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Biophysical Chemistry 127 (2007) 41 – 50

Biophysical Chemistry

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

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Received 12 October 2006; received in revised form 7 December 2006; accepted 7 December 2006
Available online 9 December 2006

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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doi:10.1016/j.bpc.2006.12.003

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.

2.4. Analysis of binding of utRNA to the Rel protein using surface plasmon resonance

The utRNA binding to the Rel protein and its cysteine mutants was assayed using BIA2000 biosensor system (Biacore). CM5 sensor chip was used to immobilize Rel WT protein and its cysteine mutants as per the manufacturer's instructions. The surface density was kept below 150 RU in each case. One channel was left blank for the measurement of background and its values were subtracted from the measured response. Experiments were performed in SPR buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% Tween 20 and 1 mM dithiothreitol. Lyophilized *E. coli* tRNA Type XXI (Sigma) was dissolved in the SPR buffer at a concentration of 20 mg/ml. The molar concentration of the stock was calculated by assuming the utRNA molecule to be 70 bases and taking 330 Da molecular weight for each base. The concentration range to assess the binding of utRNA to Rel proteins was 3.125 μM to 50 μM and the concentration of protein was ~10 μM in every case. The association was assessed by passing utRNA over the chip surface at a flow rate of 20 μl/min for 120 s after which the utRNA was allowed to dissociate by washing the surface with buffer for 120 s. Each injection was repeated at least twice. Chip was further regenerated by allowing the buffer to flow for several minutes at a flow rate of 40 μl/min. The kinetic constants, k_a and k_d were calculated using BIA evaluation software 3.1 by global analysis with a 1:1 Langmuir binding model. Ratio of k_d/k_a gave the value of equilibrium dissociation constant, K_D.

2.5. Labeling of protein with fluorophores

Purified proteins were dialyzed against the labeling buffer containing 40 mM HEPES pH 8.0, 500 mM NaCl and 5 Glycerol. After dialysis, the absorbance of the protein sample at 280 nm was recorded and the concentration was determined. Tris(2-carboxyethyl) phosphine was added in 10 fold molar excess to the protein to reduce all disulfides. Later, IAEDANS was added to the protein solution to a molar concentration ratio of 1:1. The samples were then incubated at 4 °C for 12 h in dark. Excess of dithiothreitol was added to stop the reaction and the samples were then subjected to gel filtration chromatography on Sephadex G75 to remove the unbound dye. The dye:protein ratio was calculated by measuring the absorbances at 280 nm and 336 nm and using the extinction coefficient of IAEDANS at 336 nm = 13,100 $M^{-1}cm^{-1}$. IAEDANS labeled protein was further labeled with IAF following the same procedure as for the IAEDANS labeling. The free dye was removed as described above. Dye:protein ratio was calculated by measuring the absorbances at 280 nm and 492 nm and taking IAF extinction coefficient at 492 nm = 77,000 $M^{-1}cm^{-1}$.

2.6. Measurement of lifetime and the rotational correlation time of the fluorophores in the labeled proteins

The lifetime, τ and the rotational correlation time, τ_r were measured on the Fluorolog Tau-3 lifetime system (Jobin Yvon) using the phase-modulation method for measuring lifetime [35]. The instrument was equipped with Xenon arc lamp as an illumination source. Glycogen solution, prefiltered through 0.2 μm filter, was used as reference for the measurement of lifetime. All the measurements were done at 25 °C and the absorbances of the samples at their λ_{ex} were <0.2. A minimum of 15 data points was collected and values of τ and τ_r were calculated by fitting the curves in single exponential decay using the modeling software provided with the instrument. All the measurements were done at least thrice and the values with lowest χ^2, as suggested [35], have been reported.

2.7. Measurement of R_0

Förster distance, R_0, at which the transfer efficiency is 50%, was calculated using the formula, $R_0 = 9.79 \times 10^3\ [(J)\ Q\ (n^{-4})\ (\kappa)^2]^{1/6}$ where, n is the refractive index of the medium and was taken as 1.4 [36]. Q is the quantum yield of the sample which was calculated as described earlier [36] from the absorbance of the sample at 336 nm and the area enclosed by the corrected emission spectrum of the donor when excited at 336 nm. Quinine sulfate in H_2SO_4 was taken as reference [37]. The spectral overlap integral, J, was calculated as mentioned [36] using the FORTRAN program described therein. The orientation factor, κ^2, was taken to be 0.476 as in our case, the rotational correlation time for both the donor and the acceptor was found to be more than their lifetime [38–40]. It should be noted here that the R_0 shows a sixth root dependence on the κ^2 and the refractive index, n [36].

