Electronic Supplementary Material (ESI) for Molecular BioSystems

Supplementary Information

C-di-GMP-proflavine-hemin supramolecular complex, has peroxidase activity- implication for a simple colorimetric detection

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General methods for optical measurements: The concentrations of stock solutions of c-di-GMP and proflavine were determined by measuring the absorbance at 260 nm for c-di-GMP and 445 nm for proflavine and using 21,600 $M^{-1}cm^{-1}$ and 40,000 $M^{-1}cm^{-1}$ as a molar extinction coefficients for c-di-GMP and proflavine respectively. Proflavine was purchased from Sigma-Aldrich. rGTP, rATP, rCTP, and rUTP were purchased from Promega. cGMP (Guanosine 3',5'-cyclic mono phosphate) was purchased from CALBIOCHEM. GMP (Guanosine-5'-monophosphate) was purchased from Amersco. C-di-AMP was purchased from Axxora.com. C-di-GMP was obtained as previously described [ref1].

General preparation of sample before colorimetric measurements: target (nucleotide), water, buffer solution (pH 7.9) and salt solutions were mixed, heated up to 95 ºC and kept at 95 ºC for 5 min, then cooled down to room temperature and kept at room temperature for 15 min. Proflavine was then added to the mixture and then incubated in the refrigerator at 4 ºC overnight (12 hr). After ABTS was added, the mixture was incubated at 15 ºC for 5 min (for Figures 1B and 3) or 10 ºC for 5 min (for Figure 2) before H_2O_2 was added to the mixture. The formation of the product was monitored at 415 nm immediately after the H_2O_2 was added. The incubation periods are required to allow the supramolecular hexamer to form. Initially, longer incubation times and lower temperatures (4 ^oC for incubation) and (10 or 15 ^oC for reaction) were chosen to provide sufficient time for the formation of the supramolecular complex as well as ensure that the complex remained intact. One of the reviewers suggested that we investigate if the assay could be simplified by performing the peroxidation reaction at room temperature. *Pleasingly, we found out that the assay still works if the incubation of c-di-GMP and proflavine is done for only 1 h (at 4 ^oC; done in a refrigerator found in every laboratory) and the mixture to warmed to room temperature (~ 25 ^oC) before peroxidation is done at room temperature (see Figure S7). This therefore greatly simplifies this detection assay.*

Absorbance measurements: The instrument settings were chosen as follows: 415 nm for monitoring the formation of the radical cation $ABTS^{\bullet+}$. Scanning speed was 1000 nm/min. The measurements were carried out at 15 ºC (for Figures 1 and 3)) and 10 ºC (for Figure 2). Subtracting the absorbance value before H_2O_2 was added normalized absorbance values.

Circular dichroism experiments (CD): The measurement was performed at 15 ºC. Data pitch: 1 nm, scan speed: 50 nm/min, response: 8 sec, band width: 1 nm.

Preparation of the cell lysate: *E. coli* BL21(DE3) was used for production of WspR(D70E), RocR from pVL1321 (a pET vector derivative). Strains were grown up at 30 ºC in Luria-Bertani (LB) medium (3 L) with shaking and IPTG induction was performed for 6 hrs. Final OD (600 nm) was 2.8 for BL21(DE3) pVL1321-wspR, 6.8 for BL21(DE3) pVL1321-rocR. From plating of serial dilution of the culture, each OD (600 nm) unit is equivalent to $1x10^9$ colony forming units (CFU). Cells were pelleted by centrifugation, and resuspended to final volume of 40 ml of 10 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. 100 µl of 25 mg/ml Lysozyme was added to each re-suspension. The re-suspension was sonicated (20 seconds, 3 times) to disrupt membranes and allow lysozyme to reach cell walls. 60 % perchloric acid (final concentration was 12 %) was added to the re-suspension to precipitate cellular macromolecules. The re-suspension was incubated for 10 minutes on ice and neutralized by base. The re-suspension was centrifuged and the supernatant was filtered using a 0.2 mm filter and 3 kD exclusion columns.

