The antibiotic cyclomarin blocks arginine-phosphate-induced millisecond dynamics in the N-terminal domain of ClpC1 from *Mycobacterium tuberculosis*

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*Mycobacterium tuberculosis* can remain dormant in the host, an ability that explains the failure of many current tuberculosis treatments. Recently, the natural products cyclomarin, ecumycin, and lassomycin have been shown to efficiently kill *Mycobacterium tuberculosis* persisters. Their target is the N-terminal domain of the hexameric AAA+ ATPase ClpC1, which recognizes, unfolds, and translocates protein substrates, such as proteins containing phosphorylated arginine residues, to the ClpP1P2 protease for degradation. Surprisingly, these antibiotics do not inhibit ClpC1 ATPase activity, and how they cause cell death is still unclear. Here, using NMR and small-angle X-ray scattering, we demonstrate that arginine-phosphate binding to the ClpC1 N-terminal domain induces millisecond dynamics. We show that these dynamics are caused by conformational changes and do not result from unfolding or oligomerization of this domain. Cyclomarin binding to this domain specifically blocks these N-terminal dynamics. On the basis of these results, we propose a mechanism of action involving cyclomarin-induced restriction of ClpC1 dynamics, which modulates the chaperone enzymatic activity leading eventually to cell death.

Tuberculosis (TB)3 is a major public health problem with 10 million people infected and 2 million dying from this disease each year (1). The main challenge in the treatment of TB is the long duration of therapy required for a cure, as the resistance of TB results from its ability to stay dormant for long periods of time in the host. Most antibiotics require bacterial replication for their action, and this dormant state renders *Mycobacterium tuberculosis* (*Mt*) resistant to bactericidal antibiotics. Aggravating this problem, *Mt* has become increasingly resistant to existing antibiotics, and multidrug-resistant TB is now widespread (1).

The proteolytic complex formed by the proteins *Mt*ClpP1 and *Mt*ClpP2 and their hexameric regulatory ATPases *Mt*ClpX and *Mt*ClpC1 is essential in mycobacteria and has emerged as an attractive target for anti-TB drug development. The Clp ATP-dependent protease complex is formed by two heptameric rings of protease subunits (*Mt*ClpP1 and *Mt*ClpP2) enclosing a central degradation chamber and a hexameric ATPase complex, *Mt*ClpC1 or *Mt*ClpX (2). The ClpC1/ClpX ATPases recognize, unfold, and translocate specific protein substrates into the *Mt*ClpP1P2 proteolytic chamber, where degradation occurs. *Mt*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 (Fig. 1a). The active form of ClpC is a homohexamer, and in *Mt*ClpC1 and *Synechococcus elongatus* ClpC ATP alone is essential and sufficient for efficient protein degradation in association with ClpP (3). However, *Bacillus subtilis* ClpC (BsClpC) requires the binding of both ATP and the adaptor protein MecA for the formation of the active hexamer. No homologous adaptor protein has been described in *Mt* (4), but it remains to be tested whether *Mt*ClpC1 can associate with a MecA-like protein.

Recently, Clausen and co-workers (5) demonstrated that BsClpC specifically recognizes proteins phosphorylated on arginine residues by the arginine kinase McsB. These phosphorylation sites are often found in secondary structure elements and thus are accessible only when the protein is unfolded or misfolded. This innovative work revealed a new pathway for selective degradation of misfolded proteins in bacteria, but the structural consequences of arginine-phosphate (ArgP) binding to ClpC are unclear. Indeed, although the crystal structure of...
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BsClpC NTD shows two ArgP molecules bound to the protein, no significant structural changes were observed, which is quite surprising because arginine-phosphorylated substrates (e.g., casein) can stimulate BsClpC ATPase activity and promote complex oligomerization (5).

The potential importance of MtbClpC1 as a novel drug target against TB has been emphasized by the recent findings by two independent groups that pyrazinamide (PZA)-resistant strains contain mutations (Fig. 1b) in ClpC1 (6, 7). PZA is a critical first-line TB drug used with isoniazid, ethambutol, and rifampicin for the treatment of TB and is also frequently used to treat multidrug-resistant TB (1). In addition to PZA, three natural product antibiotics that specifically target MtbClpC1 have been discovered recently: cyclomarin (8, 9), ecumicin (10), and lassomycin (11). Cyclomarin, identified and since synthesized by independent groups, is bactericidal against TB and able to kill nonreplicating bacteria (9). Despite the absence of resistance mutations, ClpC1 NTD was identified as the drug target using affinity chromatography with cyclomarin conjugated to Sepharose (9). Although the crystal structure of the NTD was identical with or without cyclomarin bound, observations in Mycobacterium smegmatis suggest that cyclomarin can increase proteolysis by the ClpC1P1P2 machine (8, 9). How cyclomarin binding to the NTD may lead to increased proteolysis is still not known. Ecumicin is another potent natural antibiotic that efficiently kills Mtb persisters, and resistance mutations in ClpC fall within its NTD (Fig. 1b). When tested in vitro, ecumicin increases ClpC1 ATPase activity severalfold while simultaneously compromising the degradation of ClpC1P1P2 substrates (10, 12). Lassomycin, an actinomycetes ribosomally encoded cyclic peptide, is yet another natural antibiotic able to kill Mtb persisters with efficiency (11). Despite differing structurally from ecumicin, lassomycin also activates ATP hydrolysis by ClpC1 ATPase, and resistant mutants map to a basic domain in the protein’s NTD (11).

