A revised mechanism for (p)ppGpp synthesis by Rel proteins: The critical role of the 2’-OH of GTP

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Bacterial Rel proteins synthesize hyperphosphorylated guanosine nucleotides, denoted as (p)ppGpp, which by inhibiting energy requiring molecular pathways help bacteria to overcome the depletion of nutrients in its surroundings. (p)ppGpp synthesis by Rel involves transferring a pyrophosphate from ATP to the oxygen of 3’-OH of GTP/GDP. Initially, a conserved glutamate at the active site was believed to generate the nucleophile necessary to accomplish the reaction. Later this role was alluded to a Mg²⁺ ion. However, no study has unequivocally established a catalytic mechanism for (p)ppGpp synthesis. Here we present a revised mechanism, wherein for the first time we explore a role for 2’-OH of GTP and show how it is important in generating the nucleophile. Through a careful comparison of substrate-bound structures of Rel, we illustrate that the active site does not discriminate GTP from dGTP, for a substrate. Using biochemical studies, we demonstrate that both GTP and dGTP bind to Rel, but only GTP (but not dGTP) can form the product. Reactions performed using GTP analogs substituted with different chemical moieties at the 2’ position suggest a clear role for 2’-OH in catalysis by providing an indispensable hydrogen bond; preliminary computational analysis further supports this view. This study elucidating a catalytic role for 2’-OH of GTP in (p)ppGpp synthesis allows us to propose different mechanistic possibilities by which it generates the nucleophile for the synthesis reaction. This study underscores the selection of ribose nucleotides as second messengers and finds its roots in the old RNA world hypothesis.

A hyperphosphorylated guanosine nucleotide called (p)ppGpp shields bacteria from environmental stress and also plays a crucial role in bacterial pathogenesis; particularly in virulence, biofilm formation, and antibiotic resistance (1–3). There has been a special interest in understanding the role of (p)ppGpp in bacterial physiology because of its exclusive presence in bacteria and chloroplasts of plants but not in humans (4). Therefore, enzymes that metabolize (p)ppGpp form distinct targets to develop new age anti-bacterials. Before now, efforts in this regard have found limited success and at best resulted in low potency inhibitors (5–7). To develop effective inhibitors, it is essential to understand the catalytic and regulatory mechanisms in detail. Rel proteins, with varying length and sequence composition, have been reported from bacteria and chloroplasts; all of which are placed under the RelA/SpoT homolog superfamily. Of these subgroups, monofunctional Rel, bifunctional Rel, and SAS have been extensively studied and classified based on the number of domains they possess and their associated catalytic activities (4). Irrespective of the length, the number of domains, and the mode of regulation, the active site responsible for the synthesis activity is common to all enzymes of these subgroups. Crystal structures of Rel proteins bound to substrates at the synthesis active site are available. They are (i) GDP-bound structure of the N-terminal domain of Rel from Staphylococcus equisimilis (⁸RelSeq), (ii) AMP-CPP (nonhydrolyzable ATP analog) bound structure of SAS1 (RelQ) from Bacillus subtilis, and (iii) the structure of SAS2 (RelP) from Staphylococcus aureus bound to GTP and AMP-CPP together (i.e. the precatalytic structure). These provide great insights into the active-site architecture (8–10); substrates GTP/GDP, ATP, and the catalytic metal ion (Mg²⁺) bind at distinct locations at the synthesis active site. The synthesis reaction is accomplished by transferring a diphosphate (P₂O–Pγ) from ATP to the 3’-OH oxygen of GTP/GDP, resulting in the formation of pppGpp or ppGpp. Based on a comparison of the active sites of the synthetase domain of Rel and the palm domain of DNA polymerase (Pol) β, it was believed that the conserved Glu⁹23 at the active site in ⁸RelSeq would activate the 3’-OH of the guanosine nucleotide for a nucleophilic attack on the β-phosphate of ATP (Fig. 1A) (8). Glu³21 of ¹⁹RelSeq corresponds to Glu¹³⁹ in SAS1 and Glu¹⁷⁴ in SAS2. While reporting the AMP-CPP bound SAS1 structure, the authors reiterated that Glu¹³⁹ in SAS1 would activate the 3’-OH of GTP for nucleophilic attack (9). However, the last reported precatalytic structure of SAS2 (with both substrates bound together) does not support this mechanism and instead presents a metal ion promoted activation with inadequate details of the deprotonation mechanism (Fig. 1B) (10).

Moreover, the structural basis for the selection of nucleotide substrates in Rel still needs to be addressed rigorously. For example, monofunctional Rel preferentially utilizes GDP over GTP as the acceptor of the pyrophosphate, whereas bifunctional Rel favors GTP over GDP (11–13). This preference for one guanine nucleotide over another has been found to be associated with the conserved sequence motif RXK(D/E)XDD in the synthesis domain (13). In addition, a recent study suggests that RelP (SAS2) from Enterococcus faecalis can also use GMP...
as an acceptor nucleotide to form pGpp, in addition to GTP and GDP (14). Similar pyrophosphorylated guanosine variant, pGpp formation by RelA from *Escherichia coli* was also reported previously (13). Interestingly, apart from the above-mentioned guanine nucleotide, ITP too has been reported to act as substrate to accept the pyrophosphate (11, 12, 15). Although different guanine (or even inosine) nucleotides are accepted as substrates, it is intriguing that dGTP/dGDP do not form hyperphosphorylated products (11, 12, 15). On the other hand, ATP and dATP can both act as donors of the pyrophosphate (11, 12). To the best of our knowledge, no study has so far discussed the reasons for the discrimination between GTP and dGTP by Rel proteins.

In this study, we revisit the pyrophosphate transfer mechanism proposed for the (p)ppGpp synthesis reaction. With careful analysis of the available substrate-bound structures of Rel family proteins, we question the proposed mechanism explaining the activation of the 3’-OH for a nucleophilic attack on the β-phosphate of ATP. We further attempt to understand why Rel does not utilize dGTP as a pyrophosphate acceptor, although it is similar to GTP. The structural analysis combined with biochemical studies presented here, confirm that the 2’-OH of GTP is not essential for binding at the active site. However, the inability to utilize dGTP as a substrate by different Rel homologs suggests an important role for 2’-OH in catalysis. Here, we investigate possible roles of 2’-OH of GTP for the catalytic reaction in Rel and show that it is an essential catalytic element. This work yet again underscores the selection of ribose nucleotides as second messengers and finds its roots in the old RNA world hypothesis.