2.8. Measurement of distance between the fluorophores and steady-state anisotropy

For the measurement of distances in the presence of utRNA, both the mono- and the doubly labeled proteins were incubated with increasing concentrations of utRNA at 25 °C for 10 min. The absorbance of the fluorophore (IAEDANS) was kept below 0.05 at its excitation wavelength (336 nm) to avoid inner filter effect. The emission was monitored at 470 nm in each case and was corrected for inner filter effect (after utRNA addition) using the following expression, $F_{corr} = F_{obs} \times 10^{(Aex + Aem)/2}$, where, F_{corr} and F_{obs} are the corrected and observed fluorescence intensities respectively. A_{ex} and A_{em} are the absorbances of the samples at 336 nm and 470 nm respectively. The distance between the two fluorophores was calculated using the formula [41], $r = R_0\ ((1/T) - 1)^{1/6}$ where, T is the transfer efficiency that is calculated by measuring the intensities of the fluorescence of the IAEDANS in the presence (F_{DA}) and the absence (F_D) of the IAF at 470 nm using the expression, $T = 1 - (F_{DA}/F_D)$. For steady state anisotropy, the IAEDANS labeled proteins were incubated at 25 °C for 10 min with increasing concentrations of utRNA.

The anisotropy was measured using the excitation and the emission wavelengths 336 and 480 nm respectively. Likewise, the anisotropy of Rel CTD was measured in the presence of increasing concentrations of pppGpp. All the experiments were repeated at least four times and the data presented here is the average.

2.9. Gel filtration chromatography

Rel CTD was incubated with increasing concentrations of pppGpp at 25 °C for 10 min and then 300 μl of it was loaded on to Superdex 200 10/300 GL Tricorn column (GE Healthcare) connected to Amersham Pharmacia ÄKTA FPLC system at a flow rate of 0.4 ml/min and was detected at 280 nm. Before run, the column was equilibrated with the running buffer and was calibrated using β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and lysozyme (14 kDa). All the proteins for calibration were procured from Sigma and were used without further purification. The Rel CTD elution peak gave a molecular weight of ~41 kDa (calculated weight ~39 kDa). Retention time of pppGpp was used as a marker for the accuracy of the column during each run. For observing denaturation in the presence of urea, Rel CTD was incubated with increasing concentrations of urea and was loaded on to the column pre-equilibrated with the running buffer containing same amount of urea.

2.10. Circular dichroism spectroscopy

The effect of pppGpp was assessed on Rel CTD 4,5 using circular dichroism spectroscopy as described earlier [6]. Briefly, the protein was treated with high concentrations of DTT to solubilize the aggregates and was dialyzed against the CD buffer containing 10 mM HEPES–KOH buffer pH 7.9, 150 mM KCl, and was filtered through a 0.2 μm filter (Sartorius). CD spectra were recorded on a Jasco J-715 spectropolarimeter at 22 °C using a path-length of 0.2 cm, bandwidth 2.0 nm and a response time of 2 s. 5 scans with each concentration of pppGpp were recorded at a speed of 50 nm/min, averaged and buffer subtracted. Data in terms of the mean residue ellipticity at 222 nm were calculated.

3. Results

3.1. C692S mutation in Rel WT protein does not alter protein–tRNA interaction

We have earlier shown that the Rel protein of *M. smegmatis* is composed of various domains. The HD and the RSD domains present in the N-terminal half of the protein have catalytic activities and are meant for the hydrolysis and the synthesis of pppGpp respectively (Fig. 1). On the other hand, TGS and ACT domains present in the C-terminal half have regulatory functions. Deletion of the C-terminal half (i.e. both TGS and ACT domains) resulted in higher synthetic activity [6]. It was therefore interesting to find out how these domains regulate the activity of the protein. It has been suggested that, TGS domain

