the FEBS Journal

Mycobacterium tuberculosis ClpC1

Characterization and role of the N-terminal domain in its function

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Keywords
chaperone; heat shock proteins; HSP100; protein aggregation; protein refolding

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(Received 31 July 2008, revised 7 October 2008, accepted 10 October 2008)

doi:10.1111/j.1742-4658.2008.06738.x

Caseinolytic protein, ClpC is a general stress protein which belongs to the heat shock protein HSP100 family of molecular chaperones. Some of the Clp group proteins have been identified as having a role in the pathogenesis of many bacteria. The *Mycobacterium tuberculosis* genome demonstrates the presence of a ClpC homolog, ClpC1. *M. tuberculosis* ClpC1 is an 848-amino acid protein, has two repeat sequences at its N-terminus and contains all the determinants to be classified as a member of the HSP100 family. In this study, we overexpressed, purified and functionally characterized *M. tuberculosis* ClpC1. Recombinant *M. tuberculosis* ClpC1 showed an inherent ATPase activity, and prevented protein aggregation. Furthermore, to investigate the contribution made by the N-terminal repeats of ClpC1 to its functional activity, two deletion variants, ClpC1Δ1 and ClpC1Δ2, lacking N-terminal repeat I and N-terminal repeat I along with the linker between N-terminal repeats I and II, respectively were generated. Neither deletion affected the ATPase activity. However, ClpC1Δ1 was structurally altered, less stable and was unable to prevent protein aggregation. Compared with wild-type protein, ClpC1Δ2 was more active in preventing protein aggregation and displayed higher ATPase activity at high pH values and temperatures. The study demonstrates that *M. tuberculosis* ClpC1 manifests chaperone activity in the absence of any adaptor protein and only one of the two N-terminal repeats is sufficient for the chaperone activity. Also, an exposed repeat II makes the protein more stable and functionally more active.

Chaperone proteins are vital proteins required by many bacteria during normal growth and also under conditions of severe stress to maintain cell viability. Chaperone proteins assist in the proper refolding of proteins or the assembly of proteases that process proteins that cannot be altered conformationally [1,2]. Heat shock proteins act as chaperones and interact with hydrophobic residues exposed in unfolded polypeptides to facilitate their correct folding, prevent protein aggregation and translocate them across cell membranes [3]. Increased expression of heat shock proteins is triggered by a range of stress conditions, and is also induced in both the host and pathogen during the process of infection [4].

Heat shock protein, HSP100 or caseinolytic protein (Clp) is a highly conserved family of molecular chaperones, and members of this family have been shown to exist in a variety of organisms from *Escherichia coli* to humans [5–11]. Clp family members possess ATPase activity and have been grouped as Class I or II based on the presence of two or one highly conserved nucleotide-binding regions [12]. Class I proteins, ClpA–E and L, all have two distinct nucleotide-binding domains (NBDs) or AAA+ modules, whereas Class II proteins,

Abbreviations
Clp, caseinolytic protein; NBD, nucleotide-binding domain.

ClpX and Y, have only a single AAA+ module [12]. ClpA, X and C associate with the oligomeric peptidase, ClpP to form an ATP-dependent protease [6,13,14]. HSP100/Clp family members have a protein-unfolding activity dependent on ATP hydrolysis, and translocate folded and assembled complexes, as well as improperly folded and aggregated proteins for degradation by ClpP [15]. They also disaggregate and refold aggregated proteins [16]. ClpC, a Class I protein is found in a diverse range of organisms including photosynthetic cyanobacteria, the chloroplasts of algae and higher plants and most Gram-positive eubacteria [5,7,9,17,18]. ClpC proteins are the most highly conserved subgroups within the Clp family, although little is known about their specific functions. ClpC consists of two AAA+ domains, the first of which contains an additional N-domain, homologous to the N-domains of ClpA or ClpB, and a linker domain homologous to, but half the size of, the linker domain of ClpB [19]. The N-terminal region contains two 32-amino acid repeats I and II, which are almost identical across all species [17]. The linker domain consists of a coiled-coil structure, which is inserted into the smaller C-terminal sub-domain, D1 of NBD1 [20].

Some Clp proteins, which act as both chaperones and proteolytic enzymes, have been identified as having a role in the pathogenesis of *Yersinia* and *Salmonella typhimurium* [21–23]. Clps have been linked to the tight regulation of virulence genes, and cell adhesion and invasion in the pathogen *Listeria monocytogenes* [24–26]. It has recently been demonstrated that partial disruption of heat-shock regulation in *Mycobacterium tuberculosis* has an important impact on virulence, as it impairs the ability of the bacteria to establish a chronic infection [27].

The *M. tuberculosis* genome has revealed the presence of heat shock proteins ClpP1, ClpP2, ClpC1, ClpX and ClpC2, annotated at the Pasteur Institute TubercuList server (http://genolist.pasteur.fr/TubercuList/) as Rv2461c, Rv2460c, Rv3596c, Rv2457c and Rv2667 respectively. These proteins may be important in the pathogenesis of *M. tuberculosis*. In this study, we cloned, expressed and characterized a general stress protein ClpC1, Rv3596c, of *M. tuberculosis*. *M. tuberculosis* ClpC1 has an inherent ATPase activity and also functions like a chaperone *in vitro*. Furthermore, we investigated the role of the N-terminal domain of *M. tuberculosis* ClpC1 in its structure and function. Most Clp proteins, including ClpC have been shown to be essential for growth. The Clp proteins in *M. tuberculosis*, like many other bacteria, may also be involved in its pathogenesis and an understanding of their mode of action could be useful in exploring them as drug targets.

