Supporting Information

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SI Materials and Methods.

***Escherichia coli* RtcA Purification for Crystallization Experiments.** Mutations C308S and H309N were introduced into the *rtcA* ORF of expression plasmid pET-His_{10}RtcA-(2-339) (1) by two-stage overlap extension PCR. The pET-His_{10}RtcA-(C308S-H309N) plasmid was transformed into *E. coli* BL21-Codon Plus (DE3). To prepare RtcA-(C308S-H309N) for crystallization experiments, a 2 L bacterial culture was grown at 37 °C in Luria–Bertani broth containing 0.1 mg/mL ampicillin until the A_{600} reached 0.6–0.8. The culture was chilled on ice for 30 min and then adjusted to 0.1 mM isopropyl β-D-thiogalactoside and 2% (v/v) ethanol. Incubation was continued at 17 °C for 16 h with constant shaking. Cells were harvested by centrifugation and stored at −80 °C. All subsequent procedures were performed at 4 °C. The cell pellet was suspended in 100 mL of buffer A (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10% sucrose) and lysozyme was added to 0.2 mg/mL. After mixing gently for 1 h, the suspension was sonicated to reduce viscosity and insoluble material was removed by centrifugation at 30,000 × *g* for 45 min. The soluble lysate was mixed for 90 min with 6 mL of a 50% slurry of His60 Ni Superflow resin (Clontech) that had been equilibrated in buffer A. The resin was recovered by centrifugation and resuspended in 20 mL of buffer B (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10% glycerol). The cycle of centrifugation and resuspension of the resin was repeated thrice, after which the resin (3 mL) was poured into a column. The column was washed serially with 20 mL of buffer C (50 mM Tris-HCl, pH 7.4, 2 M KCl) and 20 mL of buffer B containing 25 mM imidazole. The bound proteins were eluted stepwise with 3 mL aliquots of 100, 200, 300, 400, and 500 mM imidazole in buffer B and collected in tubes containing NaCl and ATP (or AMP) such that their final concentrations were 400 mM and 20 mM, respectively. The elution profile was monitored by SDS-PAGE. Fractions containing His_{10}RtcA-(C308S-H309N) were pooled and mixed with 4 mL of DEAE-Sephacel resin (GE Healthcare) that had been equilibrated in buffer B containing 10 mM ATP (or AMP). The mixture was poured into a column and His_{10}RtcA-(C308S-H309N) was recovered in the flow-through fraction, which was then concentrated by centrifugal ultrafiltration to 2.5 mg/mL. This material was then gel-filtered through a 120 mL 16/60 HiPrep Sephacryl S-100 HR column (GE Healthcare) in buffer D (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mM ATP or AMP, 5% glycerol) at a flow rate of 1 mL/min. The peak RtcA-containing fractions were pooled and concentrated by centrifugal ultrafiltration.

RNA Substrate for 3′-Phosphate Cyclization in Vitro. A 3′-phosphorylated RNA oligonucleotide labeled with ^{32}P at the penultimate 3′ phosphate was prepared by T4 RNA ligase 1 (Rnl1)-mediated addition of [5′-^{32}P]**p**Cp to an 19-mer synthetic oligoribonucleotide. The [5′-^{32}P]**p**Cp donor was generated via enzymatic phosphorylation of unlabeled 3′-CMP by T4 polynucleotide kinase-phosphatase (Pnkp) mutant D167N (kinase active, but devoid of 3′ phosphatase; purified according to ref. 2) in the presence of [γ^{32}P]ATP. A kinase reaction mixture (20 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM 3′ CMP, 0.1 mM [γ^{32}P]ATP (130 μCi), 0.1 mg/mL BSA, and 10 μg/mL Pnkp-D167N was incubated at 37 °C for 60 min. The Pnkp was inactivated by heating the mixture at 95 °C for 3 min. The mixture was then adjusted to 40 μL and, in the process, supplemented with 100 μM fresh unlabeled ATP, 50 μM 19-mer oligoribonucleotide 5′-rUGGCUCCGAUAUCACGCUU, and 0.2 mg/mL Rnl1 (purified according to ref. 3). The T4 ligase reaction mixture was incubated for 1 h at 37 °C and the products were resolved by electrophoresis through a 40-cm 20% polyacrylamide gel containing 8 M urea. The ^{32}P-labeled 20-mer product (5′-$_{HO}$UGGCUCCGAUAUCACGCUU**p**Cp) was located by autoradiography and excised from the gel. The oligonucleotide was eluted by soaking the gel slice overnight in 0.4 mL of 1 M ammonium acetate, 0.2% SDS, 20 mM EDTA. The eluted oligoribonucleotide was recovered by ethanol precipitation and resuspended in 10 mM Tris-HCl (pH 6.8).