**Results and Discussion**

**Substrate-bound Rel structures do not clarify the catalytic mechanism for (p)ppGpp synthesis**

Diverse Rel homologs from bacteria and plants with distinct catalytic and regulatory domains are known (4). However, very few crystal structures are available, and that too of the catalytic domain alone. Despite the difference in their sequence length and domain architecture, bacterial bifunctional Rel and SAS proteins have a conserved active site for (p)ppGpp synthesis (Fig. 2A). The atomic structures of Rel homologs reveal that a characteristic fold, constituting five anti-parallel β-strands surrounded by α-helices, forms the (p)ppGpp synthetase domain (Fig. 2B) that shares structural similarity with the nucleotidyltransferase superfamily (8). Additionally, crystal structures of Rel bound to its substrates have further revealed that the β1–α1–β2 region (Fig. 2B, olive green) of the synthetase fold provides stabilizing interactions to ATP, whereas the β3–loop–β4 (Fig. 2B, purple) structure creates the site for GTP/GDP binding. To compare the structural differences between the synthesis domains of Rel homologs in different substrate-bound states, we superimposed the crystal structures of GDP-bound NRel_seq (PDB code 1VJ7, chain A), AMP-CPP–bound SAS1 (PDB code 5F2V, chain O), and the precatalytic-state structure of SAS2 (bound to both GTP and AMP-CPP together) (PDB code 6EWZ, chain A) (substrates have been deliberately omitted for clarity) (Fig. 2C). The synthesis domain of NRel_seq superposes well with SAS1 and SAS2, with root-mean-square deviations of 1.060 and 0.69 Å, respectively. Despite high structural similarity, significant differences could be observed in the synthetase fold. In the structure of SAS1 bound to AMP-CPP, the guanine nucleotide is absent, and the loop connecting β3 and β4, which is known to stabilize GTP, is unstructured (Fig. 2C and Fig. S1B). Similarly, it can be noted that the helix connecting β1 and β2 reorients in SAS1 and SAS2 to accommodate AMP-CPP compared with the structure of NRel_seq that is bound to GDP alone (Fig. 2C and Fig. S1). These observations highlight substrate-induced restructuring of the active site elements in Rel. More evident in the superposed structures is the relatively open conformation of the active site of SAS2, where both the substrates are present together, emphasizing conformational changes in the synthetase active site caused by substrate binding. Such rearrangements are believed to be essential, not only for accommodating the substrates at the active site but also to bring the catalytic elements together (8, 9). Therefore, we further analyzed the position of the other elements of the active sites that are believed to play important roles for catalysis.

While reporting the GDP-bound NRel_seq structure, the authors suggested rearrangements of the active site region caused by the binding of the second substrate. It was suggested that this rearrangement to a catalytically favorable conformation would accomplish the transfer of the pyrophosphate from ATP to the 3’-OH of GTP/GDP (8). Further, the details of the mechanism of pyrophosphate transfer were proposed by drawing similarities to the active site of DNA Polβ. In DNA Polβ,
the 3’-OH of one substrate (nucleotide) attacks the phosphodiester linkage of the second substrate (another nucleotide), resulting in the transfer of a dNMP to the 3’ position. In the case of Rel, the reverse would happen; instead of dNMP, the pyrophosphate would be transferred to the acceptor group. Both of these enzymes use Mg$^{2+}$ for catalysis. Therefore, on comparing the active sites of the then-available crystal structures of these enzymes, Hogg et al. (8) had proposed a mechanism for pyrophosphate transfer. According to the proposal, a proton would be transferred from the 3’-OH to a highly conserved glutamate residue from the active site (which was identified as Glu$^{323}$ in case of NRel$_{Seq}$) to activate the 3’-OH group of the acceptor nucleotide (GTP/GDP). The loss of (p)ppGpp synthesis activity upon mutating this conserved Glu in Rel proteins further strengthened this assumption (8, 16). On examining GDP-bound NRel$_{Seq}$ structure, the nearest carboxyl oxygen of Glu$^{323}$ is at a distance of nearly 6 Å from the 3’-OH of the bound GDP (Fig. S2). However, Hogg et al. (8) then suggested that rearrangement(s) at the active site will bring this catalytic residue within a hydrogen-bonding distance to the 3’-OH of the guanine nucleotide, when both the substrates bind simultaneously. This would ensure the abstraction of the proton to generate a nucleophile for the synthesis reaction to take place. As speculated, there are significant changes in the synthesis domain in response to substrate binding. However, examining the SAS2 precatalytic structure, where both the substrates are bound at the active site, the proposed proton acceptor Glu$^{174}$ (corresponding to Glu$^{323}$ of NRel$_{Seq}$) is still at a distance of 4.92 Å from the 3’-OH group of the bound GTP (Fig. 3A). The observed distance between these two groups does not support the hypothesis by Hogg et al. (8) for proton transfer. On the contrary, from the crystal structure of SAS2 it appears that, like Asp$^{107}$, the role of Glu$^{174}$ is majorly to stabilize the catalytic Mg$^{2+}$ at the active site (via water-mediated interactions) rather than activating the 3’-OH (Fig. 3B). Apparently, mutating the conserved Asp in NRel$_{Seq}$ also results in the loss of activity as
with the Glu mutant (8). A similar loss of activity is observed on mutating corresponding conserved residues (Asp317 or Glu373) in \(^3\) \(^{N}\)Rel\(_{Mtb}\). We speculate that a critical requirement is that both Asp and Glu, the conserved residues, would be needed for the proper coordination of the catalytic metal ion at the active site (Fig. 3B). Interestingly, Manav et al. (10) argue that electro-positive Mg\(^{2+}\) will promote the deprotonation of 3'-OH but provide no further views concerning the final base that will abstract the proton. Irrespective of the influence of the electro-positive nature of Mg\(^{2+}\) for deprotonation, the proton from the 3' hydroxyl group has to be transferred to an acceptor moiety. Upon careful examination of the precatalytic structure, there seems to be no such group in close proximity of the 3'-OH of GTP—not even a water molecule that can be activated by another residue. This makes it difficult to map the deprotonation mechanism (Fig. 1B). Moreover, the catalytic mechanism in DNA polymerase, which was a basis for the proposed catalytic mechanism in Rel, has been reestablished through molecular simulation studies in recent times (17). The new evidence suggests that the generation of nucleophile at 3'-OH is not by any residue from the active site; instead it is due to intramolecular hydrogen bonding (17). In view of these observations, we felt the need to revisit and critically scrutinize the current structural and biochemical data further, to understand the mechanism for (p)pppGpp synthesis. These efforts are presented below.

dGTP can compete with GTP for binding to active site

For (p)pppGpp synthesis, GTP/GDP is the acceptor of the pyrophosphate group coming from ATP. We had earlier established that the choice of GTP verses GDP as a substrate is based on the conserved sequence motif, RXKD or EXDD, that is present in the synthesis domain of Rel; whereas the former prefers GTP, the latter prefers GDP (13). Moreover, recently it has been established that GMP too can be utilized as a substrate in some cases, where it results in the formation of pGpp, a variant of hyperphosphorylated guanosine nucleotide (14). In summary, Rel proteins utilize different guanosine phosphates as a substrate to form a range of hyperphosphorylated guanosine molecules. The selectivity for the guanine base in Rel, over other nucleotides, is achieved through noncovalent interactions offered by the active-site residues (8, 10). Surprisingly it has been noted in earlier studies that the dGTP is incapable of forming a product, when provided as an acceptor nucleotide in place of GTP for the synthesis reaction (11, 12, 15, 18, 19). dGTP differs from GTP by the absence of a hydroxyl group at the 2' position, with no change in the guanine base. To the best of our knowledge, no study has discussed the molecular basis explaining this discrimination between the use of two similar nucleotides, i.e. GTP and dGTP, by Rel proteins. Because GTP and GDP-bound structures of Rel family proteins are now available, we decided to examine attributes that render selective utilization of GTP but not dGTP. Enzymes like RNA polymerase distinguish its substrate NTPs from the dNTPs by selectively providing a stabilizing interaction to the 2'-OH group of NTPs (Fig. S3A), whereas DNA polymerases have steric gates to avoid binding of NTPs over dNTPs at the active site (Fig. S3B) (20–22). Hence, we examined possible interactions at the active site of Rel that could selectively recognize the 2'-OH of GTP/GDP. The nearest residue to the 2'-OH of the GDP bound to \(^{N}\)Rel\(_{seq}\) and GTP, present in the precatalytic structure of SAS2 is a conserved Gln (Gln\(^{325}\) in \(^{N}\)Rel\(_{seq}\) and Gln\(^{176}\) in SAS2). The side-chain nitrogen atom of this Gln and 2'-O of the bound guanine nucleotide are more than 3.3 Å apart. It is not clear whether this interaction can discriminate between the binding of dGTP and GTP (Table 1 and Fig. S4). Can the absence of 2'-OH in dGTP preclude its binding to Rel? Further experiments were designed to answer this.