Results

Figure 1 shows the sequence and putative domains of *M. tuberculosis* ClpC1. It is an 848-amino acid protein and has two AAA+ modules. The monomeric protein has five distinct domains namely, the N-terminal domain (residues 3–153), D1 large domain (residues 154–350), D1 small domain (residues 351–464), D2 large domain (residues 465–722) and D2 small domain (residues 723–848). Within the N-terminal domain there are two repeats, spanning amino acids 3–38 and 78–113 respectively (Fig. 1).

The DNA encoding *M. tuberculosis* ClpC1 was cloned into a T7 promoter-based *E. coli* expression vector and expressed in BL21–λDE3 cells. The expressed protein migrated as a $\sim$ 93 kDa protein on SDS/PAGE. ClpC1 was purified to near homogeneity from the soluble fraction by a combination of ammonium sulfate precipitation, and anion and gel-filtration chromatography (Fig. 2A).

The recombinant ClpC1 was analyzed to determine if it had an inherent ATPase activity. We used radioactive ATP as the substrate and quantified the radioactive inorganic phosphate generated upon its enzymatic hydrolysis by ClpC1. *M. tuberculosis* ClpC1 was found to contain significant ATPase activity, and its specific activity was found to be 400 units·mg^{-1} protein. Furthermore, it was found to use ATP as its preferred substrate; however, it also had 80, 75 and 70% activity respectively on GTP, UTP and CTP (data not shown).

Having established that, as predicted from the primary structure, recombinant *M. tuberculosis* ClpC1 functioned like an ATPase, we investigated the contribution made by its N-terminus to its functional activity. Two deletion variants, ClpC1Δ1 and ClpC1Δ2 were generated in which, respectively, amino acids 1–38 and 1–77 were deleted from the N-terminus of *M. tuberculosis* ClpC1 (Fig. 2B). ClpC1Δ1 has the N-terminal repeat I deleted, and the intervening sequence between repeats I and II forms its N-terminus (Fig. 2B). ClpC1Δ2 contains the N-terminal repeat I and the intervening sequence between repeats I and II deleted, and the N-terminal repeat II forms its N-terminus (Fig. 2B).

The deletion mutants were also expressed in *E. coli* and purified to near homogeneity following the procedure used for wild-type ClpC1. The respective mobilities of ClpC1Δ1 and ClpC1Δ2 on SDS/PAGE were 90 and 85 kDa (Fig. 2A).

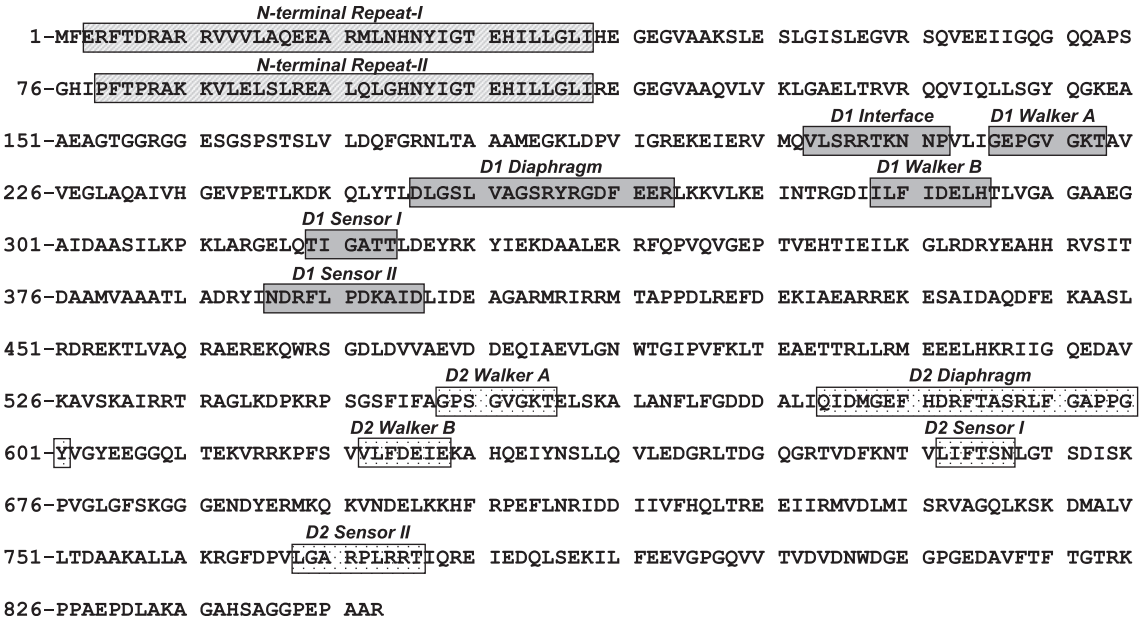

Fig. 1. Amino acid sequence of *M. tuberculosis* ClpC1. The deduced amino acid sequence of ClpC1 of *M. tuberculosis* encoded by Rv3596c is shown. The various proposed conserved regions are boxed and labeled.

The effect of deletions on the overall structure of ClpC1 was studied by CD spectral analysis of the purified proteins in the far-UV region. As shown in Fig. 3, ClpC1 showed the CD profile of a $\alpha+\beta$ protein, with broad minima between 215 and 225 nm. ClpC1Δ1 and ClpC1Δ2 also showed similar CD spectra, however, the amplitudes of the profile were different from that of ClpC1 (Fig. 3). In addition, ClpC1Δ1 had minima at 208 nm, indicating an increased helical content (Fig. 3). Therefore, ClpC1Δ1 showed an altered structure between the two deletion variants.