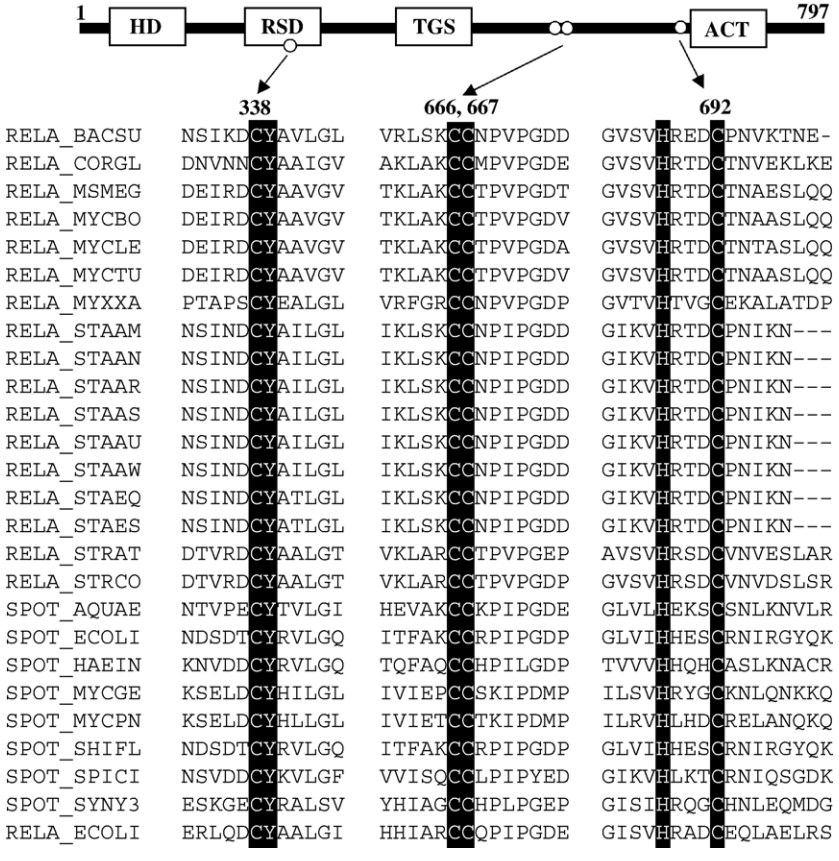


Fig. 1. Domains and multiple sequence alignment of Rel, RelA and SpoT sequences from different Gram-negative and Gram-positive bacteria showing the conservation of cysteines. Only four conserved cysteines (C338, C666, C667 and C692 with respect of Rel_{Msm}) among different Rel/Spo sequences and their flanking amino acids have been shown and marked.

has a ligand (nucleotide) binding regulatory role [42], whereas ACT domain regulates the protein activity depending upon the cellular amino acid concentration [43–45].

Upon multiple sequence alignment of proteins of Rel family, it was observed that out of the six cysteines present in the mycobacterial Rel, four (C338, C666, C667 and C692) were conserved throughout (Fig. 1). This therefore tempted us to postulate that these cysteines do play some role in the activity of the protein and its regulation. Earlier, the synthesis assays performed with the Rel WT protein in the presence of utRNA showed an increase in the activity [6]. Interestingly, one mutant in a series of triple Cys→Ser substituted proteins of Rel WT showed non-responsiveness to utRNA [6]. We concluded, by a process of elimination, that C692 (cysteine conserved in Rel protein of different organisms) played a crucial role in utRNA-mediated pppGpp synthesis.

To further investigate if this non-responsiveness behavior is because of the utRNA not binding to the protein, we probed the interaction of Rel protein with utRNA by Surface Plasmon Resonance (SPR). The interaction between Rel WT and utRNA was monitored in the concentration range between 3.125 μM and 50 μM (Fig. 2). It should be noted here that the concentrations of utRNA used in this experiment are lesser than what has been used in the pppGpp synthesis assays. Interestingly, the protein with C692S mutation showed similar affinity to the utRNA as in the case of Rel WT (Table 1). This observation immediately suggested that C692 is not required for utRNA binding to Rel.

3.2. Binding of utRNA causes a conformational change in the Rel protein that abrogates the interaction between the C692 and the residues present at the active site

The observation that C692 is not involved in utRNA binding led us to hypothesize that this cysteine residue is involved in communication with amino acids at the active site region, thereby regulating pppGpp synthesis. Therefore, in order to understand as to how utRNA mediates an interaction between the two halves of Rel, we used Förster energy transfer to measure the distance [46,47] between the two regions of the protein in the presence and absence of utRNA. C338 (present in the RSD domain) and C692 were chosen as two fixed points and were labeled with fluorescent probes to monitor the distance between them. We, at first, attempted to find out if a conformational change occurs in the protein upon utRNA binding. We, therefore, constructed cysteine mutant termed as Rel 1345, in which four of the six cysteines (C332, C492, C666 and C667) were mutated to serines. This protein was found to have very less pppGpp synthetic activity, which was because of the mutation of cysteine to serine at 332nd position as confirmed by a single cysteine mutant Rel C332S (data not shown). The protein was labeled with stoichiometric amounts of 5-[2-(iodoacetamido)ethylamino] naphthalene-1-sulfonic acid (IAEDANS) and dye to protein ratio was obtained as 1:1 (see Materials and methods). Next, the protein was labeled with increasing concentrations of 5-(Iodoacetamido) fluorescein (IAF) and quenching of IAEDANS fluorescence was monitored at 470 nm, as suggested earlier [48]. A saturation of the quenching of IAEDANS at IAEDANS/IAF molar ratio of ~1:1 suggested that at least one cysteine out of the two was labeled with IAEDANS and the other cysteine with IAF (Fig. 3). As we

Table 1
Association and dissociation constants and dissociation equilibrium constants for uncharged tRNA and different Rel proteins as measured by surface plasmon resonance

Protein	k_a ($\times 10^2$ $M^{-1}s^{-1}$)	k_d ($\times 10^{-2}$ s^{-1})	K_D ($\times 10^{-5}$ M)
Rel WT	7.48	3.50	4.67
Rel 4,5	7.60	3.22	4.24
Rel 3,4,5,6	7.81	3.10	3.97

Abbreviations used are as follows: Rel 4,5, Rel C666S, C667S; Rel 3,4,5,6, Rel C492S, C666S, C667S, C692S. (k_a, second order binding rate constant; k_d, first order dissociation rate constant; K_D, equilibrium dissociation constant).