The ability of GTP and dGTP to bind at the active site was determined in the presence of mant-GTP, a fluorescently labeled analog of GTP. The fluorescence signal of mant-GTP increases upon binding the protein and has been used to monitor guanine nucleotide binding in Rel proteins (23). A modified protocol was used here to monitor the extent of mant-GTP binding, as explained under “Experimental procedures.” In Fig. 4A, the increased fluorescence signal in the presence of \(^{N}\)Rel\(_{seq}\) (spectra in black) compared with the signal of mant-GTP alone in buffer (spectra in cyan) confirmed nucleotide binding to the protein (Fig. 4A, GTP and dGTP). Further, the binding of mant-GTP to the protein was examined in the presence of varying concentrations of GTP or dGTP. The fluorescence emission signal reduced in the presence of GTP (red, 5 \(\mu\)M); a gradual reduction with increasing GTP concentrations (5–40 \(\mu\)M) indicates a competition between GTP and mant-GTP in \(^{N}\)Rel\(_{seq}\) (Fig. 4A). Interestingly, dGTP too exhibited a similar competition as seen from the decrease in fluorescence emission with increasing concentrations of dGTP in Fig. 4A. The effect was comparable at micromolar concentrations used in the experiment. To substantiate this, we examined binding of dGTP to \(^{N}\)Rel\(_{seq}\) using isothermal titration calorimetry. The dissociation constant (\(K_d\)) of GTP and dGTP to \(^{N}\)Rel\(_{seq}\) was determined by dynamic light scattering.
was determined using ITC. Titration of nucleotides against the protein depicted significant heat change (Fig. 4B). The data were analyzed using Microcal origin 7 software and fitted to the “one-set-of-site” model. The stoichiometry was nearly 1:1 (Table 2). $K_d$ for GTP was found to be 5.6 µM, whereas for dGTP it is was 8.4 µM (Table 2). The comparable $K_d$ values confirmed that dGTP too binds $^{35}$RelSeq. To evaluate the importance of the hydroxyl group at both the 2’ and 3’ positions for GTP binding, we further titrated ddGTP (di-deoxyGTP) with $^{35}$RelSeq wherein the hydroxyl groups at both 2’ and 3’ positions are absent. This too bound the protein (Fig. 4B and Table 2), suggesting that the interactions made by the hydroxyl groups at the 2’ and 3’ positions are not important for substrate binding. We therefore infer that the guanine base is important in stabilizing

![Figure 4](image-url)

**Figure 4. Guanine nucleotide binding to $^{35}$RelSeq**. A, mant-GTP upon binding to $^{35}$RelSeq results in increased fluorescence emission. In the presence of different concentrations (5–40 µM) of GTP (left panel) and dGTP (right panel), the fluorescence emission was monitored. The color codes of the curves are as indicated outside the inset. B, the heat change profile of GTP, dGTP, and ddGTP when titrated against $^{35}$RelSeq using isothermal titration calorimetry. Binding constants were determined by using single-site binding isotherm. The upper panels represent the raw ITC data. The lower panels are the integrated peaks of heat change derived after subtracting the dilution enthalpies for respective nucleotides from the raw data.

**Table 2**

| Nucleotide | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol/°C) | $K_d$ (µM) | Stoichiometry |
|------------|----------------------|------------------------|------------|---------------|
| GTP        | $-13.6$              | $-23.2$                | $5.6 \pm 0.5$ | 0.5           |
| dGTP       | $-13.5$              | $-21.2$                | $8.4 \pm 0.8$ | 0.75          |
| ddGTP      | $-11.9$              | $-16.6$                | $10.2 \pm 0.7$ | 0.85          |
and selecting acceptor nucleotides at the active site. This establishes the fact that 2’-OH is not required for the binding of GTP to Rel but may have other roles.

**dGTP binding does not result in a hyperphosphorylated product**

If the mechanism proposed by Manav et al. (10) (Fig. 1B) were true, replacing GTP with dGTP at the active site of Rel should not disturb the elements required for the activation of 3’-OH and pyrophosphate transfer. Hence, one would anticipate that the product should form even with dGTP as the substrate, because the 2’-OH should not play any role in catalysis.

Fig. 4 further depicts how dGTP binds NRelSeq with an affinity comparable with that of GTP (in the micromolar concentration range). Therefore, we evaluated whether dGTP could be utilized as a substrate for the synthesis reaction and yield a hyperphosphorylated product. For this, after carrying out the synthesis reaction in presence of ATP and either GTP or dGTP, the nucleotides from the reaction mixture were separated using FPLC (see “Experimental procedures”). In the FPLC elution profile, where GTP was used an acceptor nucleotide, four discrete peaks were seen (Fig. 5A, upper panel). By comparing these peaks with the elution profiles generated with control nucleotides, we confirm the presence of pppGpp and by-product.
AMP, apart from the peaks corresponding to the remaining unutilized substrates (GTP and ATP) when GTP was used as one of the substrates, whereas only two peaks were observed in the elution profile obtained upon using dGTP as a substrate (Fig. 5A, lower panel). In contrast, here, peaks corresponding to substrates ATP and dGTP (but not the products) could be detected. Neither the hyperphosphorylated product–formed pppGpp nor AMP were detectable, which clearly suggests that the reaction did not take place. This result was further validated with the help of radiolabeled nucleotide-based assay. Corroborating the results of FPLC analysis, here too, only when GTP was used in the reaction, a spot corresponding to the hyperphosphorylated product (pppGpp) could be visualized on the autoradiograph; the same could not be detected when dGTP was used instead (Fig. 5B). Next, to see whether such nucleotide selectivity is a general behavior of Rel homologs, we performed synthesis reactions using \(^{\text{NRel}}_{\text{Mtb}}\) and \(^{\text{NRel}}_{\text{A}}\) (N-terminal RelA from E. coli), with both GTP and dGTP as the acceptor nucleotides. The results suggest that \(^{\text{NRel}}_{\text{Mtb}}\) and \(^{\text{NRel}}_{\text{A}}\) too cannot utilize dGTP as a substrate (Fig. S5). Moreover, \(^{\text{NRel}}_{\text{Seq}}\) activity was inhibited at higher concentrations of dGTP as noted when the (pp)ppGpp synthesis reaction (using GTP as a substrate) was carried out in the presence of dGTP. Here, the synthesis activity was measured by evaluating the amount of remaining ATP in each case using Kinase-Glo reagent (see “Experimental procedures”). Decrease in percentage activity at high concentrations of dGTP (Fig. 5C) implies that it competitively binds the active site of \(^{\text{NRel}}_{\text{Seq}}\) and inhibits product formation. Taking all of these observations into consideration, we conclude that dGTP would serve as a catalytically inactive analog because of the absence of 2’-OH, implying that 2’-OH is an important element participating in catalysis.