The ATPase activity of *M. tuberculosis* ClpC1, ClpC1Δ1 and ClpC1Δ2 was found to be very similar under standard conditions, i.e. pH 7.6, 37 °C (Table 1). These proteins were further characterized to compare their biochemical properties and functions. The enzymatic activity of the three proteins was assayed at different pH values. ClpC1 and the variants were active over a broad pH range of 6.5–12.5. The activity of all three proteins increased gradually from pH 6.5 to 10.5 and was highest at pH 10.5 (Fig. 4A). Increasing the pH further resulted in a slight decrease in the ATPase activity (Fig. 4A). To determine the optimum temperature, the activities of *M. tuberculosis* ClpC1 and its variants were assayed between 25 and 85 °C (Fig. 4B). All three proteins exhibited bell-shaped curves and were active over the temperature range studied. The optimal ATPase activity of ClpC1, ClpC1Δ1 and ClpC1Δ2 was observed between 37 and 50 °C (Fig. 4B). ClpC1, ClpC1Δ1 and ClpC1Δ2 exhibited increasing activity with increasing ATP concentrations from 2.5 to 20 mM; the activities did not change between 20 and 50 mM (Fig. 4C). All three proteins had similar K_m values for ATP, ranging between 2 and 6 mM (Table 1). Because these proteins were found to have good ATPase activity at high pH and temperature, their enzymatic activities under standard conditions, i.e. 37 °C, pH 7.6, were compared with those at 45 °C, pH 8.5. As shown in Table 1, the ATPase activity of the three proteins increased by $\sim$ 1.5-fold at high pH and temperature compared with that under the standard conditions. The ATPase activity of ClpC1, ClpC1Δ1 and ClpC1Δ2 was inhibited by ADP in a concentration dependent manner (Fig. 4D).

The effect of divalent metal ions and salt on the ATPase activity of *M. tuberculosis* ClpC1 and the two deletion variants was investigated. In the absence of divalent metal ions all three proteins had very low ATPase activity, which increased with the addition of Mg^{2+}, Mn^{2+} and Ca^{2+} (Fig. 5). The optimum concentration of these metal ions was found to be 10 mM (Fig. 5). The addition of sodium chloride and potassium chloride, ranging from 0.2 to 1.6 M did not affect the ATPase activity of ClpC1, ClpC1Δ1 and ClpC1Δ2 (data not shown).

To analyze whether *M. tuberculosis* ClpC1 prevents formation of protein aggregates, the effect of ClpC1 on the heat-induced denaturation of luciferase was

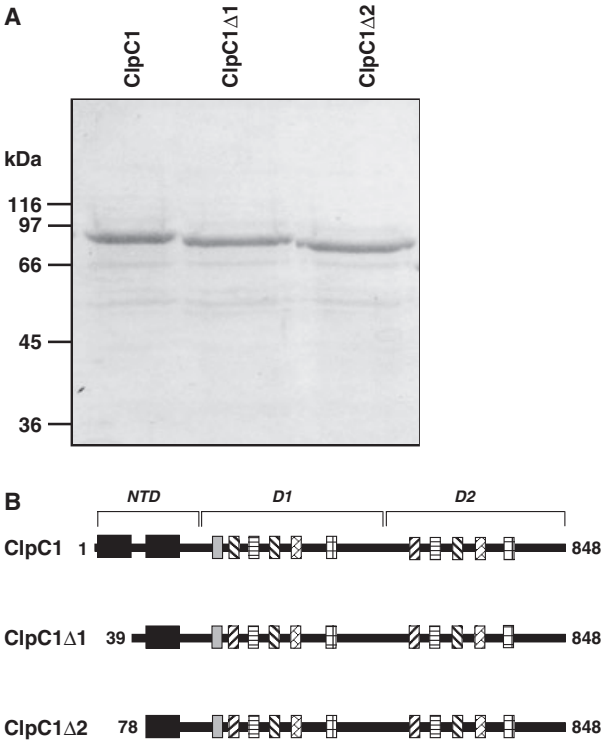

Fig. 2. Construction and purification of *M. tuberculosis* ClpC1 and its deletion mutants. (A) SDS/PAGE of purified full-length ClpC1 and deletion mutants, ClpC1Δ1 and ClpC1Δ2. (B) Full-length ClpC1 and deletion mutants, ClpC1Δ1 and ClpC1Δ2; the first and last amino acid numbers are indicated. Various conserved regions within NTD, D1 and D2 domains are (■) N-terminal repeats; (▩) interphase; (▨) Walker A; (▤) diaphragm; (▧) Walker B; (▨) sensor I; (▦) sensor II.

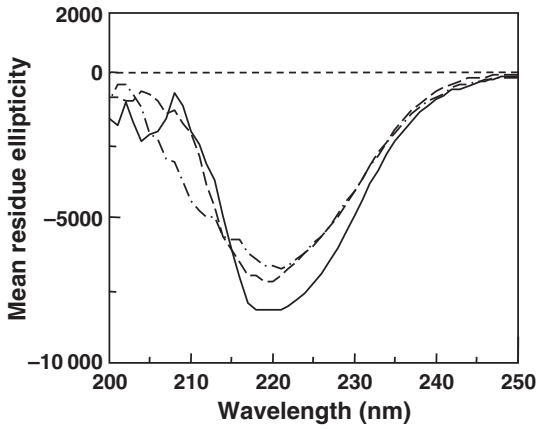

Fig. 3. CD-spectral analysis of *M. tuberculosis* ClpC1 and its deletion mutants. The spectra are presented as mean residue ellipticity, expressed in deg·cm^2·dmol^{-1}. ClpC1 (——), ClpC1Δ1 (— · —), ClpC1Δ2 (– – –).