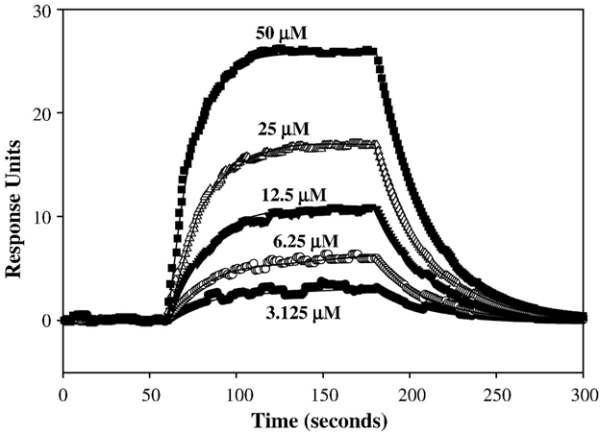


Fig. 2. Surface plasmon resonance analysis of the binding of uncharged tRNA to the Rel WT protein. An increase in the response units is suggestive of the binding of the analyte to the immobilized ligand. Various concentrations of uncharged tRNA used in the experiment are depicted. The actual experimental data along with the lines representing the global fit using Langmuir 1:1 binding has been shown. Global analysis with a 1:1 Langmuir binding model has been used for the calculation of the binding and the dissociation constants.

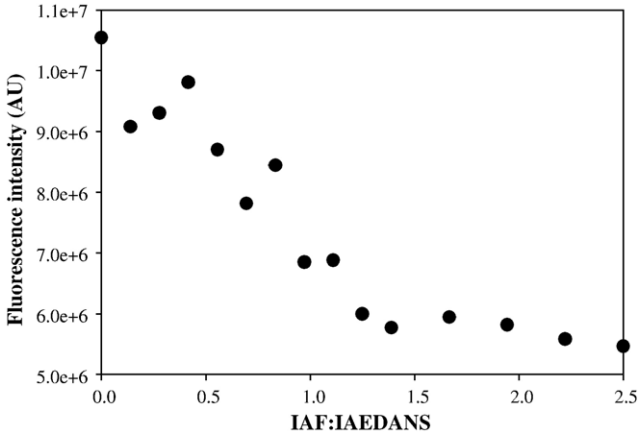


Fig. 3. Quenching of IAEDANS in a labeled protein with increasing concentrations of IAF showing a saturation of IAEDANS quenching at IAF: IAEDANS ratio of ~1:1.

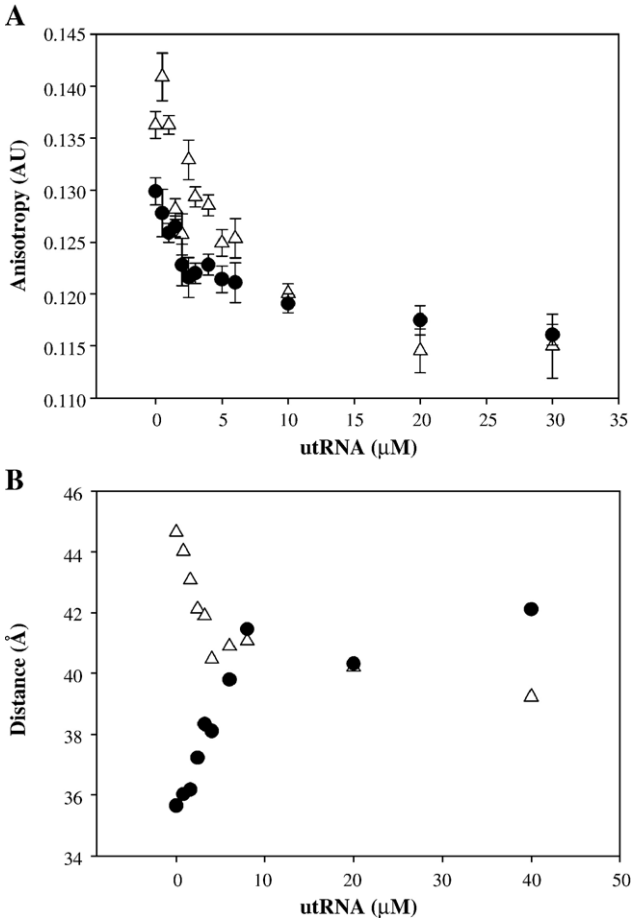


Fig. 4. a: Steady state anisotropy of IAEDANS labeled Rel 1245 (△) and Rel 1345 (●) in the presence of increasing concentrations of utRNA. b: Change in the distance between the two fluorophores IAEDANS and IAF in doubly-labeled proteins Rel 1245 (△) and Rel 1345 (●) in the presence of increasing concentrations of utRNA.