**SAS1 too cannot utilize dGTP for (p)ppGpp synthesis**

SAS are small, single-domain versions of Rel that are only capable of synthesizing (p)ppGpp. The synthesis active sites of full-length Rel and SAS1 are structurally identical, and the catalytic residues are also conserved (9). We therefore expected that with SAS1 too, dGTP should not yield a hyperphosphorylated product. When the reaction products were analyzed using FPLC-based separation, indeed SAS1, like the other Rel homologs, could not utilize dGTP as a substrate (Fig. 6A). To corroborate this, (p)ppGpp synthesis by SAS1 was monitored over a certain time period using mant-ATP (a fluorescent analog of ATP) as a substrate. Here, following the transfer of pyrophosphate from mant-ATP onto GTP, the by-product of the reaction mant-AMP would leave the active site. As the reaction takes place, the number of mant-AMP molecules bound to SAS1 should decrease with time and therefore result in a decreased fluorescence signal, because free mant-AMP, which is not bound to the protein, would not yield a signal (Fig. 6B). Fluorescence emission was monitored (until 14 min) after initiating the synthesis reaction upon adding GTP (denoted as 0 min, red spectra) to the reaction mixture containing SAS1 and mant-ATP. Over time, a gradual drop in fluorescence signal was seen, indicating the utilization of mant-ATP for product formation (Fig. 6B, GTP panel). In a control reaction repeated in the presence of EDTA, a metal ion chelator, a stable fluorescence signal was observed for the same time period (Fig. 6B, panel GTP + EDTA), implying that the reaction could not occur when Mg\(^{2+}\) was chelated. Similarly, unlike for GTP, a stable fluorescence signal that did not reduce steadily over time was observed upon adding dGTP as a substrate irrespective of whether EDTA was added or not (Fig. 6B, dGTP and dGTP + EDTA panels). This reaffirms that the reaction does not occur in the presence of dGTP (Fig. 6B). It is important to note that, when GTP or dGTP was added to the reaction mixture, a sudden dip in the fluorescence emission signal of mant-ATP may be noted (compare the spectra colored black corresponding to SAS1 with mant-ATP alone, and the spectra for 0 min in red, as soon as GTP/dGTP was added (Fig. 6B)). We assign this sudden change in the fluorescence emission to a rearrangement in the active site caused by the simultaneous binding of both the substrates (GTP/dGTP and mant-ATP), which likely changes the microenvironment around the fluorescent group. This aligns well with the discussion based on structural superimposition of \(^{\text{NRel}}_{\text{Seq}}\) with SAS1 and SAS2 bound to substrates (Fig. 2). Nevertheless, we do not completely negate the possibility of minor changes in stoichiometry of the SAS1–mant-ATP complex in the presence of GTP or dGTP. In either case, it is clear that the effects caused by binding of dGTP or GTP to SAS1 are similar. Synthesis assays carried out using dGTP/GTP and radiolabeled ATP also provided robust evidence that dGTP is not utilized as an acceptor nucleotide by SAS1 (Fig. 6C). This confirms that none of the Rel homologs synthesize the hyperphosphorylated product using dGTP. There is no study that reports the physiological presence of hyperphosphorylated deoxyguanosine (ppdGpp) nucleotides in bacteria. In vivo, the concentration difference between GTP and dGTP could partly explain the reason for using GTP/GDP for the formation hyperphosphorylated derivatives that play the roles of secondary messengers (24). On the other hand, the possibility of ppdGpp formation might be irrelevant in the physiological context.

Still, the nonexistence of such nucleotides needs to be rationalized for the sake of understanding catalytic details in Rel and to reveal the importance of 2’-OH of GTP in catalysis. As our results establish, dGTP binds at the synthetic active site, without being utilized for the synthesis reaction; we next set out to investigate the importance of 2’-OH in catalysis.

**Examining synthesis activity using 2’ modified GTP analogs**

We first sought to understand the chemical nature of the functional group at the 2’ position that would promote catalysis. Two important properties that an OH group at 2’ position can likely impart include the following: (i) The preferred conformation of sugar puckers in a nucleotide, which is altered by the presence or absence of 2’-OH. Accordingly, GTP would preferably adopt the 3’-endo conformation, whereas dGTP would prefer 3’-exo conformation (Fig. 7A). If dGTP adopts the 3’-exo conformation at the active site, the attacking –OH group will be in a retracted conformation with respect to the β-phosphate of ATP and render it incapable of attacking the phosphodiester linkage (Fig. 7B). (ii) The electronegative
Figure 6. SAS1 cannot utilize dGTP as a pyrophosphate acceptor. A, FPLC profile for the separation of nucleotides following (p)ppGpp synthesis reaction by SAS1, carried out using GTP (left panel) or dGTP (right panel) as a substrate. B, fluorescence emission spectra of SAS1 (1 μM) incubated with mant-ATP (5 μM) alone or upon adding 40 μM GTP/dGTP (0–14 min). Buffer containing 50 mM HEPES (pH 8.0), 200 mM NaCl, 100 μM MgCl₂ was used. After adding GTP/dGTP, scans were taken every 2 min. EDTA (100 μM) was added to chelate the catalytic metal ion. The color codes of the scans are as labeled in the inset below. 0 to 14 min represent the time after addition of GTP or dGTP. C, autoradiogram showing the product formed after the synthesis reaction by SAS1 with GTP (lane 2, 2 mM) or dGTP (lane 3, 1 mM; lane 4, 2 mM). Lane 1 is a control without protein, but only substrates.
oxygen of the 2′-OH, which can form a H-bond and might have a role in proton abstraction and/or in maintaining the geometry of the catalytic center. In the absence of this, either of these roles are affected, and as a consequence, the reaction would not take place.

To determine which of these properties is required for the reaction, we made use of GTP analogs modified at the 2′ position such that they preferentially possess either of the above-mentioned properties but not both (Table 3). For example, a fluorine substitution at 2′ position (2′-F dGTP) increases the propensity for 3′-endo conformation. Another modification included 2′-NH₂ (2′-NH₂ dGTP); the presence of the –NH₂ group gives H-bonding capability such that it can act as both H-bond acceptor as well as donor. In addition, this group can exchange its proton with other groups through an H-bond network. The third modification that we considered was 2′-O-methyl group (2′-O-Me dGTP), which can act as H-bond acceptor but cannot exchange H with any other group (25). We also anticipated that the bulky methoxy group at 2′ position is likely to disturb the orientations of the nucleotide substrates. With these three different GTP analogs as an acceptor nucleotide, (p)ppGpp synthesis reactions were carried out. The pyrophosphate transfer from ATP, to GTP, dGTP, and these GTP analogs was monitored in SAS1 (Fig. 8) and NRelSeq (Fig. S6) using two different methods. In one, the amount of ATP remaining after the reaction was monitored using the Kinase-Glo reagent and is represented here as the percentage of ATP utilized (Fig. 8, A and C, and Fig. S6, A and C), whereas in the other, the hyperphosphorylated product formed was detected on an autoradiogram (Fig. 8, B and D, and Fig. S6, B and D). The amount of ATP utilized by SAS1 in the presence of GTP derivatives was analyzed; evidently, dGTP and 2′-O-Me dGTP cannot react with ATP, whereas 2′-F and 2′-NH₂ analogs of GTP reacted with ATP (up to 70–80%) like the natural substrate GTP (Fig. 8A). The results were additionally reproduced using radiolabeled ATP in the synthesis assay to detect product formation, and here too like GTP, 2′-F and 2′-NH₂ analogs of GTP resulted in the formation of hyperphosphorylated product, as evident from the autoradiogram (Fig. 8B). In case of 2′-NH₂ dGTP, it appears that the hyperphosphorylated product migrated to the region corresponding to ppGpp (and not ppGpp; see lane 6 in Fig. 8B and Fig. S6B), which might be due to a difference in the charge (because of the additional NH₂ group) of the resulting nucleotide product, when compared with GTP. Similar results were obtained for NRelSeq (Fig. S6, A and B). The findings from these experiments clearly suggest that the 2′-F and 2′-NH₂ substituted analogs could act as acceptor nucleotides for the synthesis reaction (in SAS1 and NRelSeq), and the pyrophosphate transfer could take place, which was not the case with dGTP. In other words, when the 3′-endo conformation and/or H-bonding capability is restored by the specific substitution at 2′ position, catalysis occurred.

