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2′,3′-cAMP hydrolysis by metal-dependent phosphodiesterases containing DHH, EAL, and HD domains is non-specific: Implications for PDE screening

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ABSTRACT

The recent report of 2′,3′-cAMP isolated from rat kidney is the first proof of its biological existence, which revived interest in this mysterious molecule. 2′,3′-cAMP serves as an extracellular adenosine source, but how it is degraded remains unclear. Here, we report that 2′,3′-cAMP can be hydrolyzed by six phosphodiesterases containing three different families of hydrolytic domains, generating invariably 3′-AMP but not 2′-AMP. The catalytic efficiency (k_{cat}/K_m) of each enzyme against 2′,3′-cAMP correlates with that against the widely used non-specific substrate bis(*p*-nitrophenyl)phosphate (bis-pNPP), indicating that 2′,3′-cAMP is a previously unknown non-specific substrate for PDEs. Furthermore, we show that the exclusive formation of 3′-AMP is due to the P–O2′ bond having lower activation energy and is not the result of steric exclusion at enzyme active site. Our analysis provides mechanistic basis to dissect protein function when 2′,3′-cAMP hydrolysis is observed.

1. Introduction

It is well-known that RNA with 2′,3′-cyclic phosphodiester end exists as an common intermediate after cleavage by RNases, like RNase A [1]. Meanwhile, the existence of small 2′,3′-cyclic mononucleotides in biological system has remained mysterious for a long time till the recent discovery of 2′,3′-cAMP being released from rat kidney [2]. The released 2′,3′-cAMP is further broken down into 2′-AMP and 3′-AMP, which can serve as sources of extracellular adenosine [3]. How 2′,3′-cAMP is synthesized or degraded remains unknown. However, reports of phosphodiesterases (PDEs) that can hydrolyze 2′,3′-cAMP are abundant. A family of metal-independent 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase, EC 3.1.4.37) that hydrolyzes 2′,3′-cAMP into 2′-AMP was discovered in brain since 1960s [4]. Though the physiological substrate of CNPase remains unclear, recent evidence suggests that it can function as a tRNA splicing enzyme *in vivo* [5]. On the other hand, there are numerous reports of 2′,3′-cyclic-nucleotide 2′-phosphodiesterases that generate 3′-AMP (EC 3.1.4.16, see http://www.brenda-enzymes.org/php/result_flat.php4?ecno=3.1.4.16). These reports were based on *in vitro* enzymatic assays using synthetic substrates; their physiological relevance therefore remains obscure. In general, while PDEs belonging to diverse domain families have been reported to have 2′,3′-cyclic-nucleotide 2′-phosphodiesterase activity [6–9], 2′,3′-cyclic-nucleotide 3′-phosphodiesterase activity has been confined to the 2H phosphoesterase family that includes CNPase [10]. What governs this differential specificity remains a mystery for decades.

Earlier reports suggested that the 2′,3′-cyclic ribose structure is unstable due to the strain of the five-membered ring [11]. It was also shown that a small Cu^{II} complex, through an activated hydroxide group bound by the metal, could efficiently hydrolyze 2′,3′-cAMP [12]. To date, at the enzyme level, it remains unknown whether PDEs and other metallo-hydrolases, whose active sites are generally endowed with specificity towards cognate substrate, can also non-specifically hydrolyze 2′,3′-cyclic nucleotides like 2′,3′-cAMP. This would have broad implications regarding the physiological relevance of the known 2′,3′-cyclic nucleotide phosphodiesterases, as well as the recent approach of using 2′,3′-cyclic nucleotides as part of a natural substrate collection to screen cognate PDEs out of proteins with unknown functions but known structures [13]. More and more such proteins are made available by efforts of the Structural Genomics Initiatives [6,7,14], which calls for an urgent and critical assessment of the mechanism of 2′,3′-cyclic nucleotide hydrolysis.

Here, we compare the hydrolytic pattern of 2′,3′-cAMP and bis-pNPP, a widely used non-specific PDE substrate, by six phosphohydrolases (Fig. 1A), five of which have been previously characterized as metal-dependant PDEs with known substrates (Table 1). Our results demonstrate that hydrolysis of 2′,3′-cAMP into 3′-AMP by these enzymes is non-specific and provide mechanistic insight. Our results and analysis also suggest that, depending on products

Abbreviations: bis-pNPP, bis(*p*-nitrophenyl)phosphate; AP, alkaline phosphatase; CNPase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase; PDE, phosphodiesterase; PPT, phosphopantetheinyl; HPLC, high performance liquid chromatography.