were interested only in studying behavior of the entire protein *vis-à-vis* two fixed points, and also for a given donor/acceptor pair, the Förster distance, R_0 will remain constant, specific labeling of a particular cysteine was not attempted.

To find out if a conformational change occurs in the protein upon binding of utRNA, the anisotropy of the IAEDANS labeled Rel 1345 was measured with increasing concentrations of utRNA. We observed a decrease in the anisotropy upon utRNA binding. The utRNA (~23 kDa) binding increases the overall molecular weight of the protein from ~89 kDa to ~112 kDa, which, in principle, should give rise to an increased anisotropy value [49,50]. The observed reduction in anisotropy can be explained only by assuming that utRNA binding increases the total compactness of the molecule (Fig. 4A). The IAEDANS labeled Rel 1345 was further conjugated with IAF and the doubly labeled protein was used for fluorescence resonance energy transfer (FRET) experiment to measure the distance between the C338 and C692. The mono-labeled Rel 1345 conjugated with IAF was used for the measurement of spectral overlap integral, J, for the IAEDANS and IAF (Table 2). The quantum yield, Q, for IAEDANS labeled protein was measured (Table 2) keeping quinine sulfate in H_2SO_4 as reference [37]. We measured the lifetime, τ and the rotational correlation time, τ_r for the proteins labeled with either IAEDANS or IAF (Table 2) using frequency domain fluorescence spectroscopy in which case, for a given sample, frequency-dependent phase and modulation are measured [35,51]. All values were obtained after single exponential fit. The measured τ and τ_r for IAEDANS and IAF are within limits and similar values have been reported earlier [52]. The orientation factor, κ^2 was assumed to be 0.476 as the rotational correlation time (τ_r) for both donor and acceptor is more than their lifetime (τ) and therefore, the probe is not an isotropic rotor during the lifetime of the excited state [38–40]. The Förster distance, R_0, for the IAEDANS/IAF pair was measured to be 40.6 Å (when $\kappa^2=0.476$) and this value has been used for the calculation of the distances. Nonetheless, if κ^2 is assumed to be 2/3 then the R_0 comes out to be 45.5 Å which agrees well with the reported value [53–55]. However, so far we did not find any studies between these two probes with restricted rotation and κ^2, 0.476 as reported here. Nevertheless, this difference in κ^2 only alters the distance ~10% due to sixth power dependence [36,38,40].

The distance between the C338 and C692 in Rel 1345 was measured to be 35.6 Å, which increased up to 42.1 Å with increasing concentrations of utRNA (Fig. 4B) indicating, therefore, the movement of C-terminal part of the protein away from the catalytic site situated in the RSD domain, upon utRNA binding. Although this explained the enhancement in the synthesis activity of the protein by disrupting the interaction between the C692 and the catalytic site residues, did not corroborate with the anisotropy measurement (Fig. 4A) because such a movement would not have made the protein compact. We, therefore, constructed another mutant in the Rel protein termed Rel 1245 in which all the six cysteines except two (C492 and C692) were mutated to serines. It should be noted here that Rel 1245 also had similar pppGpp synthetic activity as that of Rel C332S mutant. The C492, present in the TGS domain, was chosen because it falls approximately in the middle of the other two (C338 and C692). The protein was labeled with IAEDANS and quenching of IAEDANS in the presence of increasing concentrations of IAF was monitored to confirm the labeling of at least one cysteine with IAEDANS and the other with IAF

Table 2
Parameters for the measurement of Förster distance, R_0 between IAEDANS/IAF pair, lifetime and rotational correlation time of IAEDANS and IAF

Fluorophore	τ (ns)	χ^2	τ_r (ns)	χ^2	J ($M^{-1}cm^3$)	Q	R_0, $\kappa^2=0.476$	R_0, $\kappa^2=2/3$
IAEDANS	13.52	3.09	118.77	0.201	3.868×10^{-13}	0.224	40.6 Å	45.5 Å
IAF	3.98	2.05	11.74	0.88				

Refractive index, n was taken as 1.4. (τ = lifetime; τ_r = rotational correlation time; J = spectral overlap integral; Q = quantum yield; R_0 = Förster distance.)

