GroEL Mediates Folding of *Bacillus anthracis* Serine/Threonine Protein Kinase, PrkC

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Abstract *Bacillus anthracis* causes anthrax in human and animals. Both, signaling system such as two component system and endogenous chaperone system such as GroEL–GroES help bacteria to cope with the environmental challenges. Such molecular chaperones are the stress induced proteins that help bacteria to override unfavorable conditions by their moonlighting functions. Previous reports showed that PrkC and PrpC, the Ser/Thr kinase–phosphatase pair in *B. anthracis*, control phosphorylation of GroEL and regulate biofilm formation. In this study, we show that GroEL is involved in the folding of PrkC to active form. The proteins (GroEL, PrkC and PrpC) were expressed and purified by affinity chromatography. Purified GroEL was used for refolding of denatured PrkC and PrpC and observed that GroEL refolds PrkC but not PrpC as measured by their enzymatic activity. We also observed that purification of GroEL with six histidine tag using Cobalt-Agarose resin yielded superior quality GroEL protein with negligible contamination of non-specific proteins. Thus, cobalt resin can be a better choice for purification of many histidine tagged proteins, where Ni-NTA does not work very well.

Keywords *Bacillus anthracis* · GroEL · PrkC · Signaling · Protein folding

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Bacillus anthracis is the causative agent of anthrax which makes spores in stress conditions. The disease is initiated when spores enter in the host, germinate in macrophages and start producing anthrax toxin. During the life cycle (vegetative cell and spore), *Bacillus* comes across various environmental stresses where heat shock proteins (hsps) play an important role in their survival [1]. Hsps comprise a set of molecular chaperones that transiently assist in repairing of misfolded and partially unfolded proteins. Folding of proteins is an error prone mechanism that requires a thermodynamic balance to drive the protein towards its native state [2]. The GroEL–GroES chaperonin system is one of the most well studied examples of molecular chaperone in bacteria which is also conserved in *B. anthracis* [3].

Homo-oligomeric GroEL forms two heptameric rings stacked back to back forming a cylindrical structure that takes the specific substrate proteins into a central cavity [4]. The structure of GroEL is divided into three subunits with apical, intermediate and equatorial domains. The exposed hydrophobic residues on the misfolded or unfolded substrate proteins are captured by the apical domains of GroEL thus enclosing the substrate into a cage like structure. A ring like cofactor, GroES, then covers this central cavity. When the folding process is complete, the folded protein and GroES gets dissociated from GroEL [5]. GroEL is one such immunogenic Hsp, which is located on the exo-sporium and upon immunization provides protection against *B. anthracis* infection [6]. GroEL in combination with protective antigen (PA) enhanced protective efficacy of PA against anthrax spore challenge [7].

B. anthracis possess an intricate intracellular signaling machinery to survive in extreme environmental stress conditions [8–11]. The kinase phosphatase pair, PrkC and PrpC are the conserved mediators of bacterial signaling

system which regulate critical cellular processes like sporulation, germination, cell growth and virulence in *B. anthracis* [8, 9]. Previous studies have shown that PrkC mediated phosphorylation of GroEL is associated with biofilm formation in *B. anthracis* [3]. GroEL has specificity for its substrates as it assists in the folding of nearly 10% of the newly synthesized polypeptides. Substrate proteins interact with GroEL not only during the initial folding process but also for maintenance of conformation upon exposure to different environmental conditions during their lifetime [12]. Here, we studied whether PrkC and PrpC is a substrate of GroEL–GroES system in *B. anthracis*. We purified GroEL from *B. anthracis* and analyzed its role in folding of heat denatured PrkC and PrpC proteins by measuring their enzymatic activity.