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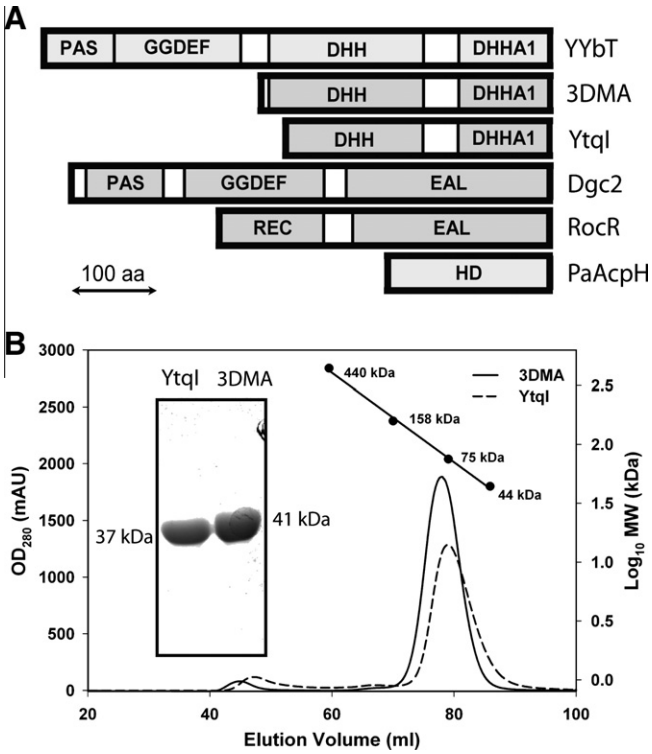

Fig. 1. (A) Domain composition of the six enzymes. (B) Characterization of YtqI and 3DMA by size exclusion chromatography. Inset: SDS–PAGE gel of purified proteins.

generated, 2′,3′-cAMP can be used as a probe to screen for enzymes that work on 2′,3′-cyclic phosphodiester structure under physiological context or merely PDEs in general.

2. Materials and methods

2.1. Materials

The 2′,3′-cyclic nucleotide substrates, their respective linear nucleotide standards, bis-pNPP and pNPP are from sigma. Calf intestine aphosphatase (AP) was from Roche and was used according to manufacturer's instruction. Custom synthesized YtqI and 3DMA genes were obtained from GenScript Corporation (NJ, USA).

2.2. Protein cloning, expression and purification

The genes encoding 3DMA and YtqI proteins were cloned into PET28(a+) (Novagen) between the NdeI and XhoI restriction sites, resulting in two N-terminal His_6-tagged recombinant constructs. The plasmids were transformed into *Escherichia coli* strain BL21 (DE3). Subsequent expression and purification procedures were the same as used for YybT [15], except a new lysis buffer was used [50 mM Tris (pH 8.0), 150 mM NaCl, 5% Glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM ethylenediaminetetraacetic acid].

2.3. PDE activity assay against 2′,3′-cyclic nucleotides

For RocR and DGC2, enzymatic reaction condition used was: 100 mM Tris–HCl (pH 8.0), 20 mM KCl and 25 mM $MgCl_2$. For YybT, YtqI, 3DMA, and PaAcpH, reaction condition was the same as described for assaying the c-di-AMP hydrolysis by YybT [15]: 100 mM Tris–HCl (pH 8.3), 20 mM KCl, 0.5 mM $MnCl_2$. Reactions were stopped by adding 1/10 volume of 0.5 M EDTA and the progress of 2′,3′-cAMP hydrolysis was monitored using an HPLC system with reverse phase XDB-C18 column (4.6 × 150 mm) [mobile phase: 20 mM triethylammonium bicarbonate (pH 7.0, pH adjusted with acetic acid), 9% methanol, 0.9 mL/min], as was adapted from Ref. [16]. Initial velocity at a certain substrate concentration was obtained from a series of reactions with varying incubation time. The kinetic parameters k_{cat} and K_m were obtained by fitting the initial velocities at various substrate concentrations to the Michaelis–Menten equation using the software Prism (GraphPad). HPLC condition used was the same for 2′,3′-cGMP, but slightly modified (methanol concentration changed to 6%) for 2′,3′-cCMP and 2′,3′-cUMP.