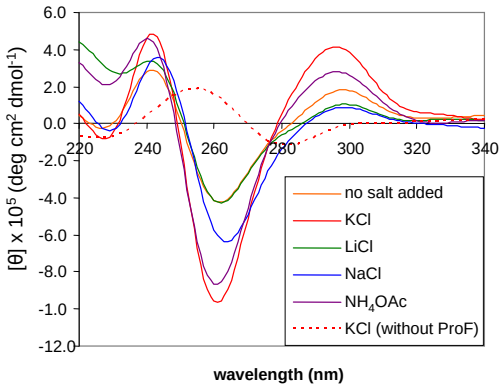
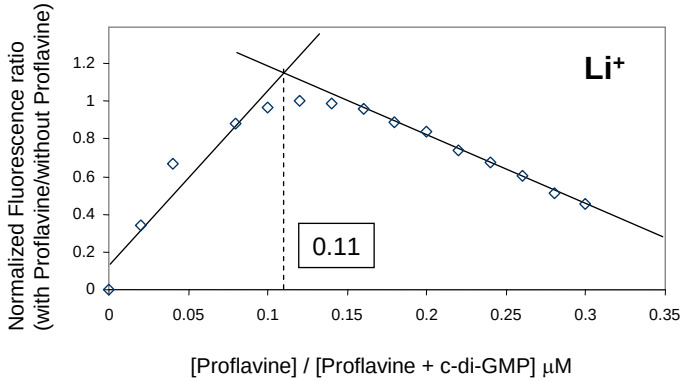


Figure S1. CD data of c-di-GMP, proflavine and hemin nucleotidezyme in the presence of different metals. [c-di-GMP] = 40 μM, [Hemin] = 0.5 μM, [Proflavine] = 10 μM, [metal] = 250 mM. Buffer: 50 mM Tris-HCl (pH 7.9) at 15 ºC.



cation	Value of mole fraction of Proflavine matching with point of intersection	Stoichiometry between Proflavine and c-di-GMP
K^{+} ref4)	0.18	1 : 4.56
Li^{+} this work)	0.11	1 : 8.09

Figure S2. Job plot. Total [Proflavine] + [c-di-GMP] was fixed at 50 μM. Buffer: 50 mM Tris-HCl (pH 7.9) containing 250 mM LiCl. Ex. 445 nm, Em. 508 nm.