Next, to evaluate the importance of these properties, we decided to attenuate the effect of the substituted groups. For

Table 3. GTP analogs, substituted group at the 2′ position and properties associated with these groups

| S. No. | GTP analogs | Group at 2′ position | Property conferred |
|-------|-------------|----------------------|--------------------|
| 1     | GTP         | OH                   | 3′-Endo conformation |
| 2     | dGTP        | H                    | 3′-Exo conformation  |
| 3     | 2′-F dGTP   | F                    | 3′-Endo conformation  |
| 4     | 2′-NH₂ dGTP | NH₂                  | Can accept and donate H-bond |
| 5     | 2′-O-Me dGTP| O-CH₃                | Can accept H-bond but cannot transfer proton |

Figure 7. Different pucker conformations of the ribose and 2′-deoxyribose nucleotides. A, guanine ribose (top panel) and guanine 2′-deoxy ribose (bottom panel) nucleotides are represented to show the difference in ring pucker of the ribose sugars in these nucleotides. A preferred 3′-endo or 3′-exo conformation can be observed, which depends on the presence or absence of the hydroxyl group at the 2′ position. B, represented here is the superposition of guanine base and ribose sugar of GTP (pink sticks) onto the GTP (brown sticks) from the substrate-bound co-crystal structure of SAS2, PDB code 6EWZ. The difference in the orientation and distance of the 3′-hydroxyl of GTP and of dGTP with respect to the β-phosphate of ATP (thin lines) is shown.
example, NH$_2$ would be protonated in acidic conditions, and it will only be able act as a hydrogen-bond donor but not as an acceptor. However, the 3'-endo conformation of 2'-F analog of GTP would still prevail, and hence the effect on the utilization of this nucleotide would be unaltered if the conformation alone is sufficient for the reaction to take place. Hence, we performed activity assays at acidic pH to distinguish and identify the property of the group at the 2'-position of GTP that is obligatory for catalysis. Acidic pH conditions under which Rel homologs still retained synthesis activity were screened, and to minimize the effect of the buffer component at different pH, we used Bis-Tris propane at both the alkaline (pH 8.5) and acidic pH (pH 6.3). SAS1, in the presence of GTP, even at low pH could still utilize ATP, but neither 2'-F nor 2'-NH$_2$ analogs reacted with ATP (Fig. 8C), suggesting that the reaction did not take place at acidic pH. In line with this, when the synthesis assay was performed using radiolabeled ATP too, product could not be detected on the TLCs for any of the GTP analogs, whereas in the presence of GTP, the product can be seen unambiguously (Fig. 8D). Similarly, for NRel$_{Seq}$ too, the product formed at higher pH, but not at acidic pH (Fig. S6, C and D). Hence, 2'-F and 2'-NH$_2$ analogs of GTP can act as pyrophosphate acceptor, but this ability is lost at acidic pH, which is not the case with GTP. As anticipated, at acidic pH, the NH$_2$ group gets protonated and loses the ability to accept the H-bond, which would not trigger catalysis. On the other hand, even at acidic pH, the 2'-OH GTP can act as the H-bond acceptor and hence promote catalysis. The 2'-F analog of GTP does not result in the product at acidic pH, confirming that the 3'-endo conformation alone is not sufficient. Moreover, despite the known difference in sugar pucker conformation between ribose and deoxyribose nucleotides, the crystal structures of DNA polymerases report that the substrate deoxynucleotides adopt a 3'-endo conformation at the active site; one reason for this is the coordination interaction between the 3'-OH of deoxynucleotide and the metal ion (26). If so, in Rel, dGTP too could be held in a similar conformation through metal ion coordination for the reaction to take place. However, this does not seem to happen. Hence, the difference

**Figure 8.** (p)ppGpp synthesis for SAS1 using 2'-modified GTP analogs and the effect of pH. The effect of different substitutions at the 2'-position of GTP on the synthesis activity of SAS1 was evaluated using alkaline buffer, pH 8.5 (A and B), and acidic buffer, pH 6.3 (C and D). The amount of ATP utilized (%) by different GTP analogs was obtained from the luminescence signal monitored using the Kinase-Glo reagent (A and C). The hyperphosphorylated product formation was monitored using [$\gamma$-32P]ATP at two different pH conditions, and the autoradiograms are shown (B and D). B and D, lanes 2–6 represent different GTP analogs used in the reaction. Lane 1, control without protein; lane 2, GTP; lane 3, dGTP; lane 4, 2'-O-methyl dGTP; lane 5, 2'-F dGTP; lane 6, 2'-NH$_2$ dGTP. Note that the position of hyperphosphorylated product differs in lane 6 because of the difference in charge, compared with the products formed in other lanes (see text).
in sugar pucker conformation is less likely to be the reason for not using dGTP as a substrate. In addition, the 2'-F dGTP, despite a ribose nucleotide in the preferred 3'-endo conformation, could still not be utilized as efficiently as GTP for the synthesis reaction. This further supports the aforesaid notion. However, the loss of product formation at acidic pH by GTP analogs suggests that the hydrogen bond acceptor ability of 2' group is critical for the synthesis reaction. Interestingly, the H-bond acceptor characteristic of organic F is debated widely, and it has been noted that it can take part in H-bonding (27, 28). Hence, we predict a possibility that the product formation observed for 2'-F dGTP in the alkaline condition could be a result of the H-bond acceptor characteristic of the organic fluorine. This suggests that the hydrogen-bonding interaction made by the 2'-OH is important for catalysis. The environment around the 2'-OH is shown in Fig. 9.

To gain a preliminary idea of the differences in molecular interactions and active-site conformations between dGTP- and GTP-bound states, we employed computational studies. To understand the role of 2'-OH of GTP for catalysis, we performed short hybrid quantum mechanics/molecular mechanics (QM/MM) simulations; a short 7-ps simulation employing the precatalytic structure of SAS2 equilibrated with GTP or dGTP along with ATP offered a preliminary idea of the differences in molecular interactions. It was observed that dGTP was stable at the active site during the QM/MM equilibration (Fig. 10A). However, certain differences in the conformation of GTP/ dGTP and their interaction with the active-site residues appeared significant to rationalize why dGTP cannot serve as a substrate for ppGpp synthesis. First, the conformation of the ribose ring of dGTP differs from that of the GTP, although the active-site residues and ATP superimpose very well (Fig. 10A). The conformational change of ribose ring in dGTP results in increased distance (termed \( d_1 \)) between the 3'-OH oxygen (the attacking group) and the scissile phosphate (P\(_b\)) of ATP compared with that for GTP (Fig. 10B). In the GTP-bound state, \( d_1 \) is largely stabilized at 3.4 Å, whereas in the dGTP-bound state, \( d_1 \) is largely stabilized at 3.7 Å and shows a broad distribution.