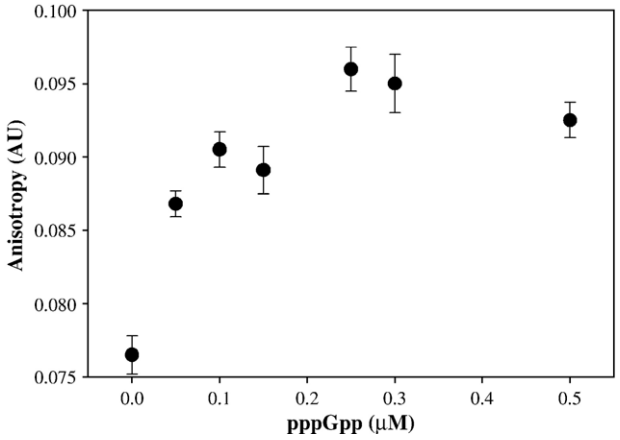


Fig. 5. Steady state anisotropy of IAEDANS labeled Rel CTD 45 in the presence of increasing concentrations of pppGpp.

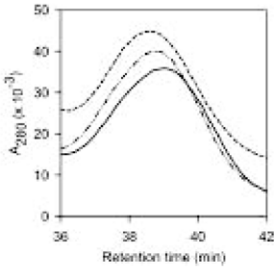
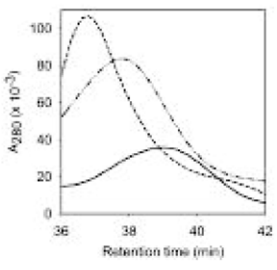
(data not shown). The anisotropy of Rel 1245 in the presence of increasing concentrations of utRNA was monitored. The decrease in the anisotropy was again suggestive of the total compaction of the protein molecule (Fig. 4A). The IAEDANS labeled Rel 1245 was further labeled with IAF and the distance between the two cysteines was measured in the presence of utRNA. Interestingly, the distance, in this case between the two cysteines was found to be 44.6 Å, which decreased with increasing concentrations of utRNA to 39.2 Å (Fig. 4B). The two distance measurements thus, suggested that the C-terminal part of the protein (C692), upon utRNA binding, moved away from the RSD domain (C338) but towards the TGS domain (C492) thereby making the molecule denser.

3.3. Binding of pppGpp to the C-terminal part of the protein makes it more unstructured: a negative feedback?

The fluorescence data motivated us to propose the movement of the C-terminal part of the protein away from the active site in the RSD domain thus enhancing the synthesis of pppGpp in the presence of utRNA. We, however, had previously noticed that the amount of enhancement in the synthesis of pppGpp upon utRNA binding was only ~1.5 fold whereas the synthesis activity of Rel NTD was found to be ~5 fold higher than the Rel WT [6]. It thus indicated the presence of other mechanisms towards the regulation of the Rel activity. TGS domain has been proposed to have a regulatory role mediated by ligand (most likely nucleotide) binding [42] and therefore, the presence of it in the Rel protein could alter the activity by binding to the nucleotides. We attempted to look for possible pppGpp binding to Rel. For this purpose, we utilized Rel CTD alone as the N-terminus of the Rel accommodates HD domain required for carrying out pppGpp hydrolysis [6,34,56].

Rel CTD 45 was obtained by mutating two cysteines C666 and C667 (positions with respect to Rel WT) to serines that helped in reducing the amount of aggregation observed in Rel CTD due to the four free cysteine residues. The protein was labeled with IAEDANS in 1:1 molar ratio. It is expected that either of the two remaining cysteines at 492 and 692 or both in mixed proportions will be labeled. The labeled protein was used for measuring steady state anisotropy. With increasing concentrations of pppGpp, an increase in anisotropy was observed (Fig. 5). In order to find out if this change in anisotropy was due to the multimerization of the protein upon pppGpp binding, as suggested earlier in Rel protein of *M. tuberculosis* [56], gel-exclusion chromatography was carried out. Rel CTD 45 was incubated for 30 min at 25 °C with increasing concentrations of pppGpp and was injected in a Superdex 200 gel filtration column at a flow rate of 0.4 ml/min. A maximum shift of ~0.40 min was observed in the retention time of Rel CTD 45 in the presence of pppGpp which was suggestive of local unfolding in the protein upon binding to pppGpp (Table 3). The retention time of pppGpp was found to be 68.03±0.05 min and was used as a control. We could not detect any multimerization occurring in Rel CTD 45 upon pppGpp binding. Multimerization would result in at least a dimer in the product whose retention time will be lower. To measure the degree of unfolding mediated by pppGpp, Rel CTD 45 was incubated with increasing concentration of urea and was injected in the gel filtration column. A shift of approximately 2.22 min in the protein retention time was observed with an increase in urea concentration to up to 6 M (Table 3). However, comparing the two gel-filtration profiles, it was concluded that the unfolding mediated by pppGpp was not as drastic as with