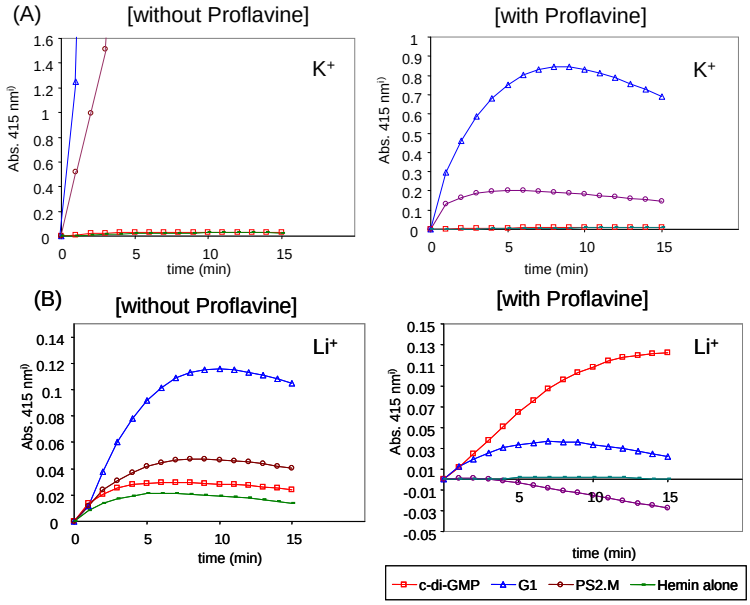


Figure S3. Comparison of peroxidation enhancement by c-di-GMP and DNA (G1 or PS2M) with ABTS as substrate and in the presence or absence of proflavine. [c-di-GMP] = 8 μM, [DNA] = 2 μM, [Hemin] = 0.5 μM, [Proflavine] = 30 μM, [cation] = 250 mM, Buffer: 50 mM Tris-HCl (pH 7.9), [ABTS] = 2 mM, [H_2O_2] = 2 mM, temperature: 15 °C. (A) K^+ was used as cation, (B) Li^+ was used as cation. For the sequence of G1 and PS2.M, see Figure S4. i) Normalized Abs.

In the presence of potassium, DNA peroxidases are significantly more active than c-di-GMP peroxidase (see Figure S3). Also, in line with expectation, the catalytic efficiencies of DNA peroxidises G1 and PS2.M are significantly higher in the presence of potassium than lithium. Proflavine reduces the catalytic proficiencies of both G1 and PS2.M DNA peroxidises but increases the catalytic efficiency of c-di-GMP peroxidase.

name	Sequence
G1	5'-GGG TA GGG C GGG TT GGG T-3'
PS2.M	5'-GT GGG TA GGG C GGG TT GG -3'

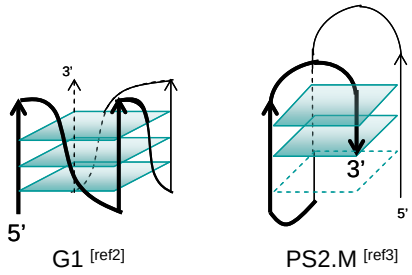


Figure S4. Sequence and structure for the DNA (G1 and PS2.M). G1 forms three G-tetrads whereas PS2.M forms two G-tetrads (see references 2 and 3).

Table S1. C-di-GMP/proflavine enhancement of hemin peroxidation (from Figure 1B of main text)

Entry	combination	Initial velocity (V_0) μM/min	V_0 (complex) / V_0 (hemin alone)
a	Only Hemin (no c-di-GMP) (with Li^+)	0.05	1.00
b	C-di-GMP + Proflavine + Hemin (with Li^+)	2.21	44.2
c	Only Hemin (no c-di-GMP) (no added cation)	0.69	1.00
d	C-di-GMP + Proflavine + Hemin (no added cation)	3.89	5.64

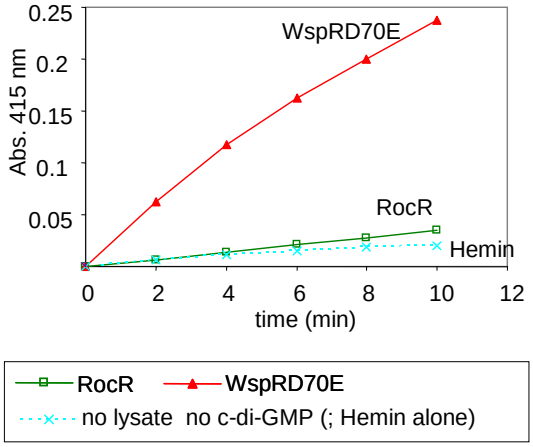


Figure S5. Detection of c-di-GMP in crude bacterial lysate (RocR: pVL791- RocR[ref5], WspRD70E: pVL791-WspRD70E[ref6]). Note, lysate is filtered to remove DNA/RNA.