1. Tanaka N, Smith P, Shuman S (2010) Structure of the RNA 3′-phosphate cyclase-adenylate intermediate illuminates nucleotide specificity and covalent nucleotidyl transfer. *Structure* 18:449–457.
2. Wang LK, Shuman S (2002) Mutational analysis defines the 5′ kinase and 3′ phosphatase active sites of T4 polynucleotide kinase. *Nucleic Acids Res* 30:1073–1080.
3. Wang LK, Ho CK, Pei Y, Shuman S (2003) Mutational analysis of bacteriophage T4 RNA ligase 1: different functional groups are required for the nucleotidyl transfer and phosphodiester bond formation steps of the ligation reaction. *J Biol Chem* 278:29454–29462.

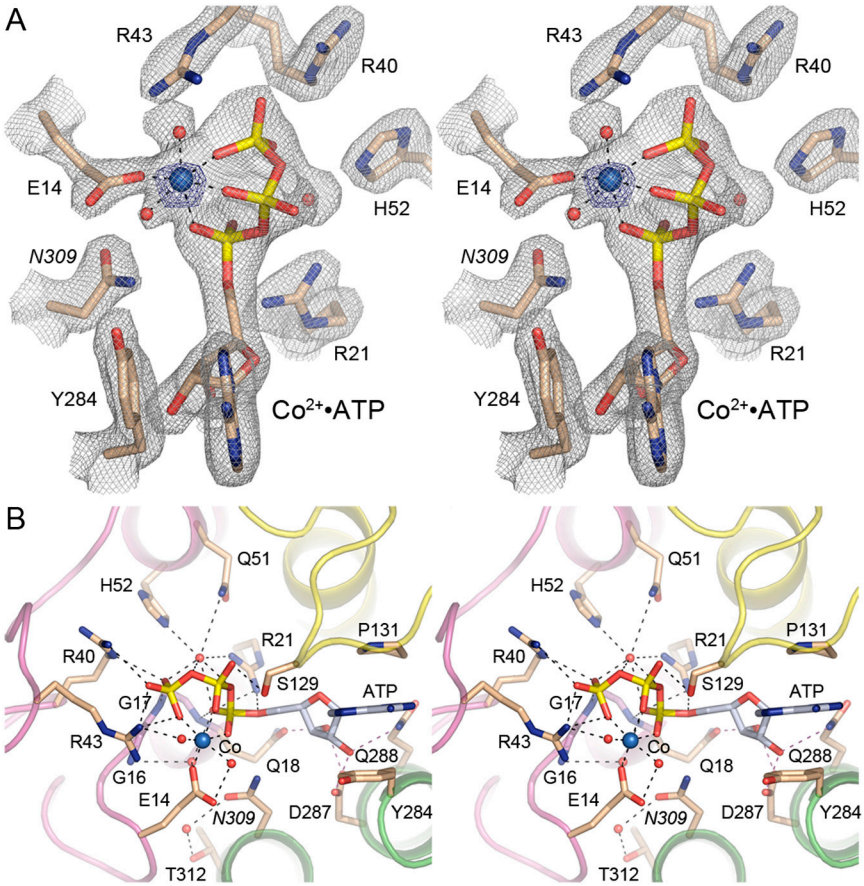

Fig. S1. Structure of an RtcA • ATP • Co^{2+} complex. (*A*) Stereo view of the electron density map of the active site. The gray mesh is the $2F_o$-F_c density of the refined model, contoured at 1.3σ. Amino acids and ATP are shown as stick models with beige and gray carbons, respectively. Waters are depicted as red spheres. The blue mesh is the anomalous difference density for the cobalt ion (blue sphere) contoured at 27σ. Contacts to the ligands in the octahedral metal coordination complex are denoted by dashed lines. (*B*) Stereo view of the atomic contacts to ATP and cobalt in the RtcA active site.