2.4. Activity assay against bis-pNPP and pNPP

Enzymatic reaction conditions were the same as those used for 2′,3′-cAMP. Hydrolysis was followed using a Shimadzu UV-1700 spetrophotomer to monitor *p*-nitrophenol formation at 410 nm. The extinction coefficient used to calculate *p*-nitrophenol concentration was 18,300 M^{-1} cm^{-1}. A control with the enzyme storage buffer only was used for baseline correction. Due to the solubility issues of bis-pNPP, high substrate concentration could not be reached to accurately determine the k_{cat} and K_m for all enzymes. Catalytic efficiency was calculated as $1/slope_{(double\text{-}reciprocal\ plot)}$.

2.5. Computational modeling

The modeling of 2′,3′-cyclic nucleotide (taken from PDE id 1JH7) into of the EAL domain PDE BlrP1 (PDB id 3GG0) and the CNPase (PDB id 1WOJ) was done in Pymol (DeLano Scientific). The scissile phosphate groups were aligned by pair-fitting 1: the phosphorus atom under attack; 2: the leaving oxygen atom.

2.6. Statistical analysis

Correlation analysis was performed using the software Prism (GraphPad). Nonparametric correlation (spearman) with one-tailed *p* value was determined.

Table 1
Steady-state kinetic parameters for the six proteins against 2′,3′-cAMP and bis-pNPP.

Enzyme	PDE fold	Cognate substrate	Ref.	2′,3′-cAMP			bis-pNPP k_{cat}/K_m (min^{-1} mM^{-1})	3′,5′-cAMP hydrolysis
				k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})		
YybT	DHH	c-di-AMP	[17]	250 ± 31	15.8 ± 6.2	15.8 ± 6.5	0.55 ± 6.5	No
YtqI	DHH	nano-RNA	[32]	610 ± 30	3.5 ± 0.58	174 ± 30	110 ± 10	No
3DMA	DHH	–	– [a]	15 000 ± 300	0.85 ± 0.07	(1.7 ± 0.1) × 10^4	670 ± 20	No
DGC2	EAL	c-di-GMP	[18]	2.6 ± 0.5	0.05 ± 0.02	52 ± 14	0.51 ± 0.03	No
RocR	EAL	c-di-GMP	[15]	0.13 ± 0.01	8.7 ± 1.0	0.015 ± 0.002	0.004 ± 0.0005	No
PaAcpH	HD	PPT arm	[20]	0.21 ± 0.02	1.0 ± 0.3	0.21 ± 0.06	0.03 ± 0.01	Residual

[a] The 3DMA protein has not been characterized but its structure was solved by Northeast Structural Genomics Consortium (PDB id: 3DMA). We speculate it is an YtqI homolog.

3. Results and discussion

Among the six proteins we characterized (Fig. 1A and Table 1), YybT, YtqI and 3DMA contain the DHH domain [17]. YybT and YtqI are PDEs for cyclic-di-AMP and nano-RNA, respectively [15,18]. The 3DMA protein was uncharacterized, but its structure was solved by Northeast Structural Genomics Consortium (PDB id: 3DMA). We speculate that it is an YtqI homolog. RocR and DGC2 contain the EAL domains that specifically hydrolyze cyclic-di-GMP [19,20]. PaAcpH is a HD domain PDE [21] that hydrolyzes the phosphodiester bond between acyl carrier protein and the phosphopantetheinyl (PPT) arm [22]. Structural data showed that DHH, EAL, and HD domain proteins are all metalloenzymes that require divalent metal ion to assist hydrolysis, at least in part to activate the attacking water nucleophile [23–25].

For YybT, RocR, DGC2, and PaAcpH, purified recombinant proteins were obtained according to published procedures described before [15,19,20,22]. For YtqI and 3DMA, dimeric proteins were obtained as described in material and methods and used for enzymatic assays (Fig. 1B). All six proteins displayed bis-pNPP hydrolysis activity with the assistance of divalent metal ions, in agreement with their PDE function, but their catalytic efficiency varied up to 10^5 fold (Table 1).