Because of their high molecular weights and structural complexity, these natural products are challenging for structure–activity relationship studies, but compounds with similar modes of actions may be very attractive as drug candidates. Comprehending the mechanism of action of these compounds will provide valuable insights for the development of more effective TB drugs. Unfortunately, the intrinsic flexibility and exchange dynamics between different oligomeric states of ClpC1 have thus far impeded structural studies. For example, crystallization of BsClpC was only possible upon the removal of flexible loop regions, rendering the protein nonfunctional and, at the same time, underlining the importance of dynamics for the function of these complexes (13). For this purpose, nuclear magnetic resonance (NMR) and small angle X–ray scattering (SAXS) offer important advantages in investigating protein conformation in solution and testing the effects of ligands.

Here we have studied the interaction of these potent new antibiotics with ClpC1 by using state-of-the-art NMR and SAXS to elucidate their mode of action. A proper comprehension of the ways these drugs influence ClpC1 mechanism may also clarify how the family of AAA+ ATPases functions upon substrate binding.

Results

Drug binding to ClpC1 NTD

It is now widely accepted that the conformational heterogeneity of proteins can be an important factor in ligand binding and drug mechanisms of action (14). Indeed, in the case of cyclomarin, no significant changes in the ClpC1 NTD X-ray structure were observed upon ligand binding. Therefore, it was proposed that hidden unexplored conformations could be the basis for the compound’s specific actions (8). Although the linking of conformational heterogeneity or dynamics to protein function is a difficult task, NMR is a powerful method for elucidating such phenomena.

Full-length MtbClpC1 contains 849 residues that form a functional hexamer of 561 kDa in the presence of ATP. The large size of this complex, together with its low solubility and expression, prohibits NMR studies of the full-length protein even when perdeuterated and specifically methyl-labeled samples are used. To circumvent this problem, we cloned and expressed separately the MtbClpC1 domains NTD (1–145 aa), D1 (165–493 aa), D2 (494–849 aa), NTD-D1 (1–493 aa), and D1-D2 (165–849 aa). With the exception of the NTD, NTD-D1, and D2 constructs, all of the others yielded insoluble proteins when expressed. Moreover, the D2 domain when purified did not show any detectable ATPase activity and did not form oligomers in the presence of ATP, whereas the NTD-D1 construct was soluble when expressed in ArticExpress cells at 4 °C but precipitated when ATP was added.

Mutations in the NTD of MtbClpC1 have been shown to confer resistance to cyclomarin, ecumicin, lassomycin, and PZA, indicating a pivotal role for this domain (Fig. 1b). This 16-kDa domain is easily accessible for solution-state NMR even without perdeuteration. Therefore, we focused our work on MtbClpC1 NTD and tested the effects of the different antibiotics on its structure in solution. MtbClpC1 NTD behaved as a homogeneous monomeric protein upon size-exclusion chromatography (Fig. S1a) and dynamic light scattering (DLS) (Fig. S1a), resulting in high-quality NMR spectra with excellent peak dispersion, indication of a well-folded, globular protein (Fig. 2a). Two sets of assignment experiments were done. At pH 7.5, we were unable to assign loop regions because of amide exchange, but at pH 6.0 we were able to assign 95% of all residues, allowing the mapping of almost the entire protein structure. ClpC1 NTD consists of eight helices that fold as two repeats of a four-helix motif sharing 58% identity (Fig. 2b). A 14–amino acid loop between helices 4 and 5 connects the two motifs (8). Analysis of the backbone 1H, 13C, and 15N chemical shifts with TALOS+ (15) showed that the predicted secondary structure elements in the NTD in solution are highly similar to the elements in the crystal structure (Fig. 2b).

We proceeded by testing the effect of cyclomarin binding on MtbClpC1 NTD spectra. When cyclomarin was added to the MtbClpC1 NTD, substantial changes in the spectrum were observed (Fig. 2a). Similar changes were observed with the analogue desoxycyclomarin (Fig. S1b) (16). In fact, given the amplitude of chemical shift perturbations (CSP), a new set of assignment experiments was required to identify the
shifted residues. The large magnitude of the CSP (Fig. 2c) observed is likely a consequence of the rich content of aromatic residues in the cyclomarin molecule and the corresponding ring-current effect. As the chemical shift is very sensitive to a change in the local chemical environment, we were able to map the compound binding site to the region between helices 1 and 5 (Fig. 2d) in agreement with the X-ray structure of this domain in the presence of bound cyclomarin (8). Analysis of the chemical shifts in backbone amides CO, Cα, and Cβ with TALOS+ (15) showed that the predicted secondary structure elements in the cyclomarin-bound NTD in solution are highly similar to the elements in the crystal structure (Fig. S1d) excluding major changes in the domain secondary structure.