Table 1. ATPase activity of *M. tuberculosis* ClpC1 and variants under different conditions. Data represent mean ± SE of three independent experiments. Numbers in parentheses indicate fold activity as compared with that at 37 °C, pH 7.6.

	ATPase activity (nmol P_i released·mg protein^{-1}·min^{-1})		K_m (mM)
Protein	37 °C, pH 7.6	45 °C, pH 8.5	ATP
ClpC1	376 ± 42	567 ± 73 (15)	5.6 ± 1.4
ClpC1Δ1	532 ± 39	863 ± 63 (16)	3.8 ± 0.3
ClpC1Δ2	571 ± 63	980 ± 93 (17)	1.7 ± 0.3

investigated. Luciferase is a highly heat-labile protein and aggregated quickly at 43 °C (Fig. 6A). The addition of ClpC1 with ATP reduced the heat-induced aggregation of luciferase in a concentration-dependent manner (Fig. 6A). ClpC1 without ATP had no effect on the heat-induced aggregation of luciferase, indicating that the ATPase activity of ClpC1 was required for its chaperone activity (Fig. 6A). The addition of BSA in place of ClpC1 failed to prevent luciferase aggregation (data not shown). Unlike wild-type ClpC1, the addition of ClpC1Δ1 with ATP did not prevent the aggregation of luciferase; instead an increased, concentration-dependent aggregation was observed (Fig. 6B). The increased aggregation was because of the aggregation of the ClpC1Δ1 protein itself at high temperatures (Fig. 6D). Like the wild-type protein, addition of ClpC1Δ2 with ATP significantly reduced the heat-induced aggregation of luciferase in a concentration-dependent manner (Fig. 6C). Compared with the wild-type protein, the ClpC1Δ2 variant was found to be slightly more active in preventing the aggregation of luciferase. ClpC1Δ2 without ATP had no effect on the heat-induced aggregation of luciferase (Fig. 6C). There was some aggregation of ClpC1 and ClpC1Δ2 in the presence of ATP at 43 °C (Fig. 6D). However, a very rapid and high aggregation of ClpC1Δ1 with ATP was observed at 43 °C (Fig. 6D). In the absence of ATP, only ClpC1Δ1 aggregated at 43 °C (data not shown). In addition to measuring aggregation as a change in turbidity, we also assayed luciferase activity prior to and after heating it in the absence and presence of *M. tuberculosis* ClpC1 and its variants. As shown in Table 2, there was ~ 70% loss in luciferase activity upon heating it to 43 °C. Addition of ClpC1 and its variants to luciferase during heating prevented the loss of activity; however, the prevention was not 100% (Table 2).

We also investigated whether *M. tuberculosis* ClpC1 could reactivate heat-inactivated luciferase *in vitro*. As shown in Fig. 7, without any additions, the heat-treated luciferase recovered only ~ 10% activity over time,

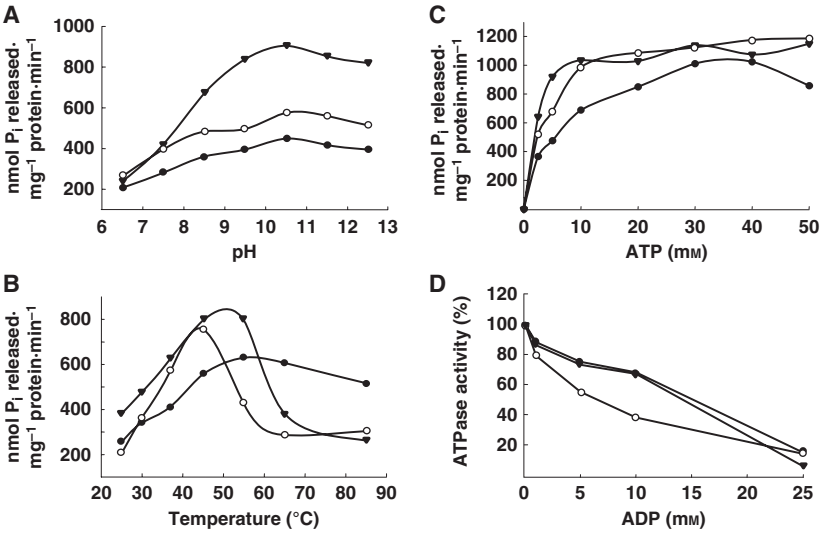

Fig. 4. ATPase activity of *M. tuberculosis* ClpC1 and its deletion mutants. The ATPase activity of proteins was assayed as described. (A) pH dependence, (B) temperature dependence, (C) steady-state kinetics with ATP, (D) effect of ADP. (●) ClpC1, (○) ClpC1Δ1 and (▼) ClpC1Δ2.

whereas in the presence of ClpC1 and ClpC1Δ2 ~ 30% activity was recovered. Although ClpC1Δ1 was found to not prevent aggregation it was able to reactivate luciferase, however, it had a reduced activity compared with ClpC1 and ClpC1Δ2 (Fig. 7). BSA was not active in reactivating inactive luciferase (Fig. 7).

The oligomeric status of ClpC1 and its deletion variants was analyzed by size-exclusion chromatography in the presence or absence of ATP or potassium chloride. As shown in Fig. 8A, ClpC1 eluted as a monomeric protein, and upon addition of ATP a significant fraction was in the hexameric form. In the presence of 1 M KCl, only monomeric ClpC1 was obtained (Fig. 8A). ClpC1Δ1, in the absence and presence of ATP eluted as hexameric or larger oligomers, and upon addition of salt the larger oligomers were destabilized to hexameric and smaller oligomeric species (Fig. 8B). ClpC1Δ2 also eluted in the hexameric form, which upon addition of ATP shifted towards higher oligomeric species (Fig. 8C). The larger oligomers of ClpC1Δ2 were destabilized to hexamers upon addition of salt (Fig. 8C).

