 as well as one of the two 3D classes of ecumicin-bound MtbClpC1 are strikingly similar to the apo MtbClpC1 structure, and have missing N-terminal domains (NTDs), which contain the binding sites for both cyclomarin and ecumicin. The role of the NTD in ClpC1’s mechanism of action remains largely unknown, but data obtained with other AAA+ ATPases suggest that it may transfer the polypeptide substrate to D1 and the D1 pore. Curiously, truncating part of the ClpC1 NTD, residues 1-78, has been shown to paradoxically activate the refolding capability of ClpC1 (13). Considering our data, it seems however unlikely that cyclomarin would induce a stable conformational change in the NTD, as for example occurs with MecA binding in other species, where an additional ring adjacent to the D1 ring is clearly observed both in X-ray and cryo-EM structures.

The second 3D class of ecumicin-bound MtbClpC1, containing roughly 40% of the imaged particles, reveals three globular domains above the D1 N-termini, most likely corresponding to three NTDs. Additional densities corresponding to the other NTDs are not observed, strongly suggesting that they remain flexible. Interestingly, the position of the putative NTDs in our map of ecumicin-bound MtbClpC1 is identical to a structure of
an NTD trimer described for a hyperactive *E. coli* ClpB MD mutant engaged with casein (26). In this structure, obtained at much higher resolution (2.9 Å), the NTD trimer forms a substrate entrance channel, positioning the casein polypeptide above the translocation pore. Additionally, trimerization of the NTD of *Mtb*ClpC1 upon addition of ecumicin was recently demonstrated in a crystal structure of the ecumicin-ClpC1NTD complex (15). The relative position of the NTDs in the ecumicin:ClpC1NTD trimer is similar to the NTD trimer in casein-bound *E. coli* ClpB, and therefore fits equally well in our low resolution map. Interestingly, contrary to the casein:ClpBNTD trimer, the orientation of the individual NPA binding sites of the NTDs in the ecumicin:ClpC1NTD trimer is facing outwards rather than inwards. However, the lower resolution of the ecumicin-bound *Mtb*ClpC1 map we obtained here (8.6 Å) does not allow us to accurately assess the ecumicin binding site or the relative conformations of the NTD domains.

How can the structures we obtained help in understanding the mechanism of action of cyclomarin and ecumicin? As described previously, albeit binding to similar regions of the NTD, cyclomarin and ecumicin result in different mechanistic consequences. ITC results as well as X-ray crystallographic studies have shown that only one molecule of cyclomarin binds to the NTD (7, 11), forming a bridge over a hydrophobic ridge dominated by the aligned phenyl rings of phenylalanines F2 and F80. It is still unclear if cyclomarin, like rufomycycin, forms a covalent adduct with the NTD N-terminal methionine. Quite striking is the fact that cyclomarin binding results in a dramatic increase in the stability of the NTD, and this is reflected in an increase of circa 15 °C in thermal stability as well as an important restriction of the domain dynamics induced by ArgP binding (7).

Ecumicin is a tridecamer depsipeptide with a larger scaffold than cyclomarin and an extended tail of three amino acids important for binding to the protein NTD (see Supplementary Fig. 1). Furthermore, it has a different binding stoichiometry were 2 ecumicin molecules are accommodated by one NTD (15). Interestingly, in the crystal packing of the ecumicin:ClpC1NTD complex, clusters of three dimers arrange themselves in a looser hexameric arrangement. Wolf *et al.* suggested that the presence of two ecumicin molecules may help to stabilize this aggregate via hydrophobic binding (15). It is
speculative to consider that a similar NTD trimer occurs in solution. Yet, the structure of ecumicin-bound ClpC1 shown here could represent such a complex, where ecumicin stabilizes the interaction between three NTD subunits. Still, several mechanism-based questions can be addressed here, particularly how the formation of an NTD trimer upon ecumicin binding can result in ATPase activation, inhibition of GFPssra degradation and a mild activation of FITC-casein degradation. As described above, due to its high mobility, very little is known about NTD function. Nevertheless, two roles for the NTDs in ClpA have been proposed (27). One of the proposed roles is to work as an antenna to capture substrates via an initial weak interaction, transferring them to the second, stronger binding site for unfolding, and subsequent translocation to the D1-D2 pore. In this way, the NTDs would not be fundamental for catalysis, but would make the processing of some challenging (folded) substrates more productive. This hypothesis is consistent with the observation that removal of half of the ClpC1 NTD appears not to affect firefly luciferase refolding by ClpC1 (28) or that casein degradation by ClpA (29) is independent of the NTD. In contrast to these unfolded substrates, impairment of the NTD can drastically affect the degradation of (folded) GFPssra by ClpA (30). Another putative role for the NTD would be to function as an ‘entropic brush’ in order to prevent unspecific interactions with the pore binding sites. Resulting from these two proposed roles, impairment of NTD function should likely affect the degradation of folded proteins like GFP more than unfolded proteins such as FITC-casein. The results presented here seem to corroborate this hypothesis, since a strong inhibition of GFPssra degradation and a mild activation of FITC-casein degradation by MtbClpC1 is observed after addition of ecumicin. However, it is still not clear how the formation of NTD oligomers may explain the strong ATPase activation observed after addition of ecumicin.