Based on the location of the resistance mutations (Fig. 1b), ecumicin was proposed to target the *Mtb*ClpC1 NTD as well (10). For this reason, we tested whether we could observe similar effects for ecumicin as we had for cyclomarin using NMR. Compared with cyclomarin, ecumicin caused only modest spectral changes (Fig. S2). These small perturbations did not indicate a strong binding of ecumicin to the *Mtb*ClpC1 NTD and were inconsistent with previous biochemical data, where ecumicin was found to be a potent inhibitor of *Mtb*ClpC1 degradation of casein but a stimulator of *Mtb*ClpC1 ATPase activity (result confirmed here (Fig. S2d)) (10). Consequently, we tested ecumicin binding to the NTD using isothermal titration calorimetry (ITC). Although we were able to confirm the nanomolar $K_d$ for cyclomarin (8) (Fig. S3), we were unable to obtain a saturation curve with ecumicin under the conditions used (up to 100 μM ecumicin (Fig. S3)). In contrast to cyclomarin and ecumicin, the addi-
Figure 2. NMR assignment and cyclomarin-binding site of MtbcClpC1 NTD. a, $^{1}H-^{15}N$ correlated backbone amide spectrum of apo- (black) and cyclomarin-bound (blue) MtbcClpC1 NTD. 95% of amide resonances of the apo-bound and 79% of the cyclomarin-bound protein spectrum have been assigned. b, TALOS+ predicted helix propensity derived from NMR assignments of apo-MtbcClpC1 NTD. In dark blue predicted helix, in white predicted loop and as a gray background helical parts in the X-ray structure of MtbcClpC1 NTD (PDB code: 3wdb). The secondary structure in solution and in the crystal seem to be identical. c, combined chemical shift difference between apo- and cyclomarin-bound MtbcClpC1 NTD $^{1}H-^{15}N$ HSQC spectra. Chemical shift differences are mapped on the structure shown in d. d, chemical shift differences from c plotted on the structure of cyclomarin-bound MtbcClpC1 NTD (PDB code: 3wdc). The assigned backbone amides are shown as spheres and unassigned residues as gray cartoons. Chemical shift differences are plotted in a spectrum from blue to white, where blue indicates a strong effect. Cyclomarin is shown as yellow sticks.
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The addition of ArgP to MtbClpC1 NTD resulted in strong perturbations of its backbone amide NMR spectrum (Fig. 3d). This effect was specific to ArgP because no significant changes were observed with unmodified arginine or phosphoserine (Fig. S4). In contrast to the rather small changes in peak intensity observed upon cyclomarin or ecumicin addition, ArgP caused significant changes in peak intensity, with 45% of the peaks falling below the level of detection into the background noise (Fig. 3e). As shown below, this decrease in intensity may indicate exchange events at the affected residues. Interestingly, these residues do not map exclusively to the putative binding site of ArgP but are localized over a large part of the core of the structure, whereby helices 2, 3, 6, and 7 are most affected (Fig. 3, c and j). Using CPMG RD experiments, we were able to prove that the observed decrease in intensity is indeed caused by millisecond dynamics (Fig. 4). Surprisingly, these dynamics can be seen throughout the domain. In Fig. 4a the affected residues and the $\Delta R_{2,eff}$ are plotted on the structure of MtbClpC1 NTD. Furthermore, we assumed that the part of the protein showing the highest degree of motion were the three helices with disappearing resonances (refer to Fig. 3f).

Although CPMG RD experiments provide residue-resolved direct evidence of the existence of dynamics, they do not actually reveal what the underlying motion corresponds to. In the case of low-affinity binding, ms dynamics also can be caused by the on/off binding dynamics of the compound. Considering the ArgP $K_D$, we could calculate the population of free NTD with the concentrations of ArgP used (2 mM), resulting in full-domain saturation (calculated free NTD, 0.3%). Fitting of the CPMG RD data with the software Chemex provided an exchange rate between the ground and excited states, the population of the excited state, and the chemical shift difference between the two states. The fitted population of the excited state (5%, Fig. S5d) is not in agreement with the calculated free state of the NTD (0.3%). Moreover, the theoretical chemical shift differences of the excited state, derived from fitting of the CPMG RD data with Chemex, do not correlate with experimental chemical shift differences between apo- and ArgP-bound NTD (Fig. S5a). In conclusion, we confirmed that the excited state does not correspond to the free state of the NTD but most likely to a different conformation.

Arginine has been reported previously to reduce the melting point of some proteins but paradoxically also has been suggested as a stabilizer for protein preparations (19–22). To discard the possibility that the dynamics observed result from ArgP-promoted unfolding, we recorded the far-UV circular dichroism (CD) spectra of MtbClpC1 NTD with and without ArgP present. In both cases, the spectra were typical for $\alpha$-helical proteins with characteristic minima at $\approx 210$ and 222 nm (Fig. 5a). Additionally, we used chemical shift differences from the Chemex fitting of the CPMG RD data and compared these values with the chemical shift differences of the folded NTD and calculated random coil shifts derived from Neighbor Corrected IDP Library (nIDP) (23) (Fig. S5b). Clearly, the fitted chemical shift differences do not correlate with the calculated random coil values. Thus, ArgP does not appear to unfold the NTD or to perturb the secondary structure of the domain. Alternatively, ArgP may induce domain oligomerization,
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(a) ArgP binding site • identical • similar • not conserved

(b) Time (min)