Table 3
Retention time of Rel CTD 45 on a Superdex 200 gel filtration column in the absence and presence of different concentrations of either pppGpp or urea

pppGpp			Urea		
Conc. (μM)	Ret. time (min)		Conc. (M)	Ret. time (min)	
0.0	39.025		0.0	39.025	
4.0	38.675		1.5	37.750	
40.0	38.625		6.0	36.800	

(Conc. = concentration; Ret. Time = retention time). Insets show the elution profile of Rel CTD with 0 μM (——), 4 μM (–··–··–) and 40 μM (- - - - -) of pppGpp and with 0 M (——), 1.5 M (–··–··–) and 6 M (- - - - -) of urea.

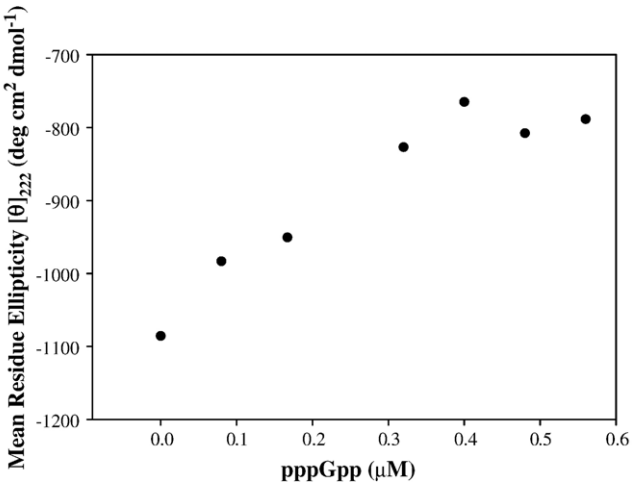


Fig. 6. Change in mean residue ellipticity of Rel CTD 4,5 with increasing concentrations of pppGpp, monitored at 222 nm.

urea at a concentration as low as 1.5 M. Circular dichroism of Rel CTD 4,5 in the presence of pppGpp further confirmed the unfolding of the protein mediated by the binding of pppGpp to the protein (Fig. 6). Together, these results suggested that the protein was becoming more unstructured in the presence of its product. A pppGpp synthesis assay by the Rel WT and Rel NTD in the presence of excess amounts of exogenously added non-labeled pppGpp demonstrated a reduction in the synthesis of pppGpp from both the enzymes as expected (data not shown). However, the degree of inhibition was more in the case of Rel WT as compared with Rel NTD thus confirming that the binding of pppGpp to the C-terminal part of the protein inhibits the enzyme synthesis activity. Moreover, at higher concentrations of non-labeled pppGpp, both the proteins showed reduced synthetic activity (data not shown), which, we believe, is due to the intramolecular crosstalk between the HD and the RSD domains as suggested earlier [34].

4. Discussion

Production of (p)ppGpp plays a crucial role in the survival of bacteria during starvation. The protein responsible for its synthesis, Rel, is being studied extensively from several other systems [9–12]. The crystal structure of Rel from *S. equisimilis* answered the long remained query of the balance between the two opposing activities of Rel by presenting an 'inter domain crosstalk' model [34]. It was suggested that due to the conformational change in the molecule, there would remain only one activity. This although explained how a futile cycle of simultaneous synthesis and hydrolysis of pppGpp can be avoided, was unable to involve the C-terminal domain in the regulation. An earlier report on Rel from *S. equisimilis* reasoned an interaction between the C-terminal and the N-terminal regions of the protein required for the regulation of the two activities [33]. This interaction was suggested for the hydrolysis of ppGpp. Upon binding of ligand such as ribosome or by deletion of the C-terminal half, this interaction will be sequestered, therefore, resulting in the enhancement of ppGpp synthesis. Our findings in the present report, however, suggest that the synthesis activity of the protein is regulated by the interaction between the RSD domain and the C-terminal part of the protein.

In *M. smegmatis*, we proposed earlier, experimenting with a series of cysteine mutants, that C692S mutation in the Rel makes it non-responsive to utRNA. The surface plasmon resonance data suggests that the C692S mutation does not alter the binding of utRNA to the protein. This immediately tempted us to speculate that there exists an interaction or, in other words, a "crosstalk" between the C692 and the pppGpp-synthesis active site present in the RSD domain. The distance between two different pairs of cysteines was measured by Förster resonance energy transfer method. As the probes showed restricted rotation in the excited state, a different value of orientation factor was used here. Although Fig. 3B shows a change in distance not more than 6 Å in either pair upon utRNA addition, we feel this is reasonable and the effect is genuine because they show concentration dependence in each case and more importantly, the magnitude of the difference is opposite. It should be emphasized that we used same κ^2 value in R_0 calculation in both cases. As change in fluorescence anisotropy is a measure of molecular dimensions, utRNA binding in principle, should increase the net change in utRNA anisotropy. However, in both the cases of Rel 1245 and Rel 1345, anisotropy showed uniform decrease in value indicating thereby that utRNA bound Rel showed more "compact-conformation" in the place of unbound Rel, which represents "open-conformation".