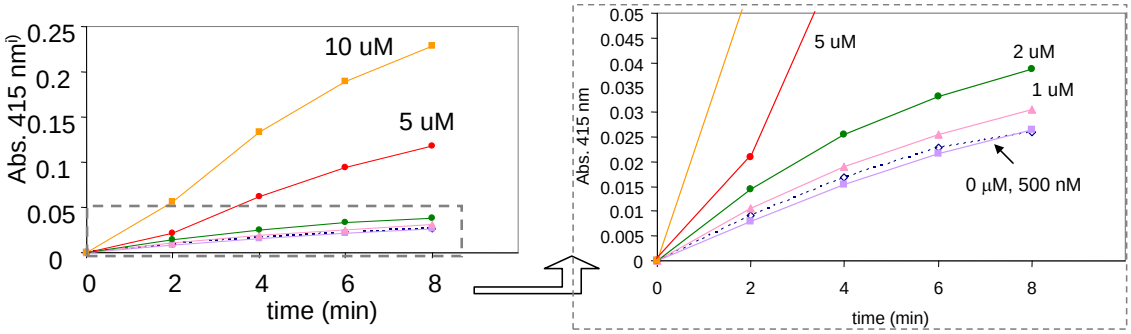


Figure S6. C-di-GMP detection in RocR lysate. Different concentrations of c-di-GMP (0, 1, 2, 5, 10 μM), [Hemin] 0.5 μM, [Proflavine] 30 μM, no metal was added. Buffer: 50 mM Tris-HCl (pH 7.9), [ABTS] = 2 mM, [H_2O_2] = 2 mM at 10 ºC. i) Normalized Abs.

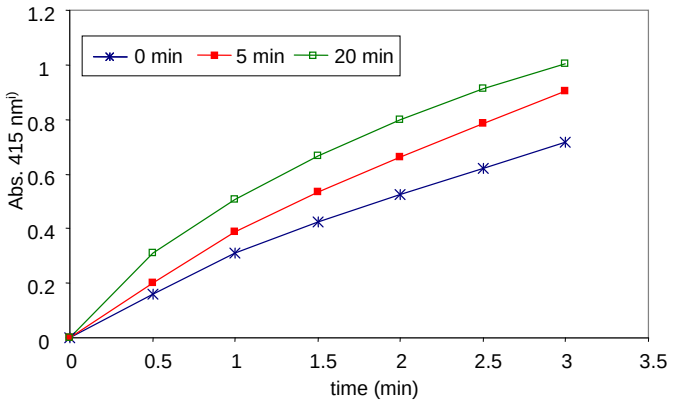


Figure S7. C-di-GMP-proflavine-hemin peroxidation at room temperature. C-di-GMP was incubated with proflavine at 4 ^{o}C for 1 h. The mixture was then warmed to room temperature and hemin was then added at room temperature. H_2O_2 was added 0, 5 and 20 min after the addition of hemin. [c-di-GMP] = 10 μM, [Proflavine] = 30 μM, [Hemin] = 0.5 μM, [ABTS] = 2 mM, [H_2O_2] = 2 mM, Buffer: 50 mM Tris-HCl (pH 7.9) containing no metal.

From Figure S7, we conclude that the detection of c-di-GMP could also be done at room temperature (25 ^{o}C).

Reference

[ref1]: I. Kiburu, A. Shurer, L. Yan, H. O. Sintim, *Mol. BioSyst*. **2008**, *4*, 518 - 520.
[ref2]: Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler and I. Willner, *J. Am. Chem. Soc.* **2004**, *126*, 7430-7431.
[ref3]: H. Lusic, M. O. Lively, A. Deiters, *Mol. BioSyst*. **2008**, *4*, 508-511.
[ref4]: S. Nakayama, I. Kelsey, J. Wang, H. O. Sintim, *Chem. Commun*. **2011**, *47*, 4766-4768.
[ref5]: H. Kulasakara, V. T. Lee, A. Brencic, N. Liberati, J. Urbach, S. Miyata, D. G. Lee, A. N. Neely, M. Hyodo, Y. Hayakawa, F. M. Ausubel and S. Lory, *Proc. Natl. Acad. Sci.U S A*, 2006, **103**, 2839-2844.
[ref6]: M. Merighi, V. T. Lee, M. Hyodo, Y. Hayakawa and S. Lory, *Mol. Microbiol.*, 2007, **65**, 876-895.