(c) Cln6 NTD

Lysozyme

(d) apo Clp1 NTD • + ArgP

(e) + cyclomarin • + cyclomarin/ArgP

(f) ArgP • disappear upon ArgP binding • not assigned
Figure 3. The effect of ArgP binding on \textit{Mtb} ClpC1 NTD. \(a\), sequence alignment of \textit{Mtb} ClpC1 NTD and \textit{Bs} ClpC NTD. Identical residues are highlighted in black and similar residues in gray. The binding site of ArgP in \textit{Bs} ClpC NTD is circled in red. \(b\), representative ITC of ArgP binding to \textit{Mtb} ClpC1 NTD (\(n/H11006\) 1.99/0.02; \(K_d/HH11006\) 5.2/0.5 \(\mu\)M; \(\Delta H/HH11006\) -4066 \(\pm/HH11006\) 59 cal/mol; \(\Delta S/HH11006\) 10.3 cal/mol/deg). \(c\), NTD pulls down lysozyme phosphorylated by McsB kinase but not untreated lysozyme (lanes 1 and 4). Cyclomarin (50 \(\mu\)M) is unable to block substrate binding, but a reduction is observed with ArgP (1 mM). \(d\), \(^{1}H/^{15}N\) correlated backbone amide spectrum of apo- (black), ArgP- (green), cyclomarin- (blue), and ArgP/cyclomarin (red)-bound \textit{Mtb} ClpC1 NTD. 95\% of the apo-, 55\% of the ArgP-, 85\% of the cyclomarin-, and 75\% of the CymA/ArgP-bound \textit{Mtb} ClpC1 NTD amide resonances are NMR-visible. \(e\), loss in peak intensity of resonances in \(^{1}H/^{15}N\) HSQC spectra upon ArgP binding with (red) or without (green) cyclomarin added. \(f\), peak height ratio of ArgP-bound \textit{Mtb} ClpC1 NTD (\(d/HH11006/HH4\), left) plotted on its structure (PDB code: 3wdb). Assigned residues are shown as spheres, unassigned residues as gray cartoons, and residues that disappear upon ArgP binding as white cartoons. Two arginine-phosphate molecules (red sticks) are placed at the putative ArgP-binding site, identical to the X-ray structure of \textit{Bs} ClpC NTD (PDB code: 5hbn).

Figure 4. ArgP induces millisecond dynamics in \textit{Mtb} ClpC1 NTD. \(a\), residues that exhibit millisecond dynamics plotted on the \textit{Mtb} ClpC1 NTD structure (PDB code: 3wdb). All assigned residues are shown as spheres. Residues that have a \(\Delta R_{2,eff}/HH11006\) of 5 are in yellow, of 15 orange, and of 30 or more red. \(b\), examples of CPMG curves for residues with a \(\Delta R_{2,eff}/HH11006\) of 5, 15, or 30. Apo\textit{Mtb}ClpC1 NTD has no ms dynamics (bottom row), and 63\% of all NMR-visible residues experience ms dynamics when ArgP is bound (top row); if cyclomarin is added before ArgP, no further ms dynamics can be observed, resulting in flat dispersion curves (middle row).
which would explain peaks disappearing by transient interactions between two ClpC1 subunits. By analyzing the protein by DLS (Fig. S1) and diffusion-ordered spectroscopy (DOSY, Fig. S5e), however, we excluded this hypothesis. Neither ArgP nor cyclomarin induced any change in \( \text{Mtb} \text{ClpC1 NTD} \) oligomerization.

Intrinsic aromatic fluorescence can be used to probe conformational changes upon ligand binding. Although \( \text{Mtb} \text{ClpC1 NTD} \) does not contain any tryptophan residues, it contains three tyrosine residues (Tyr-27, Tyr-102, and Tyr-145), which can be used as probes for domain conformational changes (Fig. S5f). When we tested the effect of ArgP on NTD fluorescence, we observed an increase in tyrosine fluorescence (Fig. S5g) associated with stabilization of the protein. NTD displayed cooperative unfolding with an apparent \( T_m \) of 69 °C; this value was increased to 79 °C in the presence of 1 mM ArgP (Fig. 5b).

An alternative approach to probing conformational changes is the use of fluorescent probes. 1-Anilinonaphthalene-8-sulfonic acid (ANS) binds to hydrophobic regions in the protein, and ANS fluorescence increases substantially when proteins undergo changes that expose hydrophobic surfaces as normally occurs during protein unfolding. ANS can, however, be used to detect subtle conformational changes, and we tested whether ArgP binding leads to changes in ANS fluorescence. Although ANS binding to \( \text{Mtb} \text{ClpC1 NTD} \) resulted in an increase in fluorescence, indicating the presence of exposed hydrophobic surface, ArgP binding did not significantly alter fluorescence (Fig. 5c). Because cyclomarin has intense intrinsic fluorescence, it could not be used in the previous fluorescence studies.

**Cyclomarin restricts ArgP-induced dynamics**

The fact that cyclomarin binding did not affect \( \text{Mtb} \text{ClpC1 NTD} \) dynamics is inconsistent with the prior proposal that cyclomarin acts by causing conformational changes in this domain (8). By contrast, ArgP leads to a significant increase in domain dynamics. We therefore tested whether cyclomarin binding could block either ArgP recognition or ArgP-induced dynamics. By adding ArgP to \( \text{Mtb} \text{ClpC1 NTD} \) prebound to cyclomarin, we observed significant changes in the \( ^1\text{H} - ^15\text{N} \)-correlated HSQC spectrum in the binding site of ArgP (Fig. S6).

This is consistent with our pulldown results, where no effect of
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Cyclomarin on arginine-phosphorylated proteins binding to the NTD was observed (Fig. 3c). However, the behaviors of ArgP-bound and ArgP/cyclomarin-bound NTD were clearly different. Several peaks that disappeared upon ArgP binding reappeared when cyclomarin was added. Instead of 45%, only 25% of all peaks disappeared when cyclomarin was added together with ArgP. Related to the spectral changes, the most striking difference between ArgP and ArgP/cyclomarin binding are the dynamic properties of the NTD. When ArgP is bound to the NTD, most observed residues exhibit μs–ms dynamics. These dynamics can however be completely abolished by the addition of cyclomarin. In ArgP/cyclomarin-bound NTD, not a single dynamic property of the NTD. When ArgP is bound to the NTD, most observed residues exhibit μs–ms dynamics. These dynamics can however be completely abolished by the addition of cyclomarin. In ArgP/cyclomarin-bound NTD, not a single residue showed μs–ms dynamics, exactly as occurs in apo MtbClpC1 NTD (Fig. 4). Cyclomarin binds to a hydrophobic “bed” formed by two phenylalanines in the symmetric axis of the domain (8), which by stabilizing the core of the domain presumably explains how it can completely block NTD domain dynamics.