Earlier studies with Rel_{Mtb} have shown that the protein shows higher synthetic activity in the presence of utRNA which increases further upon the addition of ribosome and poly(U) RNA in the system [3]. However, removing utRNA from such a reaction rendered the Rel-activating complex incapable of stimulating Rel [3], thus confirming the significance of utRNA in the Rel activation. We have used utRNA in the present work

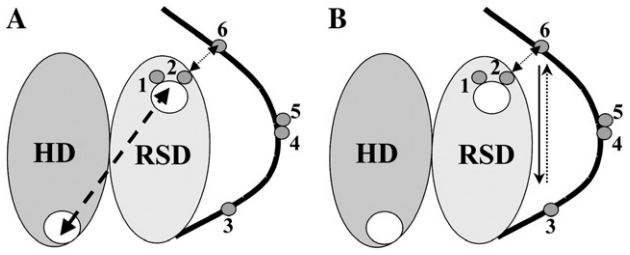


Fig. 7. Model depicting the regulation in the Rel protein by means of utRNA and pppGpp binding. The empty circles represent the two catalytic sites present in the HD and the RSD domains. Filled circles represent the cysteines (1=332; 2=338; 3=492; 4=666; 5=667; 6=692). A) Presentation of different interactions that appear in the Rel protein for the regulation of its activity. Long dashed double-headed arrow represents the interaction between the two catalytic sites, termed "inter-domain crosstalk". Double-headed dotted arrow represents the interaction between the C692 present at the C-terminus and the residues present in the RSD domain of the protein. B) Single-headed arrows represent the direction of the movement of C-terminal part of the protein upon binding to ligands. Solid arrow designates the movement in this region of the protein due to the utRNA binding and dotted arrow represents the movement upon binding of pppGpp.

in order to find out if this molecule can induce a conformational change in the Rel_{Msm}, which will then answer the activation mechanism of Rel during amino acid starvation. Negligible effect of charged tRNA alone has been shown on the Rel_{Mtb} activity [3] and hence, this molecule was not used in the present studies.

We observed that pppGpp binds to the C-terminal part (most likely TGS domain as it has nucleotide binding regulatory role) of the protein making it more unstructured. It appears that this unfolding will result in the establishment of the interaction between C692 and C338 and thus negatively regulate the synthesis of pppGpp. Moreover, the pppGpp-mediated effect on protein occurs in micromolar concentrations, indicating thereby that this process inhibits the synthesis of excessive amounts of pppGpp in the cell. A pppGpp synthesis assay with the Rel WT in the presence of exogenously added non-labeled pppGpp did show an inhibitory effect on the synthetic activity of this protein as expected. However, the degree of inhibition in this case was found to be more than that of Rel NTD at lower concentrations of non-labeled pppGpp.

With the data presented here, we propose a model for the regulation of the pppGpp synthesis by Rel protein (Fig. 7) that suggests that Cys at the C-terminal part of the protein (C692) inhibits the synthesis of pppGpp by interacting with the residues present in the RSD domain. Although we give emphasis on cysteine involvement in regulation, we do not rule out the possibility of other amino acids, present in the vicinity, required for this process. Upon binding to utRNA, the interaction between these two cysteines is lost which results in an increase in the synthesis of pppGpp. It has been reported earlier that the hydrolysis of pppGpp is drastically reduced in the presence of utRNA [3,6]. This observation can be explained with the help of an "inter-domain cross talk" model, as proposed earlier in the case of *S. equisimilis* [34] where, activation of one form (either synthesis or hydrolysis) leads to the deactivation of another. It has been shown earlier that a *trans*-complementation of Rel N-terminus with the Rel C-terminus did not give a regulated activity for both *in vivo* [33] and *in vitro* [6] proteins, although a chimeric molecule having the N-terminus from Rel_{Seq} and the C-terminus from RelA on a single polypeptide chain behaved as wild-type [33]. Using the model presented here, we reason that this observation is due to the residues present at the RSD domain and at the C-terminus, which are unable to interact in a specific manner required for the regulation during *trans*-complementation.

Acknowledgements

V.J. is supported by senior research fellow award from the Council for Scientific and Industrial Research, Govt. of India. Srilatha is acknowledged for her help in Surface plasmon resonance experiments.

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