One of the primary reasons for this difference appears to be the H-bond established between the oxygen of the 2'-OH of GTP with that of the side chain nitrogen of Gln\(^{176} \) (termed \( d_2 \)) and another with one of the nonbridging oxygens of the \( \beta \)-phosphate of ATP (termed \( d_3 \)). The histogram for the probability distribution of the distance between the oxygen of GTP 2'-OH to the side chain nitrogen of Gln (Fig. 10C) and to the nonbridging oxygen of \( P_b \) (Fig. 10D) is shown. In case of dGTP, because of the absence of 2'-OH, these interactions are lost. As explained in the previous section, the H-bond of Gln\(^{176} \) with the GTP 2'-OH, i.e. \( d_2 \), might not influence the binding affinity of dGTP. However, it appears that this interaction is necessary to anchor the substrates in a catalytically competent orientation and hold them in close proximity. This likely prohibits dGTP from acting as a substrate for ppGpp synthesis.

**Probable roles for the 2'-OH of GTP in catalysis**

On the basis of the results obtained from biochemical and computational studies, we propose two different possibilities: (i) the 2'-OH of GTP would form intramolecular hydrogen bonding with the 3'-OH either directly or indirectly (Fig. 11, A and B) that would facilitate deprotonation and act as a nucleophile. Interestingly, ribonucleotides (and not the deoxyribonucleotides); cyclic nucleotides (c-di-AMP, c-di-GMP) and (p) ppGpp that act as secondary messengers in bacteria also appear to be remnants of the old RNA world (29). The catalytic function attributed to RNA is one of the key features of the old RNA world hypothesis. It has been reported that during peptide-bond formation, in aminocyt t-RNA synthetases and in case of the reverse splicing of group III introns, the 2'-OH of the ribonucleotide (from RNA) at the reaction center has been implicated to play a catalytic role (25, 30–32). Here, the 2'-OH has been suggested to be a part of H-bond network activating the nucleophile. Therefore, it would not be surprising if the 2'-OH of GTP plays a catalytic role in (p)ppGpp synthesis too. Hence, we speculate that the 2'-OH, either directly (Fig. 11A) or indirectly through a water molecule (Fig. 11B), activates the 3'-OH...
of GTP and thereby assists deprotonation and nucleophile generation. (ii) The results from QM/MM calculations suggest that the 2'-OH establishes H-bond(s) with the nearby groups such as Gln^{176} and the P_b of ATP (Fig. 11C). This could be important in stabilizing the catalytic groups in a favorable conformation. In addition, it is likely that the H-bond between the 2'-OH and the nonbridging oxygen of the β-phosphate of ATP might play a role in stabilizing the negative charge that would build up on this phosphate group during the transition state. As a result, this interaction stabilizes the transition state. The exact role of
the 2'-OH GTP could be elucidated in future using extensive structural studies. In summary, we believe that the H-bond–
accepting capability of the 2’-hydroxyl group in GTP provides a catalytic advantage during pyrophosphate transfer reaction at
the active site of Rel.

Conclusions

Here, we present a revised mechanism for (p)ppGpp synthesis by Rel family proteins, involving the 2’-OH of GTP. Our
analysis of presently available structures shows that none of them support the proposed mechanism of activation by Hogg
et al. (8), where the 3’-OH of GTP is activated by the conserved Glu323 at the active site for nucleophilic attack. Also, none of
the structures rationalize the selection of GTP, but not dGTP, as a substrate for the synthesis reaction in Rel. Further,
dough dGTP binds the active site, it does not form a hyper-
phosphorylated product, suggesting an important role for 2’-
OH in catalysis. The follow-up studies performed using GTP
anals confirm that H-bonding interaction made by 2’-OH is
critical for the reaction to take place. In addition, preliminary
computational studies suggest that the H-bonds formed by the
2’-OH of GTP might help to keep the substrates in close prox-
imity. In the end, we present a new mechanism in which the 2’-
OH of the acceptor nucleotide is the catalytic element and pro-
vide possible models explaining its role in accomplishing the
transfer of pyrophosphate of ATP to GTP/GDP.

Experimental procedures

Structure analysis

The structures of Rel family proteins, such as GDP-bound N-
terminal Rel from S. equisimilis, NRelSeq (PDB code 1V7J),
AMP-CPP bound SAS1 (RelQ) from B. subtilis (PDB code
5F2V), and the precatalytic structure of SAS2 (RelP) from S.
aureus (PDB code 6EWZ) were acquired from the PDB. The
structures were visualized, analyzed, and superimposed, and
the figures were generated using UCSF Chimera (33).

Protein expression and purification

The recombinant construct of N-terminal Rel from S. equisimilis,
NRelSeq (amino acids 1–385) in pQE-2 was transformed into DH5α E. coli cells and grown on ampicillin-containing me-
dium. Overnight-grown inoculum was added to LB broth and
incubated at 37°C with continuous shaking. After 4 h of incu-
bation, protein expression was induced by adding 0.5 mM iso-
propyl β-D-thiogalactopyranoside, and growth was further con-
tinued for next 4 h. The cells were pelleted and then dissolved in
lysis buffer containing 50 mM sodium phosphate, pH 8.0, 300
mM NaCl, 10% glycerol, 1 mM imidazole, 1 mM DTT, 1 mg/ml
lysozyme, and 1% Triton X-100. Lysis was carried out using 15
cycles of sonication with 10-s pulses at 30-s intervals. Lysate
was clarified at 28,000 rpm (Sorvall super spin 630 rotor) for 1
h, and supernatant was applied on to the nickel–nitrilotriacetic
acid (His-trap FF, GE Healthcare) column pre-equilibrated
with wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM
NaCl, 10% glycerol, 10 mM imidazole). The column was washed
to remove unbound proteins by passing 10 column volumes of
wash buffer. Protein was eluted with elution buffer (50 mM so-
dium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, 500 mM
imidazole) in two steps. In the first step, 10% gradient of elu-
tion buffer (2–3 column volumes) were passed to remove the
impurities. In the second step, pure protein fraction was
eluted using 50% gradient of elution buffer. Protein fractions
were concentrated and finally buffer-exchanged on the
Superdex 200 (Amersham Biosciences) gel-filtration col-
umn equilibrated with 50 mM Bis-Tris propane, pH 9.0, 300
mM NaCl. Pure protein was concentrated to highest possible
concentration.