**Cyclomarin prevents ArgP inhibition of FITC–casein degradation**

The primary function of ClpC1 is to recognize certain cellular proteins and to unfold and translocate them into ClpP1P2 for degradation, although it is also able, at least in vitro, to catalyze the refolding of some proteins (2, 24). Until the discovery of ArgP in BsClpC, no recognition signal for ClpC was known. In the presence of the cofactor Meca, BsClpC catalyzes the degradation of unfolded proteins such as casein. However, ArgP (1 mM) can block Meca-dependent proteolysis, apparently because the binding site of ArgP overlaps with the contact site of Meca and the BsClpC NTD (5). This observation clearly suggests that ClpC has two alternative mechanisms for substrate selection: one for unfolded proteins containing no specific tag and dependent on the Meca adapter for efficient ClpC1 oligomerization and activation; and a second Meca-independent pathway that depends specifically on the presence of protein sequences containing phosphorylated arginines (5, 25). In contrast to BsClpC, MtbClpC1 does not require any cofactor for activity. In the presence of ATP it can, in association with MtbClpP1P2, efficiently degrade unfolded proteins such as casein (2).

Despite these differences, the mechanism of these homologous enzymes seems to be conserved between species. Thus, studying the effects of cyclomarin and ArgP on protein degradation by MtbClpC1P1P2 could yield important mechanistic insights.

One interesting question that arises is whether ArgP binding can block casein degradation as it does in BsClpC. We therefore compared the degradation of FITC–casein in the presence and absence of ArgP. ArgP caused a significant inhibition (up to 55% (Fig. 5d)) of FITC–casein degradation, but it did not completely block this process even at very high ArgP concentrations. By contrast, in the presence of cyclomarin (20 μM), no inhibition of proteolysis was observed (Fig. 5d).

**ClpC1 forms high oligomeric species in solution**

We used SAXS as a complementary method to obtain information on the effect of drug binding on ClpC1 structure. Compared with X-ray diffraction, SAXS has a modest resolution but can provide information on several global parameters: the radius of gyration; the largest intraparticle distance; the particle shape; and the degree of folding, denaturation, or disorder (26). All of these parameters can be good reporters for significant structural changes promoted by drug binding. In addition, SAXS does not require the preparation of highly concentrated deuterated samples, allowing the study of ClpC1 structure in native-like solution conditions.

Our first SAXS measurements in batch format in the presence of ATP revealed the presence of very high molecular weight species incompatible with a ClpC1 hexamer. As MtbClpC1 is inherently prone to aggregation, which is known to seriously affect SAXS data interpretation, we concluded that part of the MtbClpC1 sample could be aggregated. To overcome this problem, we turned to size-exclusion chromatography coupled to SAXS (SEC-SAXS). In this system, the sample is separated according to size and shape before SAXS measurement, thus removing protein aggregates from the protein samples. Indeed, consistent with the aggregation hypothesis, when ClpC1 was loaded on a Superose 6 10/300 GL column, the chromatograms of ClpC1 showed two distinct peaks: a small peak directly after the void volume followed by a second broad peak (Fig. 6a). The scattering signal at the second peak was relatively stable, with a radius of gyration in the range of 8 nm (Table S1) but decreasing significantly to 7.6 nm at the end of the peak, indicating either structural flexibility or overlapping oligomeric states. Surprisingly, this radius of gyration was again clearly inconsistent with a ClpC1 hexamer (radius of gyration, 5.53 nm), instead demonstrating bigger complexes. Apparently, under the conditions used (50 mM Hepes, pH 7.5, 100 mM KCl, 10% glycerol, 4 mM MgCl2, 1 mM ATP, and 1 mg/ml ClpC), the hexamer is not the dominant species, and ClpC1 appears to exist in a rather distinct molecular organization.

Recently, Carroni et al. (27), using cryo-electron microscopy (cryo-EM) and mutagenesis, showed that Staphylococcus aureus ClpC (SaClpC) exists in a decameric resting state formed through ClpC middle domains establishing intermolecular head-to-head contacts. This head-to-head contact allows the docking of two layers of ClpC molecules arranged in a helical conformation. Despite their oligomerization, these structures are, however, highly dynamic, as the peripheral subunits are likely in exchange, suggesting that higher order species can exist in equilibrium.

As the middle domain of MtbClpC1 is conserved compared with SaClpC, and to better understand how our SAXS data might relate to the resting-state cryo-EM structure, we averaged frames from the center of the peak and the trailing end. SAXS estimates the molecular mass at the center of the peak between 1100 and 1400 kDa, compatible with 12- or 14-mers (Table S1). For the tail, the estimated mass between 860 and 1200 kDa is in better agreement with 10- or 12-mers. Bead modeling based on both curves resulted in curling stone–shaped objects, whose main body matches the reported EM data in size and shape (Fig. 6b). An artifact resulting from the presence of several oligomeric populations in the sample was observed in the form of an appendix. A direct comparison of the
SAXS curve from the tail with the atomistic model gives a surprisingly good fit (χ² = 3.4 (Fig. 6c)), given that the SAXS curve represents an ensemble of states. Considering the similarities between our data and the previously reported cryo-EM structure, it is likely that *Mtb* ClpC1 forms a resting state, with a large part of the population representing even higher oligomers than decamers. This difference could derive from a concentration-dependent oligomeric equilibrium, which would explain why *Mtb* ClpC1 seems larger in our study as compared with the SEC-MALS data presented by Carroni et al. (27). In fact, cross-linking data from the same study already suggests the presence of complexes bigger than the EM decameric structure (27). Although we could not detect the hexamer in solution, the fact that under the same conditions ClpC1 is active, catalyzing the degradation of GFP-ssrA and casein in association with ClpP1P2, suggests that a part of the population exists as a hexamer.