Discussion

Clp has been linked to the tight regulation of virulence genes in the pathogens *L. monocytogenes* [23] and *S. typhimurium* [24]. The functional Clp complex is generated by an assembly of chaperone ATPases, including ClpA and ClpX, with the protease component ClpP. *M. tuberculosis* and many other Gram-positive bacteria have the ortholog ClpC in place of ClpA. In the *M. tuberculosis* genome, genes for heat shock proteins ClpP1, ClpC1, ClpX and ClpC2 have been annotated. Bearing in mind the importance of the Clp family of proteins in survival and virulence, it is of interest to understand the mode of action of these proteins in *M. tuberculosis*.

In this study, we functionally characterized the ClpC1 protein of *M. tuberculosis,* and investigated the role of its N-terminal repeats in its activity. Wild-type ClpC1 self-associates to form oligomers, contains basal ATPase activity and has chaperone activity in preventing the aggregation of luciferase and reactivating heat-inactivated luciferase. Deletion of the N-terminal conserved repeat I (amino acids 1–38) resulted in an alteration in the conformation and stability of ClpC1. Although, ClpC1Δ1 had full ATPase activity with a K_m value for ATP similar to that of the native protein, it failed to prevent heat-induced aggregation of luciferase. Apparently, the structural alteration caused by deletion of amino acids 1–38 rendered ClpC1Δ1 prone to heat denaturation. Deletion of N-terminal conserved repeat I along with the intervening amino acids linking it to N-terminal conserved repeat II did not affect the conformation of ClpC1 and the resultant protein, ClpC1Δ2, had full enzymatic and chaperone activities. The larger deletion also rendered the protein more stable. In ClpC1Δ1, the N-terminal repeat II is extended by 40 amino acids of the linker sequence between repeats I and II. In ClpC1Δ2, the N-terminal conserved repeat II is exposed and forms the terminus of the protein. It appears that an exposed N-terminal repeat is necessary for the activity of *M. tuberculosis* ClpC1; however, only one of the two repeats is sufficient.

The ClpC1 of *M. tuberculosis* is similar in its putative domain organization to that in *Bacillus subtilis*, *L. monocytogenes*, *Corynebacterium diphtherae* and *Mycobacterium bovis* (data not shown). In *L. monocytogenes,* ClpC has been shown to be important for virulence and survival in macrophages, and in *B. subtilis* it

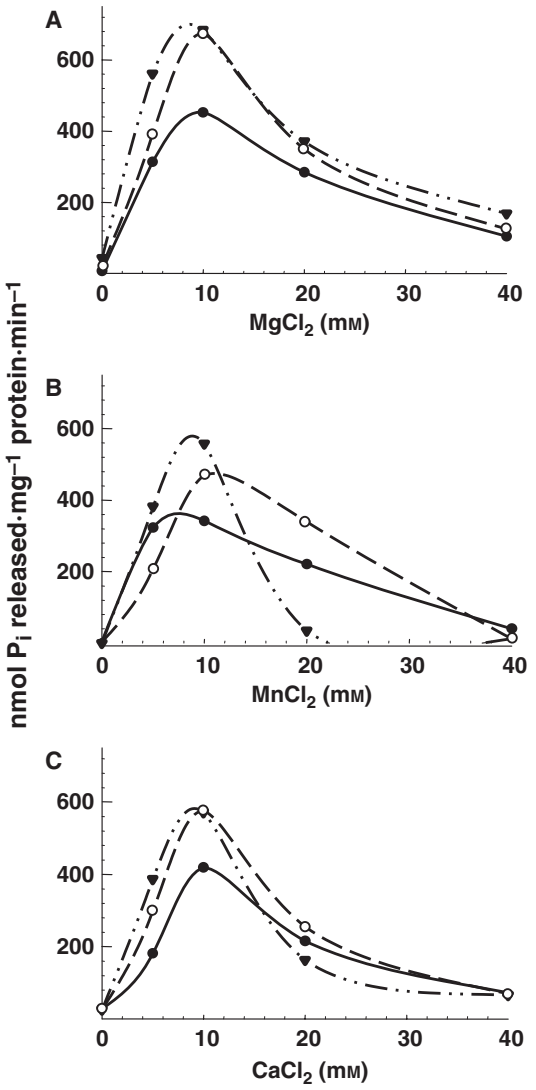

Fig. 5. Effect of divalent metal ions on the ATPase activity of *M. tuberculosis* ClpC1 and its deletion mutants. ATPase activity of proteins was assayed as described and effect of various divalent ions was studied. (A) $MgCl_2$, (B) $MnCl_2$, (C) $CaCl_2$. (●) ClpC1, (○) ClpC1Δ1 and (▼) ClpC1Δ2.

controls the competence gene expression and survival under stress conditions [26–29]. For the chaperone activity of *B. subtilis* ClpC, an adaptor protein is necessary for its interaction with the substrate, however, no adaptor protein is needed for the chaperone activity of *E. coli* ClpA and ClpX [30,31]. Recently, cynobacterial *Synechococcus elongatus* ClpC protein has been shown to display intrinsic chaperone activity without any adaptor protein; although its protein refolding activity was enhanced in the presence of MecA protein from *B. subtilis* [32]. ClpC from *S. elongatus* and *M. tuberculosis* have 80% sequence similarity with all the key determinants conserved. In this study, we also observed that *M. tuberculosis* ClpC1 displays chaperone activity without any adaptor protein.