In summary, we provide here the first high resolution structure of MtbClpC1, an important drug target, in its apo, cyclomarin and ecumicin bound states. The ecumicin-bound ClpC1 structure presented here allow us to suggest a model for antibiotic action based on the formation of stable ecumicin-bound NTD oligomers, which would block folded substrate degradation by the ClpC1P1P2 complex.
Acknowledgments

"The authors acknowledge the support of the European Union (EU) and Horizon 2020 through iNEXT-Discovery Proposal: 871037 (Access project PID: 15695), and the CRIOMECORR project (ESFRI-2019-01-CSIC-16) to the cryoEM CNB-CSIC facility." This work benefited from access to the Instruct Image Processing Centre, an Instruct-ERIC centre. Financial support was provided by Instruct-ERIC (PID 15689)."

Experimental Procedures:

**Biochemistry.** Mutations F444A, E288A and E626A were introduced into a pet20-ClpC1 plasmid using NZYMutagenesis kit. The primers used are listed in table S1. Mutations were confirmed by DNA sequencing (Eurofins) using the T7 forward primer. ClpC1 and mutants were expressed and purified as previously described (7). ClpC1 ATPase activity and FITC-casein and GFPssra degradation by ClpC1P1P2 complex were measured as described previously (18). DSF measurements were executed in a Pierce Light Cycler 96 using a 34-95 °C temperature ramp using 5 μM NTD, 12.5 μM cyclomarin and ecumicin and 166 μM ArgP in Hepes pH 7.4 50 mM NaCl 100 mM. The T_m was calculated using the first derivate of the respective curves.

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<th>Mutation</th>
<th>Forward Primer</th>
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<td>5’GGCGGCTTTCTGCGGTCTGGGCGTCG-3’</td>
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<td>E626A</td>
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</table>

**CryoEM sample preparation**
Cryo-EM grids of Apo *M. tuberculosis* ClpC1 (*Mt*ClpC1), *Mt*ClpC1 in complex with Cyclomarin and *Mt*ClpC1 in complex with Ecumicin, were vitrified using a Vitrobot Mark IV (FEI). Quantifoil Cu/Rh 1.2/1.3 300 mesh grids were previously glow-discharged for 30 s at 15 mA. Aliquots of 3 μl of the different samples were added onto the grids, blotted for 3 s at 4 ºC and 95% humidity and plunged into liquid ethane.

**CryoEM data collection**

Screening and data acquisition of all samples were performed using a 200 kV FEI Talos Arctica equipped with a Falcon III direct electron detector at the Centro Nacional de Biotecnología (CNB) cryo-EM facility. A total of 944 movies of Apo *Mt*ClpC1, 2840 movies of *Mt*ClpC1 + cyclomarin and 1520 movies of *Mt*ClpC1 + ecumicin were acquired at a nominal magnification of 120,000x (corresponding to a pixel size of 0.855 Å/pixel), with a defocus range of −1.2 to −3.1 μm. Movies were fractionated to 60 frames with a total exposure of 40 s (Apo *Mt*ClpC1 and *Mt*ClpC1 + ecumicin) or 30 s (*Mt*ClpC1 + cyclomarin), with a total dose per movie of 34.3 e/Å² (Apo *Mt*ClpC1), 36.9 e/Å² (*Mt*ClpC1 + cyclomarin) and 32.2 e/Å² (*Mt*ClpC1 + ecumicin)

**CryoEM data processing**

Collected movies (Apo dataset: 944, cyclomarin-bound dataset: 2840, ecumicin-bound dataset: 1520) were motion corrected and dose weighted using MotionCor2 (31). Further data processing was performed using cryoSPARC 3.3.1 (27). Initial CTF estimation on the imported aligned and dose weighted micrographs was performed using Patch CTF estimation. For each dataset, particle picking was performed using crYOLO (32). Imported particle stacks were extracted using a box size of 500 pixels downsampled to 250 pixels (2x binned), corresponding to a pixel size of 1.71 Å/pixel. Extracted particle stacks were cleaned using several rounds of iterative 2D classification and 2D class selection. Cleaned particle stacks were used as an input for *Ab Initio* model generation and subsequent Non-Uniform (NU) 3D refinement. For Apo and cyclomarin-bound datasets, a final NU 3D refinement was performed on unbinned particles from the pre-final 3D refinement, extracted using a box size of 500 pixels, corresponding to a pixel size of 0.885 Å/pixel. For the Apo dataset, the final NU refinement was performed using optimized per-group CTF parameters, while for the cyclomarin-bound dataset both per-group CTF parameters as well as per-particle defocus were optimized. For the ecumicin-bound dataset, particles selected after 2D classification
were used as an input for *Ab Initio* model generation and hetero refinement using 2 classes, and each class was separately refined using a final NU 3D refinement. Maps for the Apo and cyclomarin-bound datasets as well as Class 1 of the ecumicin-bound dataset were post-processed using DeepEMhancer for model building purposes (33).

