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Structure-function comparisons of (p)ppApp *vs* (p)ppGpp for *Escherichia coli* RNA polymerase binding sites and for *rrnB* P1 promoter regulatory responses *in vitro*

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ABSTRACT

Precise regulation of gene expression is crucial for bacteria to respond to changing environmental conditions. In addition to protein factors affecting RNA polymerase (RNAP) activity, second messengers play an important role in transcription regulation, such as well-known effectors of the stringent response: guanosine 5′triphosphate-3′diphosphate and guanosine 3′, 5′-bis(diphosphate) [(p)ppGpp]. Although much is known about importance of the 5′ and 3′ moieties of (p)ppGpp, the role of the guanine base remains somewhat cryptic. Here, we use (p)ppGpp's adenine analogs [(p)ppApp] to investigate how the nucleobase contributes to determine its binding site and transcriptional regulation. We determined X-ray crystal structure of *Escherichia coli* RNAP-(p)ppApp complex, which shows the analogs bind near the active site and switch regions of RNAP. We have also explored the regulatory effects of (p)ppApp on transcription initiating from the well-studied *E. coli rrnB* P1 promoter to assess and compare properties of (p)ppApp with (p)ppGpp. We demonstrate that contrary to (p)ppGpp, (p)ppApp activates transcription at this promoter and DksA hinders this effect. Moreover, pppApp exerts a stronger effect than ppApp. We also show that when ppGpp and pppApp are present together, the outcome depends on which one of them was pre-incubated with RNAP first. This behavior suggests a surprising Yin-Yang like reciprocal plasticity of RNAP responses at a single promoter, occasioned simply by pre-exposure to one or the other nucleotide. Our observations underscore the importance of the (p)ppNpp's purine nucleobase for interactions with RNAP, which may lead to a better fundamental understanding of (p)ppGpp regulation of RNAP activity.

1. Introduction

In order to cope with changing environmental conditions, bacteria have evolved several different responses, often relying on a variety of noncanonical ribonucleotides as second messengers. These nucleotides are distinct by means of structural features as well as by the fact that they are not present as pathway intermediates and their sole function appears to be regulation.

One class of these analogs arises by 3′,5′-cyclization of a single phosphate for each ribonucleotide; so far, examples include mononucleotides (cAMP and cGMP) as well as homomeric and heteromeric dinucleotides (c-di-AMP, c-di-GMP, c-GAMP). The mechanisms of action of these global regulators in bacteria range from affecting transcriptional regulators that bind to DNA (*e.g.* CRP and CRP-like proteins affected by cAMP, cGMP and c-di-GMP [1–4]), to binding to specific proteins other than transcriptional factors or riboswitches (*e.g.* c-di-GMP [5] and c-di-AMP [6]).

Another class of second messengers consists of noncyclic guanosine derivatives with 3′ and 5′ hydroxyls blocked by linear phosphate chains; these analogs are guanosine 5′-triphosphate-3′diphosphate [pppGpp] and guanosine 3′,5′-bis(diphosphate) [ppGpp], collectively termed (p)ppGpp [7, 8]. This class of nucleotides is ubiquitous in bacteria and plant plastids [8]. These molecules are effectors of the bacterial stringent response, characterized by inhibition of the rRNA and tRNA synthesis under different starvation and stress conditions [8]. The way (p)ppGpp causes the stringent response differs for Gram

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positive and negative bacteria. For example, in *Bacillus subtilis* (p)ppGpp indirectly regulates rRNA by inhibiting GTP biosynthesis, which limits rRNA transcripts that start with GTP. In *Escherichia coli,* a similar outcome is brought on by direct interactions of (p)ppGpp with the RNA polymerase (RNAP) leading to ribosomal RNA promoters' (*rrn*) transcriptional inhibition. Still, not all promoters are inhibited by this action, for example promoters for amino acid biosynthesis genes are activated [8, 9, 10]. Although (p)ppGpp is capable of exerting these effects on its own (particularly in the case of transcriptional inhibition), it has been shown that an RNAP secondary channel binding protein, DksA, may amplify its action (especially true for instances of transcriptional activation) [10–12]. Still, there are instances where DksA and (p)ppGpp do not act in synergy but instead exert antagonistic effects [13–16].

Despite the vast amount of information available so far, until very recently it was largely unknown how each moiety of the (p)ppGpp molecule contributes to transcriptional regulation. It is almost certain that the 3′diphosphate plays a crucial role here since GTP and GDP do not display such regulatory effects. The nucleotide's 5′ moiety also plays a role, as pppGpp and ppGpp were shown to affect the *E. coli* transcription to a different extent, with ppGpp being more potent than pppGpp [17, 18]. Indeed, guanosine pentaphosphate phosphatase (GppA) exists in *E. coli,* whose presence can be viewed as a means of fine tuning regulation. The importance of the 5′ γ-β and 5′-β phosphates was also demonstrated with yet another analog, pGpp, found to be synthesized in *Enterococcus faecalis* [19]. It was shown to inhibit *E. coli rrnB* P1 model promoter to a much lesser extent than either pppGpp or ppGpp, regardless of the presence of DksA *in vitro* [19].

Recent reports had pointed to two ppGpp binding sites on *E. coli* RNAP. Site 1 is DksA-independent, which is localized at the junction of the ω and β′ subunits [17, 20, 21], while site 2 is DksA-dependent and locates at the binding interface between β′ rim-helix of RNAP and DksA [22, 23]. Both pppGpp and ppGpp were shown to bind to sites 1 and 2 in a similar manner [17, 23], but it is uncertain whether potential differences in affinities might explain differences in their regulatory potency.

A recent crystal structure of the *E. coli* RNAP and DksA/ppGpp complex revealed a crucial role of the guanine base for accommodating ppGpp at both sites [23]. For site 1, the guanine base is recognized by the β′ subunit amino acid residues (sandwiched by side chains of R362 and I619, and involved in polar interactions with H364 and D622), while both diphosphate groups establish salt bridges with basic residues (R3 and R52) of the ω subunit. For site 2, the guanine base interactions involve both the β′ rim helix residues (Y679, I 683, D684 and N680) and DksA (L95).

So far, all noncanonical regulatory nucleotides seem to have purines as nucleobases. Evidently the cyclic class of analogs exists as adenine or guanine derivatives. Therefore, we ask if the same would be true for (p)ppGpp-like alarmones, *i.e.* what are the functional consequences of swapping the guanine of (p)ppGpp for adenine, and how nucleobase determines the (p)ppNpp-RNAP interactions.

2. Materials and methods

2.1. X-ray crystal structure determinations of the E. coli RNAP σ^{70}-holoenzyme in complex with (p)ppApp

Crystallizations of *E. coli* RNAP containing RpoB-H526Y or RpoB-S531L substitutions were performed as previously described [24]. To form co-crystals of RNAP with (p)ppApp, RNAP crystals were transferred to the cryoprotection solution (0.1 M HEPES (pH 7.0), 0.2 M $MgCl_2$, 30% PEG400, 10 mM DTT) supplemented with 1 mM (p)ppApp and incubated overnight at 22 °C followed by flash-freezing in liquid N_2.

The X-ray dataset was collected at the Macromolecular Diffraction at the Cornell High Energy Synchrotron Source (MacCHESS) (Cornell University, Ithaca, NY) and the data were processed by HKL2000 [25]. The resolution limit for crystallographic dataset were determined based on $CC_{1/2}$ (> 30%) rather than R_{merge} and $< I > /\sigma I > 2$ criteria, since this approach prevents loss of useful crystallographic data for structure refinement as found in a recent study [26]. The structures were solved by molecular replacement using the suite of programs PHENIX [27]. Strong Fo-Fc maps corresponding to (p)ppApp were observed after the rigid body refinements. Structures of (p)ppApp were fitted into the extra density maps to continue the refinement. The program Coot [28] was used for manual adjustment of the models during refinements. The structures were refined by using the Phenix suite of programs for the rigid body and positional refinements with non-crystallographic symmetry and reference structure restraints to avoid over-fitting the data. Final coordinates and structure factors of RNAP-ppApp complex were submitted to the PDB depository with ID code listed in Table 1.

Table 1
Data collection and refinement statistics.

RNAP complex with	ppApp
PDB code	6BYU
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a (Å)	186.3
b (Å)	203.7
c (Å)	308.3
Resolution (Å)	30–3.6
Total reflections	792,327
Unique reflections	135,308
Redundancy	5.9 (6.0)
Completeness (%)	100 (100)
I / σ	10.1 (2.16)
R_{sym} (%)	21.7 (101.8)
$CC^{1/2}$	(0.478)
Refinement	
Resolution (Å)	30–3.6
R_{work}	0.244
R_{free}	0.311
No. of atoms	54,996
B factors	153.5
R.m.s deviations	
Bond length (Å)	0.004
Bond angles (°)	0.775
Clashscore	14.2
Ramachandran favored, %	90.4
Ramachandran outliers, %	1.53

Data sets were collected at MacCHESS F1 line, Ithaca, NY.
Highest resolution shells are shown in parentheses.

2.2. In vitro transcription

For the *rrnB* P1 promoter, *in vitro* transcription was performed basically as described in [12]. Briefly, the reactions were carried out in 20 µl (final volume) at 30 °C, using 10 nM linear template spanning −180 to +109 bp (DNA template total length 289 bp, full length transcript 109 nt) and 30 nM *E. coli* RNAP holoenzyme (Epicentre Technologies or New England Biolabs), in a buffer containing 50 mM Tris-acetate, pH 8.0, 10 mM Mg-acetate, 10 mM β-mercaptoethanol (β-ME), 10 µg/ml BSA, 90 mM potassium glutamate (KGlu), 100 µM ATP, GTP and CTP, and 10 µM UTP (10 µCi/reaction [α-^{32}P] UTP), and either 250 µM (p)ppGpp or 250 µM (p)ppApp, unless stated otherwise. RNAP was preincubated (25 °C) with ppGpp or (p)ppApp for 8-min prior to the addition of KGlu, and this was followed by 8-min incubation at 30 °C with the DNA template and DksA, if present, unless stated otherwise. The reactions were initiated by adding NTP substrates and terminated after 10 min by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). For single round reactions, heparin was added to 100 µg/ml together with the NTPs. Samples were analyzed on 7 M urea,