We proceeded to test for whether the natural product antibiotics or ArgP targeting of ClpC1 could affect the distribution of ClpC1 between a resting state and an active hexameric form. The addition of ecumicin (20 μM), cyclomarin (20 μM), and ArgP (200 μM) to the SEC buffer led only to small changes in the averaged SAXS curve (Fig. S8 and Table S1). As the curve was not stable, the differences were too small to allow any statement about local structural rearrangement. It appears however that the natural antibiotics tested do not affect the ClpC1 oligomer equilibrium significantly.
Discussion

The structural characterization of AAA+ proteins involved in molecular recognition and unfolding is usually a complex task. Whereas their intrinsic heterogeneity normally results in difficult crystallization, their oligomeric organization and large size makes their study by NMR challenging. So far, no X-ray structure is available for MtbcClpC1, and as reported here, the full-length protein and its domains have intrinsically low solubility. Adding further complexity to the study of the system, we show here that MtbcClpC1 can exist in an equilibrium between different oligomeric states. The existence of a resting decameric state formed by the association of head-to-head contacts between coiled-coil middle domains of ClpC was recently described by Carroni et al. (27). The middle domains were proposed to repress the activity of ClpC by forming a highly dynamic resting state that blocks substrate binding or ClpP interaction. Quite striking is the observation that a single point mutation in the middle domain can disrupt the resting state and result in the formation of an active hexamer even in the absence of the MecA adaptor (27). Consistent with the conservation of the key residues between MtbcClpC1 and SaClpC (27), our SAXS data suggest that a similar structure is predominant versus the active hexameric form in MtbcClpC1. However, contrary to the case of S. aureus or B. subtilis, where MecA is proposed to modulate this equilibrium, in the case of Mtbc it is not clear how the distribution between the resting state and the hexameric form occurs. N-terminal domains are packed between middle domains in this resting state and are suggested to play a role in the complex stability by fluctuating between a hidden and an exposed position available to adaptors or substrates. One hypothesis is that the equilibrium could be modulated by substrates or natural product antibiotics. However, we were able to show that the addition of cyclomarin, ecumcin, or ArgP ligand appears not to shift ClpC1 distribution. With the current data, we cannot exclude the possibility that binding of a bulkier substrate shifts the equilibrium toward a state where the NTDs are not constrained, thus activating the unfoldase.

Whereas natural product antibiotics appear not to influence ClpC1 oligomerization equilibrium, we were unable to obtain convincing evidence for ecumcin or PZA binding to the isolated MtbcClpC1 NTD. This result is intriguing, as mutations in the NTD domain have been associated with resistance to ecumcin and PZA (10). With regard to ecumcin, we and others (10) have been able to demonstrate biochemical activity, namely the activation of ATPase. Most likely, ecumcin requires full-length ClpC1 or an oligomeric structure for binding and ATPase activation. Possibly the binding site is located at the interface of the NTD and D1 domain, which could explain ATPase activation by ecumcin. In this case, disrupting part of the binding site, the N-terminal interface in the resistant mutants, might be sufficient for reduced binding affinity in vivo. Small CSP were evident on the NTD NMR spectrum for ecumcin; in the regions where resistant mutations are located, suggesting that this region could be part of the putative binding site (Fig. S2). In the case of PZA, an efficient “dirty drug” with multiple reported cellular targets, we cannot exclude the possibility that resistance derives from the modulation of protein homeo-

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Figure 7. MtbcClpC1 exists in equilibrium between a resting state and a functional hexamer. MtbcClpC1 forms a resting state in equilibrium with the active hexameric form. The phosphorylation of arginines marks proteins for degradation by using ClpCP machinery. Phosphorylated arginines bind to the NTD, where they induce millisecond dynamics that could facilitate either contact between different NTDs or transfer of the substrate to the D1 domain pore. Although cyclomarin binding does not change the structure of the NTD or substrate binding, it restricts ArgP-induced dynamics.

stasis for any of the other targets, for example preventing or increasing substrate degradation (28).

Using NMR, we showed that although cyclomarin binds with high affinity to the ClpC1 NTD, the domain dynamics are not modified. MtbcClpC1 NTD is a rather rigid domain and showed no millisecond dynamics in the apo-state. Also, cyclomarin binding does not result in the peak broadening or loss of intensity that might indicate the presence of an alternative state. These observations rule out the existence of hidden conformations not captured by previous X-ray studies (8).