3.1. Kinetic evidence that hydrolysis of 2′,3′-cAMP into 3′-AMP is non-specific

We initially observed hydrolysis of 2′,3′-cAMP in our screening for potential substrate of YybT (Fig. 2A, B, and E). The end product was exclusively 3′-AMP. No hydrolysis was observed when 3′-AMP or 2′-AMP was used as substrate, in agreement with the lack of reactivity against phosphatase substrate described previously [15]. Since the cognate substrate of YybT is c-di-AMP and 2′,3′-cyclic nucleotides are prone to hydrolysis [11,12], we tested the hypothesis that 2′,3′-cAMP is a non-specific substrate for PDEs and found that all six proteins actively hydrolyzed 2′,3′-AMP into 3′-AMP in the presence of Mg^{2+} or Mn^{2+} ions. We believe that the PDE active sites are responsible for 2′,3′-cAMP hydrolysis, as we found that, for multi-domain proteins RocR and YybT, mutations of catalytic residues (E352A for RocR and D420A for YybT) that were crucial for their PDE activity [15,20] diminished the hydrolysis of 2′,3′-cAMP to below 0.1%. We also observed significant hydrolysis of 2′,3′-cGMP, 2′,3′-cCMP, and 2′,3′-cUMP by YybT and the other enzymes, generating nucleotide 3′-phosphates as products. The less than 10-fold difference in rate (Fig. 2F) suggested that hydrolysis was due to the instability of 2′,3′-cyclic ribose structure and was not base-specific. Indeed, the natural substrate

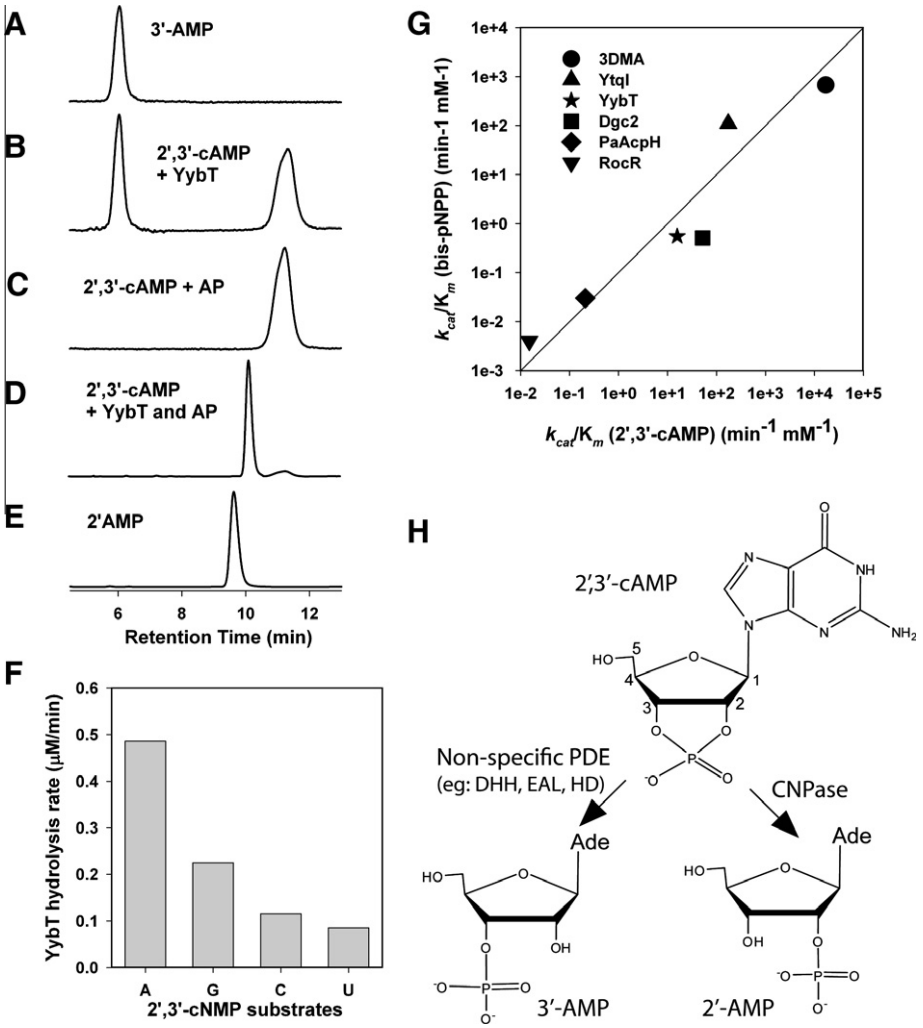

Fig. 2. (A) HPLC trace of 2′,3′-cAMP hydrolysis by AP, YybT and a combination of AP and YybT. The standard retention times for 3′-AMP, 2′-AMP, Adenosine, and 2′,3′-cAMP are: 6.0 min, 9.4 min, 10.1 min, and 11.2 min. (D) Cross-comparison of the catalytic efficiency of the six proteins against 2′,3′-cAMP (*x*-axis) and bis-pNPP (*y*-axis). (E) Scheme of 2′,3′-cAMP hydrolysis.

for PaAcpH bares no structural resemblance to 2′,3′-cAMP other than the phosphodiester moiety [22], suggesting that interaction with other parts of 2′,3′-cAMP is not necessary for catalysis by PaAcpH. Meanwhile, AP did not hydrolyze 2′,3′-cAMP after prolonged incubation (Fig. 2C), but generated adenosine when used in combination with YybT (Fig. 2D), suggesting that 2′,3′-cAMP is susceptible to PDEs only and not any metal-assisted phosphohydrolase.