The mycobacterial genome has revealed genes for both ClpX and ClpC; however, it has not been established how the ClpP protease complex must operate in *M. tuberculosis*. Recently, the crystal structure of tetradecameric ClpP1 of *M. tuberculosis* has been solved and unlike many other ClpP proteins it has been found to lack peptidase activity [33]. Compared with its orthologs, the structure of *M. tuberculosis* ClpP1 reveals a partly disordered handle domain, a slightly rotated arrangement of the monomers and an extended α helix at the N-terminus [33]. The structure of *M. tuberculosis* ClpP1 shows an alternative arrangement of the tetradecamer that may correspond to a different intermediate in the mechanism of action of caseinolytic proteases [33]. It is possible that *M. tuberculosis* ClpP1 is active upon its association with ATPases ClpC/X and in this context the unique properties of ClpC1 may be important for this interaction.

In conclusion, we demonstrate that ClpC1 of *M. tuberculosis* manifests chaperone activity *in vitro*, in the absence of any adaptor protein or cofactor. In addition, we observed that an exposed N-terminal repeat at the N-terminus is important for the interaction of *M. tuberculosis* ClpC1 with the substrate, however, only one of the two repeats is sufficient for the chaperone activity.

Experimental procedures

Cloning of *M. tuberculosis* ClpC1

Genomic DNA, extracted from *M. tuberculosis* strain $H_{37}R_v$ was used as the template to amplify DNA coding for ClpC1 by PCR. The sequence of *M. tuberculosis* ClpC1, open reading frame Rv3596c was used to design PCR primers. The amplified DNA was cloned between *Nde*I and *Hin*dIII sites in a T7 promoter-based expression vector, pVex11. The sequence was confirmed by DNA sequencing. Two deletions mutants, ClpC1Δ1 and ClpC1Δ2 encoding ClpC1 having the N-terminal repeat I (amino acids 1–38) or N-terminal repeat I along with the intervening sequence between repeats I and II (amino acids 1–77) deleted, respectively, were also constructed by PCR.

Expression and purification of recombinant *M. tuberculosis* ClpC1

E. coli BL21 cells, transformed with the plasmid containing DNA encoding *M. tuberculosis* ClpC1 were grown in super broth at 30 °C and induced with 1 mM isopropyl thio-β-D-

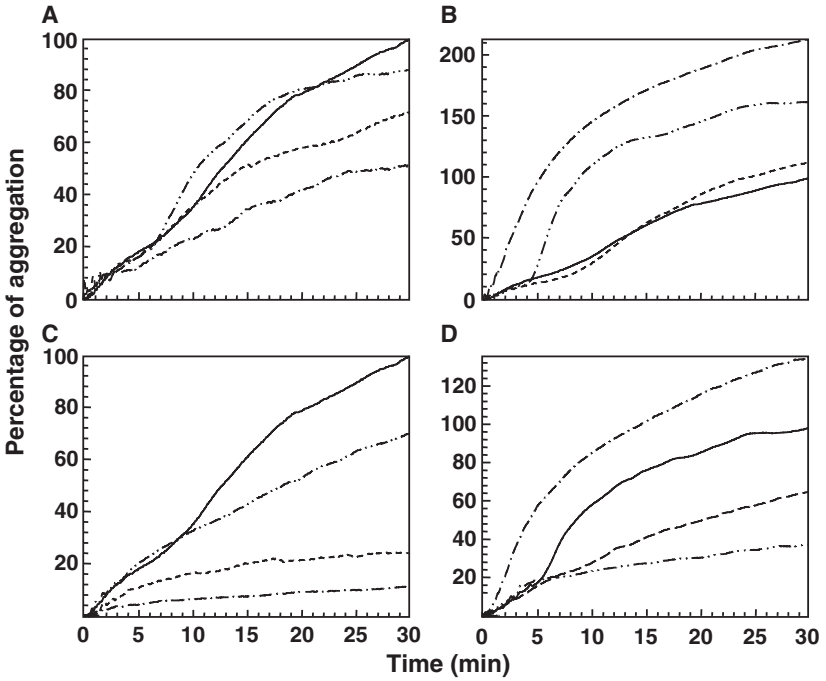

Fig. 6. Prevention of aggregation of luciferase by *M. tuberculosis* ClpC1 and its deletion mutants. Luciferase aggregation was assayed in a buffer with or without Clp proteins at 43 °C by following turbidity at 320 nm. (A), (B) and (C) represent data for ClpC1, ClpC1Δ1 and ClpC1Δ2, where various lines demonstrate reaction with (——) luciferase + ATP; (— — -) luciferase + 1 μM Clp + ATP; (—·—) luciferase + 2 μM Clp + ATP; (—··—) luciferase + 2 μM Clp – ATP. (D) The aggregation of Clp proteins in the presence of ATP at 43 °C (——) luciferase alone; (— — -) 1 μM ClpC1; (—·—) 1 μM ClpC1Δ1; (—··—) 1 μM ClpC1Δ2.

Table 2. Prevention of heat induced inactivation of luciferase by *M. tuberculosis* ClpC1 and variants. Luciferase, 5 nm was heated at 43 °C for 15 min without or with the indicated protein. Luciferase activity was assayed using a kit from Promega as described in Experimental procedures.

Protein	Activity (%)
Luciferase (unheated)	100
Luciferase (heated)	28
Luciferase (heated) + 0.15 μM ClpC1	50
Luciferase (heated) + 0.50 μM ClpC1	57
Luciferase (heated) + 0.15 μM ClpC1Δ1	43
Luciferase (heated) + 0.50 μM ClpC1Δ1	50
Luciferase (heated) + 0.15 μM ClpC1Δ2	51
Luciferase (heated) + 0.50 μM ClpC1Δ2	60

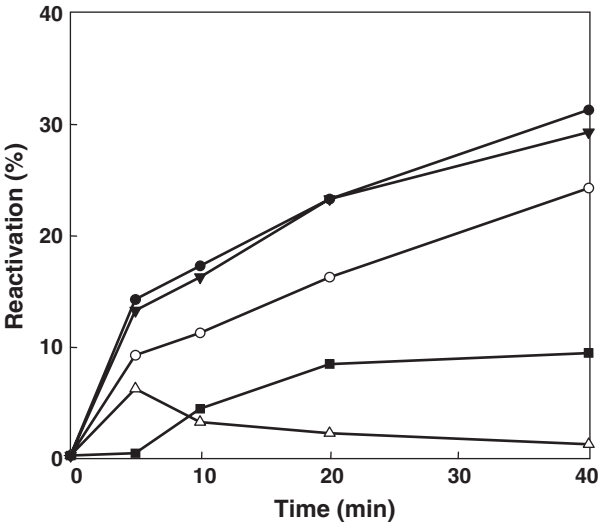

Fig. 7. Reactivation of heat aggregated luciferase by *M. tuberculosis* ClpC1 and its deletion mutants. Luciferase, 5 nm was heated at 43 °C for 15 min. Subsequently, the indicated proteins were added and the mixture was incubated at 25 °C. Samples were drawn periodically and luciferase activity assayed using a kit from Promega. (●) ClpC1, (▼) ClpC1Δ1, (○) ClpC1Δ2, (△) BSA, (■) No addition.

galactopyranoside for 3 h. Cells were lysed by incubation on ice for 45 min in a lysis buffer containing 50 mM Tris/Cl, pH 7.8, 200 mM KCl, 5 mM dithiothreitol, 10% (w/v) sucrose, 30 mM Spermidine–HCl and 1 mg·mL^{-1} lysozyme. To ensure complete lysis, the concentration of salt in the mixture was increased to 1 M, and it was incubated at 42 °C for 5 min. The lysate was centrifuged at 40 000 ***g*** for 30 min at 4 °C. The supernatant was further centrifuged at 100 000 ***g*** for 1 h at 4 °C. The supernatant was dialysed against buffer A, composed of 50 mM Tris/Cl, pH 7.6, 100 mM KCl, 5 mM dithiothreitol, 10% (v/v) glycerol and 0.01% Triton X-100, and applied onto a Q-Sepharose column equilibrated with the same buffer. The bound proteins were eluted with a salt gradient from 0.1 to 1 M KCl in buffer A using a GE AKTA-Basic chromatography system. The ClpC1 protein containing fractions were pooled, and the proteins in the pool were further fractionated by ammonium sulfate precipitation. ClpC1 precipitated at 40% ammonium sulfate, and was further purified using a Superdex-200 (GE Healthcare, Piscataway, NJ, USA) column equilibrated with buffer A. The fractions

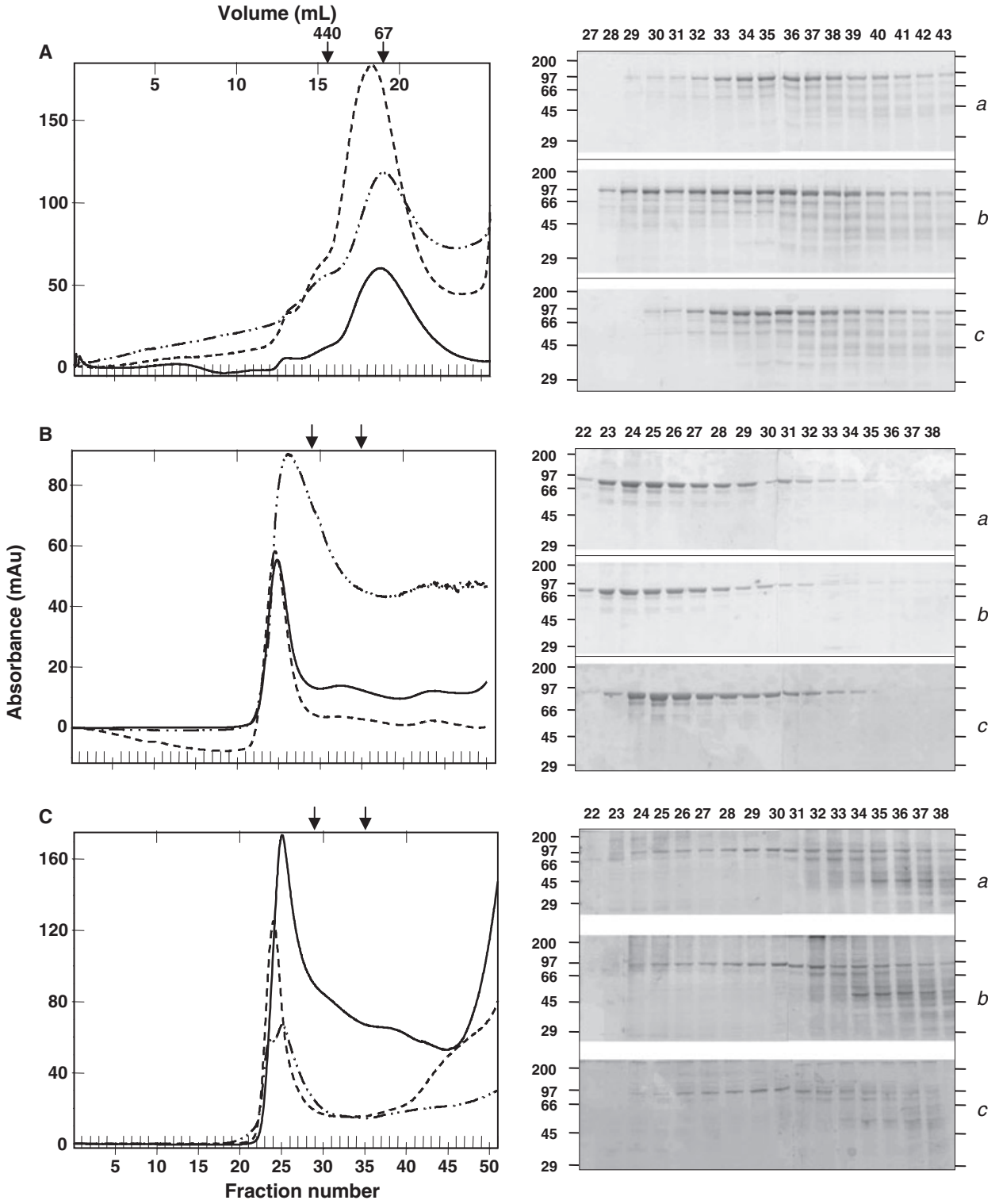

Fig. 8. Determination of oligomeric status of *M. tuberculosis* ClpC1 and its deletion mutants by gel filtration. The proteins were run on a 1 × 30 cm Superdex 200 column. The elution profiles of ClpC1, ClpC1Δ1 and ClpC1Δ2 are shown in (A), (B) and (C). Proteins were run in the absence (——) or presence of 15 mM ATP and 10 mM $MgCl_2$, (—·—·—) or 1 M KCl (— — —). The elution positions of protein standards, Ferritin (440 kDa) and BSA (67 kDa) are marked by arrows. The fractions from the columns were analyzed by SDS/PAGE; (a) no ATP, (b) +ATP, (c) +ATP and KCl.

containing homogenous ClpC1, as visualized using SDS/PAGE, were pooled and protein was quantified by the Bradford method using Coomassie Brilliant Blue plus reagent from Pierce (Rockford, IL, USA) [34]. The deletion mutants of ClpC1 were also similarly expressed and purified.

ATPase assay

For a standard assay, 5 μg protein was incubated in a 50 μL reaction mixture containing buffer A, 10 mM ATP containing [^{32}P]ATP[γP] and 10 mM $MgCl_2$ at 37 °C for 30 min. The reaction was stopped by adding 50 μL of chilled activated charcoal, 100 mg·mL^{-1} in 1 M HCl. The mixture was incubated on ice with intermittent shaking for 10 min, and centrifuged at 4 °C at 15 000 ***g*** for 15 min. Radioactivity in the supernatant was measured in a liquid scintillation counter, and the concentration of released P_i calculated using the specific activity of the substrate.

CD spectroscopy

For CD spectral analysis, 50 μg of protein, was dissolved in 1 mL of 50 mM Tris/Cl, pH 7.6, 33 mM KCl, 1.7 mM dithiothreitol, 10% (v/v) glycerol and 0.003% Triton X-100, and spectra were recorded in the far-UV range (200–250 nm) at 30 °C using a JASCO J710 spectropolarimeter. A cell with a 1 cm optical path was used to record the spectra at a scan speed of 200 nm·min^{-1} with a sensitivity of 50 mdeg and a response time of 1 s. The sample compartment was purged with nitrogen, and spectra were averaged over 10 scans. The results are presented as mean residue ellipticity.

Gel-filtration chromatography

To analyze the oligomeric status of proteins, they were applied onto a 1 × 30 cm Superdex-200 column equilibrated with buffer A. The columns were run using a GE AKTA-Prime chromatography system with a constant flow rate of 0.5 mL·min^{-1}. If mentioned, 15 mM ATP and 10 mM $MgCl_2$, or 1 M KCl was added to the column running buffer.

Prevention of aggregation of luciferase

The aggregation of luciferase was monitored in a buffer containing 50 mM Hepes/KOH, pH 7.6, 10% (v/v) glycerol, 5 mM dithiothreitol, 10 mM $MgCl_2$ and 25 mM KCl at 43 °C at 320 nm in a UV spectrophotometer equipped with a Peltier temperature programmer. ClpC1 proteins with or without 10 mM ATP were added in the reaction, wherever indicated.

To study the effect of heat treatment on luciferase activity, the native firefly luciferase (Promega, Madison, WI, USA) was dissolved in 1× lysis buffer (Promega) and the activity assayed as per the manufacturer's instructions. Fifty microliters of the luciferase assay reaction mixture contained 0.005 μM luciferase, 10 mM ATP and 10 mM $MgCl_2$. The mixture was incubated without or with ClpC1 and its variants at 43 °C for 15 min. At the end of incubation, 50 μL of luciferase assay substrate was added to each reaction mixture. Luciferase activity, the quantity of light produced by the catalysis of substrate luciferin, was measured using a Luminometer.

Reactivation of heat aggregated luciferase

Luciferase was denatured by incubating at 43 °C for 15 min. To measure reactivation of luciferase, in a 50 μL reaction, 0.005 μM heat-denatured luciferase was incubated with 0.25 μM of ClpC1 and its variants followed by incubation at 25 °C for 40 min. The refolding of denatured luciferase by ClpC1 proteins was analyzed at different time points by assaying the luciferase activity. As controls, similar reactions were carried out without any addition or addition of BSA to heat-denatured luciferase.

Acknowledgements

This work was supported by grants to the National Institute of Immunology, New Delhi from the Department of Biotechnology, Government of India. NPK and PR thank Department of Biotechnology for a Project Assistantship. DS thanks the Council of Scientific and Industrial Research, India for a senior research fellowship.

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