Finally, our finding that ArgP binding induces millisecond dynamics in the MtbcClpC1 NTD domain is a new and important clue about ClpC1 mechanisms, particularly when no alternative conformations were reported in the X-ray structure of BscClpC with ArgP-bound and because no structural changes were observed with arginine or phosphoryserine. Although we excluded unfolding and transient binding as potential explanations for the observed dynamics, we were unable to pursue structural determination because approximately half of the residues in the ArgP-bound NTD are NMR-invisible. ArgP binding results in a significant increase in tyrosine fluorescence and a dramatic change in the stability of this domain. Although the increased fluorescence could result from subtle changes in tyrosine side chains in a region densely packed with aromatic residues (three Phe and two Tyr residues (Fig. S5)), the increased stability could derive from ArgP binding preferentially to the folded state. The exact relationship between ArgP-induced dynamics and the functional cycle of MtbcClpC1 is currently unclear. Do the dynamics promote target binding through ArgP recognition by allowing multiple transient interaction sites with the incoming substrate? Conformational heterogeneity and dynamics in substrate-binding sites have been proposed to increase substrate recognition efficiency and, at the same time, to facilitate substrate handover to downstream elements by making a multitude of transient weak interactions with the substrate, which can be easily broken (29). In fact, a single phosphorylated arginine in a protein has been shown to be sufficient for efficient ClpC-mediated degradation. Thus
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ClpC’s molecular recognition mechanism must be highly efficient, for example 1 mM free ArgP does not completely block protein binding, which may appear inconsistent with the micromolar $K_d$ that we and others report for ArgP binding to the isolated NTD (5). Nevertheless, the fact that cyclomarin cannot block arginine-phosphorylated protein binding appears to contradict the hypothesis that dynamics are fundamental for substrate association to the NTD; this does not exclude, however, that they are relevant for subsequent steps, for example substrate release to the D1 pore (Fig. 7). Another possibility is that the observed conformational dynamics could modulate the positioning of the NTD related to the D1 pore. Indeed, studies with the type II eukaryotic homologue p97/Cdc48 ATPase complex, which shares the NTD-D1-D2 architecture with ClpC1, have stressed the mechanistic relevance of the interface between NTD-D1 and the denominated up/down equilibrium of the highly mobile NTDs (30). In p97/Cdc48, NTDs have been shown to adopt, depending on the nucleotide bound, either a coplanar (down) or elevated (up) position with respect to the D1 domain (30). Increasing the lifetime of the NTDs “up” state and thereby holding the substrate next to the D1 pore would promote substrate recognition, whereas supporting the down state would prevent substrate recognition, nevertheless exposing the D1 domain pore. In *Escherichia coli* ClpA, a close bacterial homologue of ClpC, the removal of NTD is known to seriously impair recognition of substrates bearing the ssrA-targeting sequence but to have only a modest effect on the degradation of unfolded proteins (31). In other words, NTDs may work as recognition domains for certain substrates while at the same time blocking the D1 domain and preventing free access for unfolded proteins. This differential effect on certain substrates was the basis for the suggested role for the NTDs as an “entropic brush” that prevents nonspecific degradation of proteins by blocking access to the D1 ring (32). In our view, the competition between recognition of ArgP-labeled and unfolded protein can simultaneously explain the inhibition of casein degradation by ArgP and the effect of cyclomarin, which is able to completely abolish this inhibition of casein hydrolysis.

Altogether, our work shows that ArgP binding to *Mtbc*ClpC1NTD leads to widespread domain millisecond dynamics and that cyclomarin is able to block this process. Although it is surely not the absence of ClpC1 NTD dynamics that kills TB but likely its functionally derived consequences (for example blocking the ArgP pathway), this work sheds light on the ClpC mechanism. Rather than a static interaction, the binding of ArgP-labeled proteins to ClpC must be understood as a highly dynamic process. Cyclomarin is therefore a unique example of a drug whose mode of action relies on the restriction of protein dynamics induced by substrate binding.

**Experimental procedures**

**Materials**

ArgP was obtained from Sigma. Cyclomarin and desoxycyclomarin were synthetized as described previously (33). *Mtbc*ClpC1, *Mtbc*ClpP1, and *Mtbc*ClpP2 were expressed and purified as described previously (2). *Bacillus stearothermophilus* McsB was cloned into a pet28a+ vector and expressed and purified as described previously (5). For the *Mtbc*ClpC1 domains, NTD corresponding to residues 1–145, D1 corresponding to residues 165–493, D2 corresponding to residues 494–849, NTD-D1 corresponding to 1–493, and D1-D2 corresponding to 165–849 were cloned into a pet28a+ vector by GenScript. Unless otherwise noted, the purification protocol consisted of an initial nickel–nitrilotriacetic acid affinity chromatography step, taking advantage of the histidine tag, followed by a size-exclusion step using a HiLoad 16/600 Superdex 200 pg column. FITC–casein, GFP-ssrA degradation, and ATP hydrolysis were measured as described previously (2). For DLS measurements, 200 μl of a 1.1 mg/ml ClpC1 NTD solution with and without ArgP (1 mM) was used.

Far-UV CD spectra were acquired on a Jasco J-810 spectropolarimeter continuously purged with nitrogen and thermostated at 20 °C. Briefly, a solution of ClpC1 NTD (5 μM) in Tris, pH 7.5, 150 mM NaCl with/without ArgP (1 mM) was used to obtain CD spectra between 205 and 250 nm.

Intrinsic tyrosine fluorescence was measured in a Varian Cary Eclipse spectrofluorimeter using a 60 μM solution of ClpC1 NTD. Samples were excited at 280 nm, and fluorescence spectra were measured from 290 to 350 nm. Samples in the presence of ANS (50 μM) were excited at 370 nm, and fluorescence spectra were measured from 400 to 600 nm.

**Degree of saturation of ClpC1 NTD**

The degree of saturation of ClpC1 NTD with ArgP was calculated using the equation,

$$\frac{[PL]}{[P]} = \frac{1}{2} \left( 1 + \frac{[L]_0}{[P]} + \frac{K_d}{[P]} - \sqrt{\left( 1 + \frac{[L]_0}{[P]} + \frac{K_d}{[P]} \right)^2 - 4 \frac{[L]_0}{[P]}} \right)$$

(Eq. 1)

where $[L]_0$ is initial ligand, $[P]_0$ is initial protein, and $PL$ is protein–ligand complex.