Kinetic measurements using 2′,3′-cAMP as a substrate revealed K_m values of sub-to-high mM range (0.045–31 mM) (Table 1). Such high K_m values are not observed for cognate PDEs of signaling nucleotides (3′,5′-c(A/G)MP, c-di-AMP, or c-di-GMP), where their K_m values are in the range of sub-to-low μM [15,19,26], suggesting non-specific binding of 2′,3′-cAMP. Importantly, we observed a correlation (spearman correlation coefficient = 0.95, $p = 0.008$) between the catalytic efficiency of individual protein against bis-pNPP and that against 2′,3′-cAMP (Fig. 2G). This correlation strongly suggests that, similar to the case of bis-pNPP, hydrolysis of 2′,3′-cAMP here is a non-specific task and its rate primarily depends on a combination of catalytic competence and active site accessibility of the PDE used. To our knowledge, no such correlation has been reported before, nor has the non-specific nature of 2′,3′-cAMP hydrolysis by enzymes.

3.2. The weaker P–O2′ bond, but not steric exclusion, was responsible for the exclusive formation of 3′-AMP

We were intrigued by the selective formation of 3′-AMP but not 2′-AMP and examined two possible explanations. First, the six PDEs belonging to three different folds all sterically hindered the binding of 2′,3′-cAMP in a conformation poised to break P–O3′ bond. We addressed this through computational docking. The EAL domain PDEs have highly conserved active sites, known structures and well-characterized catalytic mechanism [24], they are therefore selected for docking analysis. Steric hindrance is unlikely the reason here because docking of 2′,3′-cyclic nucleotide into an EAL domain revealed no steric clash between the protein and nucleotide, regardless of whether the nucleotide was modeled to generate 3′-AMP or 2′-AMP (Fig. 3A and B). As a comparison, when 2′,3′-cyclic nucleotide was docked into CNPase in a conformation for attacking the P–O2′ bond, we observed significant steric clash between the ribose, base and the enzyme (Fig. 3D). No clash was observed when the substrate was docked in a conformation for attacking the P–O3′ bond (Fig. 3C). Thus, CNPase enforces selective cleavage of the P–O3′ bond of 2′,3′-cAMP by steric exclusion, a property not shared by metal-dependant PDEs that cleaves the P–O2′ bond.

The second potential explanation is that the P–O2′ bond is energetically less stable and hence more prone to hydrolysis. This is consistent with the products generated (>95% 3′-AMP) by the small metal–hydroxide complex, which does not show steric preference and cleaves bond according to strength [12]. This would also explain why CNPase requires a steric exclusion property, since it specifically breaks the more energy-demanding P–O3′ bond. To verify, we attempted to determine the activation energy of the respective P–O bonds by monitoring spontaneous hydrolysis at a series of elevated temperatures. Unfortunately, the temperature-dependent cleavage rate cannot be accurately measured due to complications