We used an already available expression vector pYS5 which harbors a native promoter of PA that expresses the downstream gene constitutively [13]. To study the protein folding potential of native GroEL purified from *B. anthracis*, the gene for GroEL was cloned downstream of PA promoter with a His tag using primers listed in Table 1. The lysate of *B. anthracis* Sterne cells (Bas) and Bas transformed with pYS5-*groel*-His₆ plasmid (Bas His GroEL) were resolved on SDS-PAGE and subjected to immunoblotting using anti-GroEL antibodies (α -GroEL antibody; 1: 15,000 dilution and HRP-conjugated anti-mouse IgG antibody; Bangalore Genei, 1: 18,000 dilution) to confirm overexpression (Fig. 1a). Ni²⁺ and Co²⁺ immobilized on a matrix was employed to purify native GroEL. Ni²⁺-NTA matrix has a high binding affinity for the hexa-histidine tag at pH 8.0 and Co²⁺ resin display weaker yet specific interaction for hexa-histidine tag [14]. The lysate prepared from *B. anthracis* Sterne cells harboring pYS5-*groel*-His₆ was passed through the matrix

followed by washing and elution with a buffer containing imidazole. For purification from Co²⁺ matrix, the cell pellet was lysed in a buffer [1X Tris-buffered saline (TBS), 1 mM PMSF, 1 mg/mL lysozyme, and PI (Roche)] through sonication. The lysate collected after harvesting was incubated with Co²⁺ superflow resin for 3 h. After washing with wash buffer (1X TBS, 1 mM PMSF, and 10 mM imidazole) for 30 min, the protein was eluted in elution buffer (1X TBS, 1 mM PMSF, and 300 mM imidazole). To check the purity of the protein, the eluted fractions from both resins (Ni²⁺-NTA and Co²⁺ matrix) were resolved on SDS-PAGE and immunoblot was probed with anti-His antibody (Abcam 1: 18,000 dilution). The blot was developed by Super Signal® West Pico Chemiluminescent Substrate kit (Pierce Protein Research Products) according to the manufacturer's protocol [15]. The results showed that GroEL was purified to homogeneity using Co²⁺ matrix while the protein purified from Ni²⁺ matrix showed a few contaminating bands (Fig. 1b).

Our previous work has identified GroEL as a substrate of PrkC, which plays an important role in bacterial biofilm formation [3]. It is known that GroEL acts on unfolded or incorrectly folded proteins to fold them in correct conformation. PrkC is known to be a stable enzyme at relatively higher temperatures, which potentiates its role in bacterial spores. Hence, the refolding capability of GroEL can be measured on the basis of activity of its substrates after refolding. The sequences encoding *prkC* catalytic domain (*bas3713*, 1-337aa), *groes* (*bas0252*) were amplified using gene specific primers (Table 1) containing appropriate restriction sites from *B. anthracis* Sterne strain genomic DNA [16–19]. The amplified products were then digested and ligated into an *E. coli* expression vector pProEx-HTc digested with the same enzymes. The His tagged proteins

Table 1 Primers and clones

Genes	Vectors	Restriction sites	Primers (5' → 3') ^a	References
<i>prkC</i> (<i>bas3713</i> , 1-1011 bp), kinase domain	pProEx-HTc	FP-BamHI RP-XhoI	TAGGTGAAGTGGATCCTGCTGATTGGAAAACGC TGTAATTTAAAATCTCGAGTCATTATTACTTCGTTTG	[3]
<i>prpC</i> (<i>bas3714</i>)	pProEx-HTc	FP-BamHI RP-XhoI	GCGCAAAGAAGAGACGAGGGATCCAGATGAAAGCCGTGTTTCT CGTTTTCCAATCAGCACGCTCGAGTTCACCTACTTTTCGTTTGTCG	[3]
<i>groES</i> (<i>bas0252</i>)	pProEx-HTc	FP-BamHI RP-XhoI	AACAAAATGAGGAGGATCCTG TTCATGCTAAAG CCTTGGAATTTCTCGAGATTATATAATTAACCG	[3]
<i>groEL</i> -His	pYS5	FP-SpeI RP-BamHI	CCAAGGGGGTCAACTAGTTATGGCAAAAGATATTAA GGATCCTTAGTGGTGGTGGTGGTGGTGCATCATTCGCCCATAC	This study
<i>groEL</i> (<i>bas0253</i>)	pProEx-HTc	FP-BamHI RP-XhoI	AATCCAAGGGGGTGGATCCTTATGGCAAAAG TTAGGGCAAACTCGAGTTACATCATTCGCCCG	[3]