**Model building and refinement**

An initial model was generated based on the AlphaFold2 (34) prediction for monomeric *Mycobacterium tuberculosis* ClpC1 ([https://alphafold.ebi.ac.uk/entry/P9WPC8](https://alphafold.ebi.ac.uk/entry/P9WPC8)). First, parts with low per-residue confidence score (pLDDT) were removed from the AlphaFold2 ClpC1 monomer model. Next, six copies of the trimmed ClpC1 model were manually placed in the DeepEMhancer sharpened Apo ClpC1 map using rigid-body fitting in USCF Chimera (35), followed by automatic molecular dynamics based flexible fitting using NAMDINATOR (36) and subsequent manual building in Coot (37). The resulting structure was further refined in the cryoSPARC sharpened Apo ClpC1 map in Phenix, using global minimization, local grid search, ADP refinement, secondary structure and Ramachandran restraints, non-crystallographic symmetry (NCS) constraints, and using a non-bonded weight parameter of 300.

The apo structure of *M. Tuberculosis* ClpC1 was subsequently placed in the DeepEMhancer sharpened ClpC1 + cyclomarin and ClpC1 + ecumicin, Class 1 maps using rigid-body fitting in USCF Chimera, followed by manual building in Coot. Next, several cycles of refinement using the cryoSPARC sharpened maps were performed in Phenix, using global minimization, local grid search, ADP refinement, secondary structure and Ramachandran restraints, non-crystallographic symmetry (NCS) constraints, and using a non-bonded weight parameter of 300.

For ClpC1 + ecumicin Class 2, the ClpC1 + ecumicin Class 1 structure was rigid-body fitted in the map using USCF Chimera, together with three copies of an available structure of the *M. tuberculosis* ClpC1 NTD in complex with Ecumicin PDB-ID: 6PBS, (15), aligned with the *E. coli* ClpB NTD trimer found in PDB-ID 6OG3 (26).
Data Availability:
Atomic coordinates and associated structure factors have been deposited in the PDBs 8AUA, 8AUV and 8AUW.

** Acknowledgments

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References:


**Figure 1 ClpC1 resting state - hexamer equilibrium**

A) Model of the *Mtb*ClpC1 resting state created by Swiss-model using the *S. aureus* ClpC resting state as a template (PDB: 6EM9) and of the *Mtb*ClpC1 theoretical active hexameric state using the crystal structure of *B. subtilis* ClpC (PDB 3PXI). The protomers P1-P6 are shown in rainbow colours. P1 pink, P2 dark blue, P3 light blue, P4 green, P5 yellow and P6 dark red. Residue F444 is marked in light red. B) Extract of the pairwise sequence alignment of *Mtb*ClpC1 and *S. aureus* ClpC. The conserved F444 residue is marked in red. C) Size exclusion profile of wild type *Mtb*ClpC1 (black) and the two mutants *Mtb*ClpC1 F444A (blue) and *Mtb*ClpC1 F444A E288A E626A (red) in the absence and presence of ATP. D) Negative stain EM images of the hexameric ClpC1 F444A mutant.

**Figure 2 Effect of NPAs on ClpC1 activity**

A) ATPase activity of WT ClpC1 and the F444A mutant in the apo state and bound to natural product antibiotics. Degradation of FITC casein (B) and GFPssra (C) by the ClpC1P1P2 complex of apo ClpC1 and the F444A mutant in the presence of natural product antibiotics. D) Cyclomarin binding prevents the activation of ClpC1 ATPase activity promoted by Ecumicin binding.

**Figure 3 The *Mtb*ClpC1 active hexameric structure**

A) cryo-EM map of the apo *Mtb*ClpC1 hexamer bound to a substrate peptide in top, side view and with the bound substrate visible (without the protomers P1 and P6). Individual protomers are coloured independently and labelled P1-P6. B) Occupation of the nucleotide binding pockets in the *Mtb*ClpC1 hexamer with ADP (in the D1 domain orange, in the D2 domain red). From left to right hexamer in top view (substrate entry pore), side view and bottom view (interface with ClpP1P2). C) Cartoon image of the nucleotide occupation in the D1 and D2 domains and the attachment of the pore loops in D1 and D2 to the substrate. P6 is detached from the substrate in both D1 and D2. D) Top view of the ClpC1 bound substrate with poor loops attached in the typical spiral arrangement and the pore loop of P6 detached.

**Figure 4 Structural basis of the Ecumicin mechanism of action**

A) Class 1 (rainbow) and 2 (white) maps of Ecumicin bound *Mtb*ClpC1 with the percentage of particles found in each state. B) Class 2 Ecumicin bound ClpC1 map with the fitted model of the *Mtb*ClpC1 NTD (created by alpha fold) and the hexameric high resolution structure of Ecumicin bound *Mtb*ClpC1 (rainbow cartoon). C) Model of the Ecumicin bound hexameric
ClpC1 and three visible NTD domains in top, side and substrate visible view (without P1 and P6). Protomers P1-P6 are coloured individually, because of the low resolution map and the invisibility of the linker region, NTDs cannot be assigned to specific protomers and are therefore coloured in greyscale.
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Table 1 Cryo-EM data collection, processing, refinement and validation statistics
CRediT author statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: