A Conserved Arginine in Switch I is Critical for ppGpp Binding to the Prokaryotic Translational GTPase BipA

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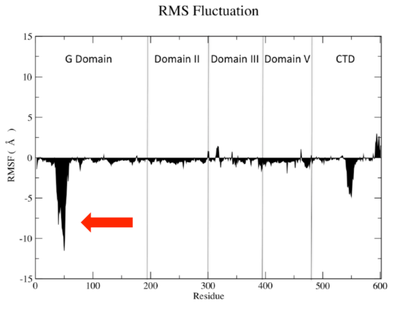
Abstract

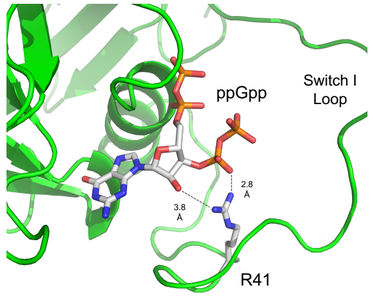
BipA is a highly conserved translational GTPase that functions in a variety of cellular processes including fitness, pathogenicity and acclimation to adverse growth conditions. Structural and biochemical studies indicate that GTP and ppGpp compete for binding to BipA to promote differential association of the protein to either the 70S or 30S ribosomal species. Exactly how guanine nucleotide binding to BipA prompts a change in the association of this protein with the ribosome is not understood.

The switch regions of most GTPases are highly flexible and cannot be observed by crystallography. Therefore, molecular dynamics simulations were used to visualize these regions of BipA in the various guanine nucleotide bound states. The ppGpp simulation suggests that a conserved arginine, located in the switch I (SW I) region of the protein, makes direct contact with the alarmone. To examine whether this residue could be a specificity determinant for ppGpp binding, steady state kinetics and isothermal titration calorimetry measurements were done to assess how an alanine substitution at this site would impact the biochemical properties of BipA. These experiments revealed that although the GTP hydrolysis and GTP binding properties of the protein were unaltered, ppGpp binding was greatly diminished. In contrast to the wild-type protein, filter binding assays show that the alanine-substituted BipA does not associate as efficiently with 30S ribosomes in the presence of ppGpp. Hydrogen deuterium exchange mass spectrometry confirm that the introduction of this mutation changes the overall dynamics of the protein away from the BipA:ppGpp state and thus selectively abolishing association with the ribosome.

MD Simulations



Equilibrium all-atom molecular dynamic simulations were performed in the NPT ensemble using the CHARMM force field with the GROMACS simulation package. Simulations were performed on the ~100 ns timescale. A comparison of the root mean square (RMSF) profiles for the apo-BipA and BipA:ppGpp revealed a decrease in the flexibility of the SW I region of the protein.



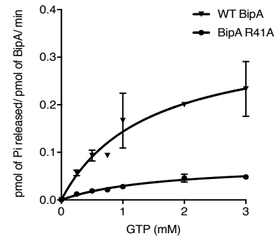
An examination of the final structure of the simulation shows that R41 located centrally in the SW I region of the protein comes into contact with 3' pyrophosphate group of ppGpp. Throughout the simulation, it interacts with either one or both of these phosphates. R41 is ~83% conserved over the BipA family, typically replaced by a lysine that still have that positive charge.

We also did a 100 ns simulation of R41A protein bound to ppGpp. During this MD run contacts are never made between the SWI region and ppGpp and, perhaps more importantly, ppGpp falls out of the guanine nucleotide binding pocket by the end of the simulation, dissociating from the protein.

References:
1. Lanzetta, P.A. et al. (1979) *Anal. Biochem.* **100**, 95-97.
2. Emsley, P. et al. (2010) *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **66**, 486−501.
3. Murshudov, G. N. (2011) *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **67**, 355−367.

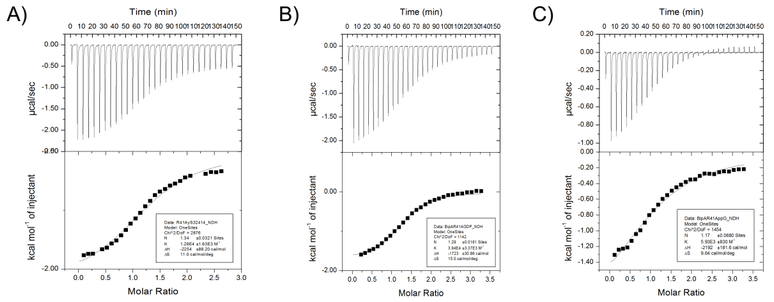
GTPase Properties

In BipA, R41 is the only conserved positively charged amino acid centrally positioned in the flexible SWI region of the protein. For example in *Escherichia coli* it sits in a negatively charged stretch of amino acids, AETQERVMDSND. This supports the hypothesis of R41 being a specific recognition element for ppGpp's negative 3′-phosphates. To determine if R41 can act as a binding determinant for ppGpp binding to BipA we inserted an alanine substitution at this site.



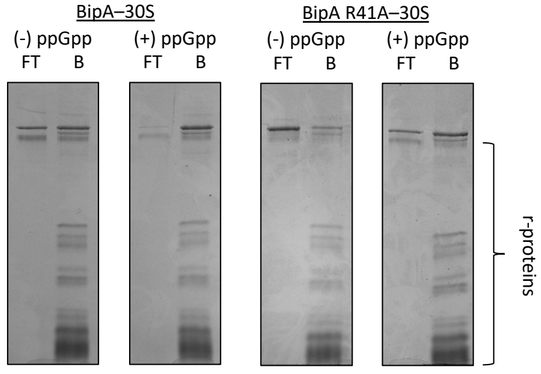
Protein	K_M (mM)	k_{cat} (hr^{-1})	k_{cat}/K_M (mM^{-1} hr^{-1})
BipA	1.4 ± 0.44	20.5 ± 3.00	14.6 ± 5.1
BipA R41A	1.6 ± 0.35	4.6 ± 0.50	2.9 ± 0.70

Steady state kinetics assays measuring the GTP hydrolysis rates of wild type and R41A BipA (1 μM) substituted protein. Kinetics were done by utilizing the malachite green phosphate assay[1]. Both proteins hydrolyze GTP to the same extent.



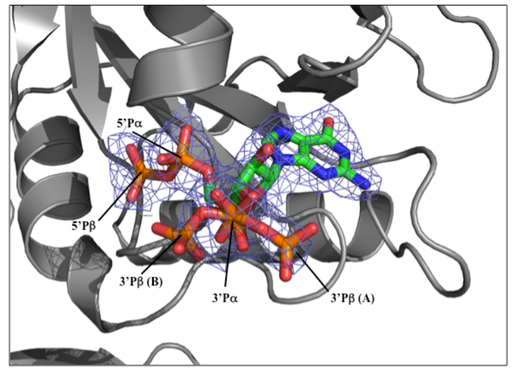
Isothermal titration calorimetry (ITC) titration curves (upper panels) and binding isotherms (lower panels) for the interaction of BipA R41A with (A) GTPgS, (B) GDP and (C) ppGpp at 15 °C in 20 mM phosphate, 100 mM NaCl, pH *7.5.*

Guanine Nucleotide	*GDP*	*GTP/ GTPγS*	*ppGpp*
BipA	22 μM	22 μM	11 μM
BipA R41A	27 μM	32 μM	178 μM



Filter binding assays with purified components were used to assess 30S ribosome binding by the R41A BipA protein. These preliminary results show that although we do observe some binding to the 30S ribosomal species in the presence of ppGpp, it is not the complete shift to the 30S:BipA:ppGpp complex observed with the wild-type protein.

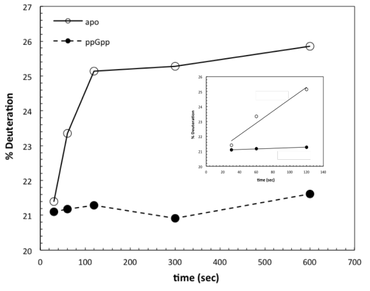
BipA ppGpp Structure



We determined the *Salmonella enterica* BipA:ppGpp structure to 2.31Å resolution. The protein crystallized in space group $P2_1$ with three molecules in the asymmetric unit. It was solved by molecular replacement using the apo BipA structural as a search model and refined to an R value of 21.6% and R_{free} of 24.5% using COOT and REFMAC[2,3].

As shown above, the structure revealed two different conformations for the pyrophosphate group esterified to the 3′ position of the ribose. As is typical for all GTPase crystallographic models, there was no visible density for the SW I region of the protein.

Solution Dynamics



HDX/ MS-MS studies indicate that the mobility of the SW I region of wild-type BipA decreases upon ppGpp binding. Raw values measured as average deuterium ions exchanged per maximum exchangeable ions and were converted to deuterium uptake as % deuteration for the peptide RVNDSNDL (residues 41-48). Results reflect change in % deuteration between apo and ppGpp bound conditions over a time course of 600 seconds.

In wild-type BipA, peptides 33-57, 41-57 and 48-57, encompassing the SW I region, display bimodal exchange pattern in the presence of ppGpp, suggesting a mixture of two conformations. This bimodal exchange pattern is not observed for the R41A protein. This suggests that the R41A mutation selectively abolishes one of these SW I conformers, probably the ppGpp binding mode, thereby leading to a much lower affinity of BipA for ppGpp (data not shown).

Summary: Switch regions of GTPases are highly flexible and therefore cannot be observed by crystallography. We used molecular dynamics simulations to visualize these residues and discovered R41, located in the switch I region of the protein, makes direct contact with the alarmone ppGpp. To biochemically verify these findings, we substituted an alanine at this position (R41A). Steady state kinetics revealed the GTP hydrolysis properties of the protein were unaltered (k_{cat} 20.5 ± 2.1 hr^{-1} and 4.6 ± 0.5 hr^{-1} for wildtype and R41A BipA, respectively). However, ITC experiments revealed that this mutation negated ppGpp binding while the protein was still able to bind GTP. HDX-MS studies validated the ordering of the SW I residues upon BipA's association with ppGpp and also point to the loss of conformational selectivity as a rationale as to why the R41A protein cannot bind the alarmone.