SAS1 construct was kindly provided by Prof. Gert Bange,
LOEWE Zentrum fur Synthetische Mikrobiologie, Philipps-
University Marburg. Protein expression and purification was
carried out as reported previously (9). Briefly, E. coli C41 cells
transformed with the SAS1 construct were grown in a LB broth
with D(+)-lactose-monohydrate. Kanamycin was added to
maintain the plasmid and aeration was provided through con-

tinuous shaking. Lysis of the harvested cells was done in (20
mM NaHEPES, pH 8.0, 250 mM NaCl, 40 mM imidazole, 20 mM
MgCl2, 20 mM KCl). The clear supernatant was obtained by
centrifugation, and protein was purified in two steps. The affin-
ity-purification step was performed using a nickel–nitrilotri-
acetic acid column with wash buffer (20 mM NaHEPES, pH 8.0,
250 mM NaCl, 40 mM imidazole, 20 mM MgCl2, 20 mM KCl)
and elution buffer (20 mM NaHEPES, pH 8.0, 250 mM NaCl, 20
mM MgCl2, 20 mM KCl, 500 mM imidazole). For gel filtration,
20 mM NaHEPES (pH 8.0), 200 mM NaCl, 20 mM MgCl2, and
20 mM KCl were used as a buffer on Superdex 200 (Amersham
Biosciences). NRelMeb and NRelA constructs reported in an ear-
lier study (23) were used here, and highly pure protein was
obtained following protein purification protocol reported in
the same study.

Competition assay using mant-GTP

Binding of GTP and dGTP to NRelSeq active site was analyzed
by monitoring their ability to compete with the fluorescently
(N-methyl-3’-O-anthranoyl–labeled, mant) labeled analog of
GTP. Experiments were performed where 1 μM of protein in 50
mM HEPES (pH 8.0), 200 mM NaCl was incubated with mant-
GTP (5 μM) alone or in the presence of increasing concentra-
tions (5–40 μM) of GTP or dGTP. Only protein in buffer and
mant-GTP alone in buffer were taken as controls. The samples
were excited at 290 nm, where tryptophan absorbs energy and
gives emission with maxima at 350 nm that coincides with the
absorbance maxima of the mant group. Hence, when protein
sample is excited at 290 nm in the presence of mant-GTP only
if the mant-GTP is bound to the protein, as a result of FRET,
the mant group is excited and produces fluorescence signal
with the maxima around 450 nm (34). Employing this protocol,
the fluorescence emission spectrum of the mant-GTP at differ-
ent conditions was scanned from 400 to 550 nm using a LS fluo-
rescence spectrophotometer (PerkinElmer) at room tempera-
ture. To avoid the effect of dilution separate samples were
prepared for every scan with the specified concentrations.
Isothermal titration calorimetry

Binding constants for the nucleotides GTP, dGTP, and ddGTP were determined by using MicroCal iTC200 (GE Healthcare Life Sciences). \( ^{175}\text{Re}_{\text{seq}} \) was dialyzed in 50 mM HEPES (pH 8.0) and 200 mM NaCl for 3 h at 4 °C, prior to the experiment. All the nucleotides were prepared to a final concentration of 1 mM in the same buffer that is used for dialysis. For each experiment 80 µM protein was loaded into the sample cell, and the respective nucleotide was titrated with 20 injections of 2 µl at an interval of 3 min. Titrations were performed at 30 °C with continuous stirring (500 rpm). Binding constant and thermodynamic parameters were obtained using Microcal Origin 7 software. Heat of dilution was determined by titrating the nucleotide into the buffer. The data were fitted to one-site binding model after subtracting the effect of dilution from the experimental data, to obtain the thermodynamic parameters \( K_a \) and stoichiometry (n). The dissociation constant (\( K_d \)) was determined as 1/\( K_a \).

Monitoring (p)ppGpp synthesis using FPLC

(p)ppGpp synthesis assay was carried out using 1 mM ATP and 2 mM GTP or dGTP. The assay buffer included 50 mM HEPES (pH 8.0), 200 mM NaCl, 5 mM MgCl₂, and 1 mM DTT. A 100-µl reaction was initiated by adding respective Rel protein (\( ^{175}\text{Re}_{\text{seq}} \) (10 µM), SAS1 (5 µM), \( ^{175}\text{Re}_{\text{Mtb}} \) (10 µM), or \( ^{175}\text{Re}_{\alpha} \) (10 µM)) and incubated at 37 °C for 30 min. Once the reaction was complete, product formation was analyzed using FPLC (BioLogic DuoFlow). A 50-µl sample was applied to the reverse phase C18 column (Waters X-Terra, 3.5-µm particle size, 4.6 mm × 250 mm) equilibrated with the mobile phase A (100 mM potassium phosphate, pH 6.0, 10 mM tetrabutylammonium hydrogen sulfate), at a flow rate of 0.7 ml min⁻¹. After applying the sample, 100% mobile phase A was allowed to pass for 8 min. The bound nucleotides were then eluted from the column by passing mobile phase B (100 mM potassium phosphate buffer, pH 6.0, 30% acetonitrile) with a linear gradient from 0 to 100% for 30 min. Buffers were filtered through a 0.2-µm filter and degassed by sonication before use. Elution profiles for different nucleotides were corroborated by running individual nucleotide controls separately.

Radiolabeled (p)ppGpp synthesis assay

Synthesis assay was carried out at 37°C in a reaction volume of 10 µl using \( ^{175}\text{Re}_{\text{seq}} \) (10 µM) or SAS1 (5 µM) with 1 mM ATP and 2 mM GTP or dGTP at the concentration of 1 mM/2 mM. 0.01 µCi of [γ-\( ^{32}\text{P} \)]ATP was used to detect the formation of pppGpp. The reaction buffer included 50 mM HEPES (pH 8.0), 200 mM NaCl, 5 mM MgCl₂, and 1 mM DTT. The reaction was stopped after 30 min by adding 2 µl of 6 M formic acid. A 5-µl reaction mixture was then spotted on the PEI-TLC sheet, and nucleotides were resolved using 1.5 M KH₂PO₄ buffer. TLC sheet was air-dried and exposed to X-ray film, and autoradiogram was developed to visualize the product spots.

Inhibitory effect of dGTP on the synthesis reaction

Inhibitory effect of dGTP on synthesis activity of \( ^{175}\text{Re}_{\text{seq}} \) was evaluated by carrying out (p)ppGpp synthesis reaction in the presence of different dGTP concentrations. A 5-µl reaction was carried out in 50 mM HEPES (pH 8.0), 200 mM NaCl, 5 mM MgCl₂, and 1 mM DTT with 1 mM ATP, 2 mM GTP as substrates and 10 µM of \( ^{175}\text{Re}_{\text{seq}} \) protein. The reactions were carried out in either the presence or the absence of the designated concentrations of dGTP. Remaining ATP in the reaction mixture on completion of the reaction was quantified with the help of a Kinase-Glo kit from Promega. After the reaction was complete, the reaction mixture was diluted 200X using Milli-Q water. In a white-bottomed 96-well plate, to a 20 µl of diluted reaction mixture equal amount of Kinase-Glo solution was added, and luminescence was measured. Here, the luminescence signal is proportional to the amount of ATP available to oxidize luciferin by the luciferase enzyme in the Kinase-Glo solution. Hence, more the luminescence signal more is the remaining ATP implying less activity of the Rel and less luminescence signal would represent more activity. To obtain the amount of remaining ATP, the value of the luminescence signal for a particular reaction sample was subtracted from the control without the protein (where all the ATP remains unutilized). The data are plotted in terms of the percentage of activity with the respective dGTP concentrations used in the experiment. The maximum difference in the luminescence was observed for the reaction performed in the absence of dGTP that was considered as the 100% activity.

