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Synthesis and hydrolysis of pppGpp in mycobacteria: A ligand mediated conformational switch in Rel

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Abstract

Bacteria respond to starvation by synthesizing a polyphosphate derivative of guanosine, (p)ppGpp, that helps the bacteria in surviving during stress. The protein in Gram-positive organisms required for (p)ppGpp synthesis is Rel, a bifunctional enzyme that carries out both synthesis and hydrolysis of this molecule. Rel shows increased pppGpp synthesis in the presence of uncharged tRNA, the effect of which is regulated by the C-terminal of Rel. We show by fluorescence resonance energy transfer that the distance between the N-terminus cysteine residue at the catalytic domain and C692 at the C-terminus increases upon the addition of uncharged tRNA. In apparent anomaly, the steady state anisotropy of the Rel protein decreases upon tRNA binding suggesting "compact conformation" *vis-à-vis* "open conformation" of the free Rel. We propose that the interaction between C692 and the residues present in the pppGpp synthesis site results in the regulated activity and this interaction is abrogated upon addition of uncharged tRNA. We also report here the binding of pppGpp to the C-terminal part of the protein that leads to more unfolding in this region.

Keywords: Stringent response; ppGpp; FRET; SPR; Conformational switch

1. Introduction

Stringent response plays a key role in the survival of bacteria under nutrient deprived conditions and is characterized by the down-regulation of stable RNA species such as rRNA and tRNA accompanied by an up-regulation of certain genes including those involved in amino acid biosynthesis [1,2]. Study of the mediators of the stringent response in mycobacteria is essential as this is one of the important pathways responsible for the maintenance of latency in mycobacteria. The key player responsible for evoking the stringent response is Rel, which synthesizes an unusual guanosine nucleotide, guanosine 3′, 5′-bispyrophosphate or (p)ppGpp, by transferring a pyrophosphate moiety from ATP to the 3′ of GDP/GTP [3–6]. These molecules (alarmones) invoke the classic 'stringent response' resulting in plethora of effects in microorganisms and plants [7], including dormancy in mycobacteria [1,2,4]. In Gram-negative bacteria such as *E. coli*, the two activities – synthesis and hydrolysis of (p)ppGpp – are carried out by two different proteins RelA and SpoT, respectively [8]. RelA lacks hydrolysis activity because of the absence of the hydrolysis domain. SpoT is involved predominantly in (p)ppGpp hydrolysis; it, however, shows low levels of alarmone synthesis [8]. In Gram-positive bacteria, a single bifunctional protein, Rel carries out both the synthesis and hydrolysis reactions [3,6,9]. RelA in Gram-negative bacteria shows ribosome dependent synthesis of (p)ppGpp [10] whereas Rel in Gram-positive organisms shows (p)ppGpp synthesis even in the absence of ribosomes [6,9,11]. The only exception, so far known, to this is *Streptomyces coelicolor*, in which the synthesis and the hydrolysis of (p)ppGpp is shown to be ribosome dependent [12].

The "alarmone" molecule, (p)ppGpp has been shown to play a vital role in the survivability of several genera of

Abbreviations: pppGpp, guanosine 3′-diphosphate 5′-triphosphate; ppGpp, guanosine 3′, 5′-bis(diphosphate); IPTG, Isopropyl β-D-1-thiogalactopyranoside; Ni-NTA, Nickel-Nitrilotriacetic acid; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; PEI, Poly(Ethyleneimine); DTT, Dithiothreitol; PMSF, Phenylmethanesulfonyl fluoride; EDTA, Ethylenediaminetetraacetic acid; IAEDANS, 5-[2-(iodoacetamido)ethylamino] naphthalene-1-sulfonic acid; IAF, 5-(Iodoacetamido)fluorescein; TCEP, Tris(2-carboxyethyl)phosphine; FRET, Fluorescence resonance energy transfer.

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bacteria and its functional significance is under extensive investigation [13–15]. It plays a distinct part in quorum sensing in *Pseudomonas aeruginosa* [16,17], symbiosis in Rhizobia [18,19], sporulation in *Myxococcus* [20,21] and the production of antibiotics in *Streptomyces coelicolor* [22]. In *Salmonella typhimurium*, the *relA*/*spoT* double mutant has been shown to be avirulent in mice models [23]. In contrast, *Vibrio cholerae* shows the expression of virulence factors and colonized suckling in mouse intestine even after the disruption of *relA* [24]. Recently, in an interesting study using diauxie, in *E. coli* as a model system, Conway laboratory has shown that deletion of *relA* causes a delay in diauxie and also results in decreased induction of RpoS and Crp regulon genes, thus showing a significant role of ppGpp in the global gene expression of an organism [25].