^aRestriction/mutation sites have been underlined

Fig. 1 Purification of GroEL using Ni^{2+} and Co^{2+} resin. **a** *B. anthracis* Sterne lysate expressing GroEL was resolved on SDS-PAGE and transferred to nitrocellulose membrane. Upper panel depicts Ponceau S stained membrane to show total protein loaded in

the well and lower panel shows image of immunoblot using anti-GroEL antibodies. **b** The Bas His GroEL purified from Ni^{2+} and Co^{2+} resins were resolved on SDS-PAGE followed by immunoblotting using anti-His antibodies

(PrkC and GroES) were over-expressed in *E. coli* BL-21 (DE3) [18–20] and affinity purified with Ni^{2+} -NTA resin (Qiagen). PrkC (0.5 μM) was denatured by heat treatment and incubated with Bas His₆-GroEL (2 μM) and GroES (1 μM) at room temperature. Enzymatic activity of PrkC was quantitated by evaluating its autophosphorylation in a kinase assay [20]. Purified PrkC was taken as a positive control and denatured PrkC (incubated with GroES alone) was taken as negative control for measuring the relative activity. The relative phosphorylation of denatured PrkC was compared considering autophosphorylation of purified PrkC as 100% (Fig. 2a). The results show that the denatured PrkC upon incubation with GroEL gets refolded and regained its activity to about 60% as compared to the purified PrkC (Fig. 2b). This result suggests that GroEL refolds PrkC as measured by kinase activity.

Our previous work also identified that *B. anthracis* phosphatase PrpC dephosphorylates GroEL which decreases its oligomerization [3]. PrpC was also analyzed as a substrate of GroEL. The gene, *prpC* (*bas3714*) was amplified using gene specific primers and purified using Ni^{2+} -NTA resin. As with PrkC, PrpC (1.8 μM) was also heat denatured and then

incubated with Bas His₆-GroEL (2 μM) and GroES (1 μM) for refolding. The refolding of PrpC protein was assessed by measuring its phosphatase activity using pNPP as a substrate. The reaction was carried out in a 96-well plate format and the plate was incubated at 37 °C for 30 min followed by measurement of absorbance at 405 nm (Microplate reader, Bio-Rad). Native PrpC was taken as positive control and GroEL–GroES mixture was taken as negative control. The results show that GroEL was unable to refold PrpC (Fig. 3).

These results suggest that GroEL may help PrkC to remain in an active conformation during the transition stages such as sporulation and infection. We purified GroEL with Cobalt beads and found that PrkC lost > 85% of activity after denaturation, which was substantially regained after incubation with native GroEL. On the other hand, PrpC did not refold in the presence of GroEL, indicating that it is not a GroEL substrate. Thus, role of GroEL in regulating signal transduction proteins involved in bacterial pathogenesis indicates utilization of chaperones by microbial pathogens in their virulence. Further, characterization of the signaling proteins may help to explore the potential role of GroEL as a drug target.

Fig. 2 GroEL mediated refolding of PrkC regains its activity. Denatured PrkC was allowed to refold in the presence of Bas His GroEL and its activity was assessed by in vitro kinase assays. Autophosphorylation of purified PrkC was taken as 100% and relative phosphorylation of denatured PrkC was calculated. After denaturation, PrkC retained only ~ 10% activity, which was regained to 60% in the presence of GroEL and GroES. All the refolding assays were performed in the presence of GroES. Upper panel **a** shows the autoradiogram whereas, lower panel **b** shows the densitometric analysis

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Authors' Contributions RV conceived and designed the experiments. YH and YS contributed materials, reagents and analysis tools. All authors have read and approved the manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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Fig. 3 Refolding of PrpC by GroEL: Denatured PrpC was incubated with Bas His GroEL in the presence of GroES and activity of PrpC was measured at 37 °C using pNPP as a substrate. Activity of purified PrpC was taken as positive control and samples without PrpC was taken as negative control

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