Time-course assay using mant-ATP

1 µM of SAS1 was incubated with 5 µM mant-ATP in 50 mM HEPES (pH 8.0), 200 mM NaCl, and 100 µM MgCl₂. The sample was excited at 290 nm, and the emission spectra were recorded in the range of 400 to 550 nm, which served as control. Synthesis reaction was initiated by adding 40 µM GTP or dGTP (0 min) and was monitored from 0 to 14 min by recording the emission spectra (400–550 nm) every 2 min upon exciting the reaction mixture at 290 nm. Progress of the synthesis reaction is monitored by comparing the emission signal of mant-ATP alone in the presence of protein to the fluorescence emission signal upon addition of GTP or dGTP. The same experiments were also performed in the presence of EDTA (100 µM) that chelates the catalytic Mg²⁺, which served as a negative control.

Synthesis activity using 2' modified GTP analogs as substrates

GTP analogs, substituted with a different chemical moiety at the 2' position of ribose, namely 2'-F dGTP, 2'-O-Me dGTP, and 2'-NH₂ dGTP, were purchased from Jena Biosciences. Synthesis reactions were carried out using Rel homologs (\( ^{175}\text{Re}_{\text{seq}} \) and SAS1) where substrate GTP was replaced with one of the above mentioned GTP analogs. To check the effect of protonation state of the substituted groups on the synthesis activity reaction was carried out at two different pH. To avoid any variation arising caused by the buffering component used to maintain a particular pH, Bis-Tris propane buffer was used because it has a wide buffer range (pH 6.0–9.5). The (p)ppGpp synthesis
activity was assayed at alkaline (pH 8.5) or acidic pH levels (pH 6.3) with the help of two methods: (i) using Kinase-Glo to monitor the amount of ATP utilized or (ii) using radiolabeled substrate nucleotide $[\gamma^{-32}\text{P}]\text{ATP}$ to monitor the product (p)ppGpp that is formed. In each case the reaction was carried out in 50 mM Bis-Tris propane (pH 8.5 or 6.3), 200 mM NaCl, 5 mM MgCl$_2$, and 1 mM DTT in the presence of 1 mM ATP and 2 mM GTP or one of the GTP analogs. 5 $\mu$L of reaction was initiated by the addition of protein (10 $\mu$M $\text{NRel}_{\text{Seq}}$ or 5 $\mu$M SAS1) at 37°C for 30 min. (i) For Kinase-Glo assay, after the reaction was complete, the samples were diluted 200 times with Milli-Q water. To the 20 $\mu$L of diluted reaction mixture, an equal amount of Kinase-Glo solution was added, and luminescence was measured. For the better appreciation of results and consistency in data representation, the amount of ATP utilized in each case is plotted here. The remaining ATP in the control samples (which gave maximum luminescence) was set to 0%, and the percentage of ATP utilized in other reaction conditions was calculated accordingly. The data plotted in this manner represent the relative amount of substrate ATP utilized with different GTP analogs. (ii) To visualize (p)ppGpp formed, radiolabeled $[\gamma^{-32}\text{P}]\text{ATP}$ was used in a separate experiments, and similar set of reactions was carried out at the exact same reaction conditions mentioned above. The reaction was stopped by adding 2 $\mu$L of 6 M formic acid. 5 $\mu$L of reaction mixture was spotted on the PEI-TLC sheet, and the nucleotides were resolved using 1.5 M KH$_2$PO$_4$ buffer. The (p)ppGpp formed in these reactions was visualized with the help of autoradiogram.

**System setup and MM MD simulations**

The initial coordinates of Rel in its precatalytic, substrate-bound state were obtained from the crystal structure of RelP (SAS2) from *S. aureus* determined in complex with AMP-CPP, GTP, and an active site Mg$^{2+}$ ion (PDB code 3EWZ) (10). AMP-CPP was replaced by ATP for modeling the precatalytic structure of Rel. This precatalytic complex of Rel was then solvated with 13497 TIP3P water molecules in a periodic box of dimensions 79 x 94 x 74 Å$^3$. The whole system was neutralized by adding 16 Na$^+$ counter ions. Additionally, 32 Na$^+$ and Cl$^-$ ions were added to the system to obtain the salt concentration of 100 mM. ATP, GTP, and the active-site Mg$^{2+}$ ion were treated using the generalized Amber force field (35). Restrained electrostatic potential–derived charges for GTP, Mg$^{2+}$, and K$^+$ ions were computed using R.E.D software (36). The protein was treated with the parm99 version of the AMBER force field (37).

The enzyme–substrate complex of Rel bound to dGTP and ATP was modeled from the Rel–ATP–GTP complex. For generating this structure, the 2'–OH of GTP substrate was replaced by a hydrogen atom using the software UCSF Chimera (33). The simulation set up of the solvated enzyme systems for the Rel–dGTP–ATP complex was the same as that of the Rel–GTP–ATP complex.

Classical MM MD simulation for the solvated Rel enzyme was carried out using the AMBER suite of programs. A 12 Å cutoff distance was used for the nonbonded interactions. The Particle mesh Ewald (PME) method (38) was used for the long-range electrostatic interactions. Following the preliminary minimization of the whole system, 1-ns NPT simulations were carried out using Langevin thermostat at 300 K and Berendsen barostat at 1 atm. This was followed by 5 ns of NVT simulation at the equilibrium volume. During the simulation, the atomic coordinates of the substrates ATP and GTP were constrained to their initial coordinates. Root-mean-square deviation of the protein backbone for these structures during MD simulation with respect to the X-ray structure was below 1.5 Å. The MM simulations were carried out using a time step of 1 fs.

**QM/MM simulations**

The coordinates of the enzyme–substrate complex, equilibrated by MM simulations, were taken for further QM/MM simulations of the Rel–GTP–ATP, as well as the Rel–dGTP–ATP complex. These hybrid QM/MM simulations were carried out using the CPMD/GROMOS interface (39, 40). The QM subsystem includes (d)GTP, ATP, a Mg$^{2+}$ ion, an active site water molecule, and the side chains of Lys$^{52}$, Lys$^{60}$, and Asp$^{79}$ (Fig. S7). The covalent bonds cleaved at the interface of QM and MM regions were saturated by the link hydrogen atoms. The QM subsystem was treated with plane wave Density Functional Theory (DFT) with the Perdue–Burke–Ernzerhof (PBE) exchange correlation functional (41), using a plane wave cutoff of 25 Rydberg and ultrasoft pseudopotentials (42). The electronic embedding scheme proposed by Laio et al. (43) was employed. The charge density–point charge interaction was considered for the interactions of MM atoms within 15 Å of the QM electron density. The rest of the MM atoms were allowed to interact with multipole expansion of the charge density interaction potential. The MM atoms included in the QM/MM interaction exclusion list interacted with the QM atoms through dynamic restrained electrostatic potential charges of the QM atoms. Separate NHC thermostats (44) were used for the QM and MM systems. The CPMD scheme (45) was employed for the dynamics of the QM part, using a fictitious mass of 700 atomic units. The MD time step for the QM/MM simulation was 0.125 fs. Hybrid QM/MM simulations of Rel–GTP–ATP and Rel–dGTP–ATP precatalytic complexes were run for 7 ps.

**Data availability**

All data are contained within the article and the supporting information. Additional information is available upon request (Balaji Prakash, bprakashit@gmail.com).

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: PDB, Protein Data Bank; ITC, isothermal titration calorimetry; AMP-CPP, adenosine 5’-([α,β-methylene]diphosphate); Pol, polymerase; QM/MM, quantum mechanics/molecular mechanics; pppGpp, guanosine-5’-(triphosphate)-3-diphosphate; ppGpp, guanosine-5’-(diphosphate)-3-diphosphate.

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