**SAXS data collection and analysis**

SEC-SAXS data were collected at ESRF BM29 (34, 35). The HPLC system (Shimadzu France) was coupled directly to the 1.8-mm flow-through capillary of SAXS exposure unit. The flow rate for all online experiments was 0.3 ml/min. The SAXS data collection was performed continuously throughout the chromatography run at a frame rate of 1 Hz with a Pilatus 1 M detector (Dectris) at the distance of 2.876 m from the capillary. The scattering of pure water was used to calibrate the intensity to absolute units (36). The X-ray energy was 12.5 keV, and the accessible $q$-range was 0.07 nm$^{-1}$ to 4.9 nm$^{-1}$. The incoming flux at the sample position was in the order of $10^{12}$ photons/s in 700 × 700 μm$^2$. A summary of the acquisition parameters is given in Table S1. All images were automatically azimuthally averaged with pyFAI (37) and corrected for background scattering by the online processing pipeline (38). For each frame, the forward scattering intensity and radius of gyration were determined according to the Guinier approximation (https://github.com/kif/freesas). For each run, regions of 20 to 80

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frames were averaged for further characterization. Data at small angles before the Guinier region were removed before further data analysis to avoid experimental artifacts.

Pair distribution functions were calculated using GNOM (39). 20 ab initio models each were calculated in C1 symmetry using DAMMIF (40) and averaged, aligned, and compared using DAMAVER (41). The scattering curve of the ClpC decamer (27) was predicted and fitted to experimental data using CRYSOL 3.0 (42).

NMR experiments

All NMR experiments were performed on Bruker Avance III spectrometers equipped with cryogenically cooled TCI probe heads and operating at magnetic field strengths corresponding to $^1$H Larmor frequencies of 850, 700, and 600, respectively. The sample temperature was set at 37 °C unless stated otherwise.

Sequence-specific resonance assignments of ClpC1 NTD

ApoClpC1 NTD was assigned in NMR buffer, pH 6 (50 mM MES, 100 mM NaCl, 5% D$_2$O) and in NMR buffer, pH 7.5 (50 mM Tris, 50 mM NaCl, 5% D$_2$O) at a protein concentration of 0.2 mM and at a $^1$H Larmor frequency of 600. The following experiments were performed: 2D $^{15}$N-$^1$H BEST HSQC, 3D BEST HNCO, 3D BEST-TROSY HNcaCO, 3D BEST HNCO, 3D BEST HNcoCA, and 3D BEST HNcoCACB (43).

The same experimental conditions were used for the assignment of ClpC1 NTD in the presence of cyclomarin, except for a lower protein concentration due to the low solubility of cyclomarin (0.2 mM ClpC1 NTD and 0.22 mM cyclomarin). DMSO controls were also measured.

For the assignment of ClpC1 NTD in the presence of ArgP and cyclomarin, $^{15}$N-$^1$H BEST HSQC, BEST HNCO, and BEST HNCA spectra were recorded at a $^1$H Larmor frequency of 850 MHz. Assignment was performed following the chemical shifts of backbone amide, C$\alpha$, and CO peaks in apo- and ligand-bound spectra. The sample conditions used were: 0.2 mM ClpC1 NTD and 2 mM ArgP or 0.2 mM ClpC1 NTD, 2 mM ArgP, and 0.22 mM cyclomarin in NMR buffer, pH 6. Data processing and analysis were performed using the NMRPipe software package (44) and CCPN software (45).

Titration of cyclomarin into MtbClpC1 NTD

For the cyclomarin titration, four titration points were measured with 0.2 mM $Mtb$ClpC1 NTD each. The DMSO content in all samples was 2%. The measured ratios between the NTD and ecumicin were 1:0, 1:1, and 1:2. Because of the insolubility of ecumicin, no higher concentrations of ecumicin could be measured.

Titration of ArgP into MtbClpC1 NTD

Five titration points were measured with 0.3 mM $Mtb$ClpC1 NTD each. The DMSO content in all samples was 0.6%. The measured ratios between the NTD and ArgP were 1:0, 1:0.5, 1:1, 1:2, and 1:10. No intensity changes were observed after a ratio 1:2 $Mtb$ClpC1 NTD:ArgP. The ratio used for the experiments was 1:1.1.

Titration of cyclomarin and ArgP into MtbClpC1 NTD

Four different samples were measured for the titration of $Mtb$ClpC1 NTD with cyclomarin and ArgP. All samples contained 4.2% DMSO and 0.2 mM $Mtb$ClpC1 NTD. We measured one reference sample, one sample with 0.22 mM cyclomarin added, one sample with 0.22 mM cyclomarin and 0.2 mM ArgP, and one sample containing 0.22 mM cyclomarin and 2 mM ArgP.

Titration of ecumicin into MtbClpC1 NTD

Three titration points were measured with 0.2 mM $Mtb$ClpC1 NTD each. The DMSO content in all samples was 2%. The measured ratios between the NTD and ArgP were 1:0, 1:1, and 1:10. No intensity or chemical shift changes were observed at any measured pyrazinamide concentration.

Three titration points were measured with 0.2 mM $Mtb$ClpC1 NTD each. The DMSO content in all samples was 2%. The measured ratios between the NTD and ecumicin were 1:0, 1:1, and 1:2.
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