In *Mycobacterium tuberculosis*, the causative agent of tuberculosis, the bacteria remain in a dormant state inside the human host (a phenomenon termed 'latency') and Rel has been shown to play a vital role in the survival of these bacilli under oxygen [26] and nutrition deprived [27] conditions. The importance of Rel in *M. tuberculosis* can be judged by the fact that the strains with deletion of *rel* gene do not show a long-term persistence both in liquid culture [28] and in animal model [29]. Furthermore, *rel* gene has been shown to be directly involved in the persistence of *M. tuberculosis* in host granulomas [30]. In *M. smegmatis*, a non-pathogenic homologue of *M. tuberculosis*, over-expression of *rel* affects the stationary phase survival of bacteria [31]. Moreover, it also alters the cellular and colony morphology [31,32]. Our studies have also shown that the Rel protein of *M. smegmatis* is bifunctional in nature, carrying out both the synthesis and the hydrolysis of pppGpp [6].

It was proposed earlier that the two catalytic activities of the bifunctional Rel protein in Gram-positive bacteria are regulated by an interaction between the N- and C-termini of the protein, connected together by a 16-residue solvent accessible hinge [33]. The crystal structure of Rel protein of *Streptococcus equisimilis* projected a "two domain crosstalk" model that explained the regulation of the two opposing activities in the protein [34]. We have previously observed that in the case of Rel from *M. smegmatis*, regulation of pppGpp synthesis is carried out by the two C-terminal domains, deletion of which increases the synthesis activity of the protein without altering the hydrolysis [6]. Rel_{Msm} also shows an increased synthesis activity upon addition of uncharged tRNA or in reducing environments. We pointed out that the mutation of a single cysteine (C692) altered levels of uncharged tRNA-mediated pppGpp synthesis [6].

In *M. tuberculosis*, Rel shows higher synthesis of pppGpp in the presence of uncharged tRNA, which increased further upon the addition of ribosomes and mRNA [3]. However, omitting uncharged tRNA from the Rel-activating complex (uncharged tRNA–ribosome–mRNA complex; RAC) did not result in any noticeable enhancement of the (p)ppGpp synthesis activity [3]. It, therefore, appears that although an intact RAC complex is required for a very high Rel synthetic activity, uncharged tRNA forms a critical and an essential part of it. It also sounds reasonable to extrapolate the role of uncharged tRNA from earlier studies that it helps RelA in recognizing amino acid starvation in *E. coli*. Charged tRNA, on the other hand, has been shown not to affect the synthesis and the hydrolysis activities of Rel_{Mtb} [3].

In the present study, we propose the role of mediator involved in 'crosstalk' between the N- and the C-terminal domains in regulating pppGpp synthesis. We suggest a model for the positive regulation of the Rel activity mediated by uncharged tRNA (hereafter denoted as utRNA) and the "feedback inhibition" brought about by pppGpp binding to the C-terminus of Rel.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

For cloning and site directed mutagenesis, *E. coli* strain DH5α and for protein expression and purification, *E. coli* strain BL21 (DE3) were used. Both the strains were grown in LB broth at 37 °C with constant shaking or on 1.5% agar plate in the presence of 100 μg/ml Ampicillin. PCR reactions were performed using Phusion DNA polymerase (Finnzyme) as per the manufacturer′s instructions. All the clones and mutants generated were confirmed by sequencing (Microsynth, Switzerland) and by trypsin digestion and mass spectrometry. Restriction enzymes were purchased from New England Biolabs. Tris(2-carboxyethyl)phosphine (TCEP), IAEDANS and IAF were procured from Molecular Probes. The *E. coli* tRNA Type XXI utRNA was procured from Sigma and was used without further purification.

2.2. Multiple sequence alignment

Rel, RelA and SpoT sequences were obtained from different bacteria (both Gram negative and Gram positive) by performing a BLAST search using Expasy BLAST tool (http://www.expasy.org/tools/blast/) and *M. smegmatis* Rel protein sequence as template and using all default parameters against the Swiss-Prot database. The name for the bacteria thus obtained was retained and the sequences obtained in FASTA format were used for multiple alignment as described previously [6].

2.3. Purification of Rel WT, Rel CTD and their cysteine mutants

Cysteine mutations were performed essentially as described [6]. Rel WT, Rel CTD and their cysteine mutants were purified as explained [6]. Briefly, pETRel constructs for different proteins were transformed in *E. coli* BL21 (DE3) and the bacteria were grown in LB broth till $OD_{600} \sim 0.6$, induced with Isopropyl β-D-1-thiogalactopyranoside (final concentration 1 mM) and were harvested, and lysed. The lysate was cleared by centrifugation at high speed and the supernatant was loaded on Ni-NTA column and the protein was purified. Imidazole was removed through dialysis and the protein was stored at 4 °C for future use.