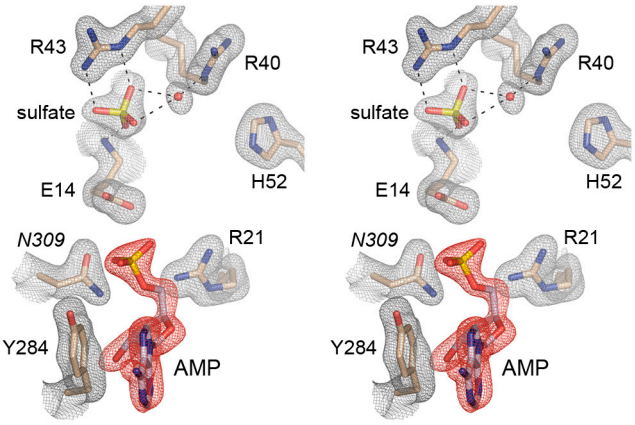

Fig. S2. Structure of an RtcA•AMP product complex. Stereo view of the electron density map of the active site. The gray mesh is the $2F_o$-F_c density of the refined model, contoured at 1.3σ. The red mesh is an omit map (F_o-F_c) of the AMP density contoured at 3.5σ. Amino acids, AMP, and a sulfate anion are shown as stick models. Atomic contacts to the sulfate are denoted by dashed lines.

Table S1. Crystallographic data and refinements statistics

	RtcA•ATP	RtcA • ATP • Mn^{2+}	RtcA • ATP • Co^{2+}	RtcA•AMP
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_1$
Unit cell dimensions @ 130 K, Å	$a = 69.2$ $b = 82.7$ $c = 52.0$	$a = 69.5$ $b = 83.1$ $c = 52.1$	$a = 69.7$ $b = 82.9$ $c = 51.8$	$a = 52.2$ $b = 79.8$ $c = 85.7$ $\beta = 103.65°$
Radiation source	NSLS X25 with Pilatus 6M Detector			
Wavelength (Å)	0.9499	0.9499	1.6051	1.282
Crystallographic data quality				
Resolution (Å)	35.49–1.58 (1.67–1.58)	27.31–1.85 (1.95–1.85)	32.37–2.10 (2.21–2.10)	36.91–1.90 (2.00–1.90)
R_{sym}, %	5.3 (37.6)	8.4 (55.5)	10.5 (51.2)	6.3 (13.0)
Unique reflections	41,491 (5,806)	25,908 (3,604)	17,859 (2,324)	52,851 (7,112)
Mean redundancy	5.5 (2.9)	5.7 (5.6)	10.0 (5.5)	2.9 (2.5)
Completeness, %	99.6 (97.2)	98.5 (96.8)	98.4 (90.0)	98.2 (91.4)
Mean $I/\sigma I$	13.1 (2.3)	12.0 (2.5)	12.5 (3.0)	10.0 (5.2)
Molecular replacement				
Protomers		1		2
Translation Z-score		49.2		11.0 / 32.5
Refinement and model statistics ($F > \sigma F$)				
Resolution (Å)	35.49–1.58 (1.62–1.58)	26.66–1.85 (1.92–1.85)	32.37–2.10 (2.23–2.10)	36.91–1.90 (1.93–1.90)
Completeness (%)	99.6 (95.0)	98.1 (96)	98.3 (90.0)	98.1 (85.0)
R_{free}/R_{work} (%)	25.3/20.0 (33.9/31.8)	25.1/18.5 (30.7/24.8)	26.3/21.0 (36.6/33.1)	23.3/18.0 (25.9/20.8)
RMSD Bonds/Angles	0.01 Å/1.282°	0.007 Å/1.164°	0.007 Å/0.845°	0.007 Å/1.104°
Ramachandran plot	97.6% favored, no outliers	99.7% favored, no outliers	97.6% favored,1 outlier: Ser218	98.7 % favored, 2 outliers: Gly159A,B
B-factors, $Å^2$, Overall/ Wilson	30.6/19.9	26.1/21.4	38.9/31.4	21.2/18.7
TLS Groups Mean Anisotropy	40.55	N/A	10.25	N/A
Model contents				
Protein residues	336	336	336	672
Heteroatoms	1 ATP 1 sulfate 1 glycerol 1 ethylene glycol 292 waters	1 ATP, 1 Mn^{2+} 1 sulfate 1 glycerol 2 ethylene glycol 306 waters	1ATP 3 Co^{2+} 1 sulfate 140 waters	2 AMP 4 sulfate, 1 chloride 1 glycerol, 1 PEG 1 ethylene glycol 731 waters
PDB ID	3TUT	3TUX	3TW3	3TV1

Standard definitions are used for all parameters. Figures in parentheses refer to data in the highest resolution bin. The refinement and geometric statistics are from PHENIX. Crystallographic data statistics are from MOSFLM/SCALA. R_{free} sets for cross validation consisted of 5% of data selected at random against which structures were not refined.