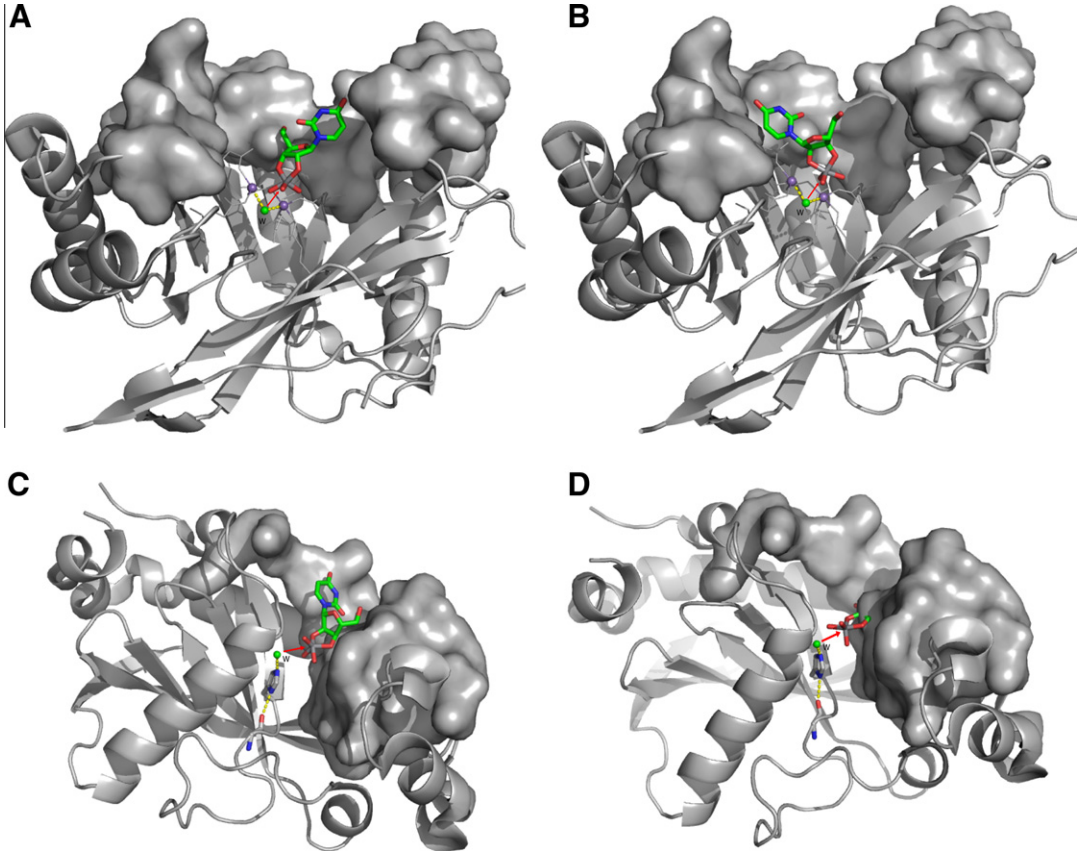

Fig. 3. (A,B) Modeling of 2′,3′-cyclic nucleotide into the active site of a EAL domain PDE. The nucleotide was modeled for in-line water attack (red arrow) on the P–O3′ bond (A) or the P–O2′ bond (B). The catalytic mechanism of EAL domain is detailed in Ref. [24]: two divalent metal ions (cyan spheres) coordinated by side-chains of active site residues (gray lines) activate the attacking the water nucleophile (green sphere). (C,D) Modeling of 2′,3′-cyclic nucleotide into the active site of human brain CNPase The nucleotide was modeled for in-line water attack (red arrow) on the P–O3′ bond (C) or the P–O2′ bond (D). The catalytic mechanism of CNPase is detailed in Ref. [36]: a conserved histidine residue activated by another main-chain carbonyl group in turn activates the attacking water (green sphere). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

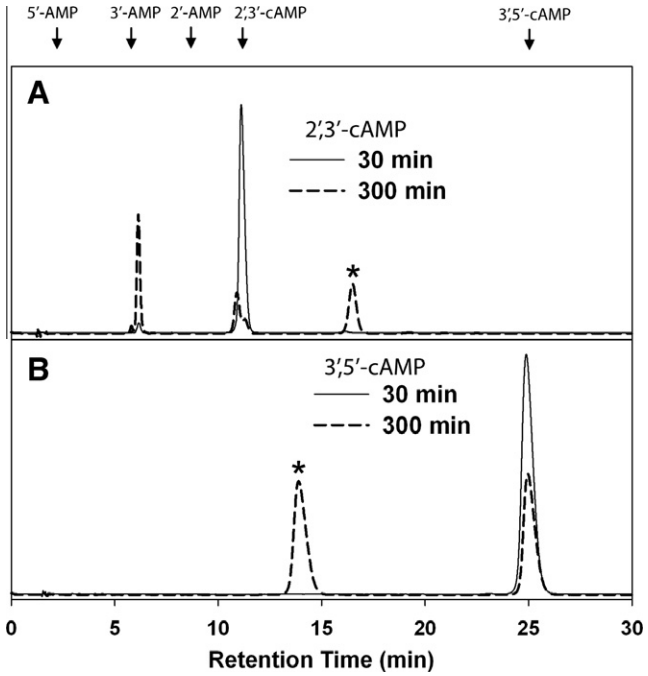

Fig. 4. HPLC trace of 2′,3′-cAMP (A) and 3′,5′-cAMP (B) incubated at 80° without any enzyme. Buffer conditions: 100 mM Tris, pH 8.3, 20 mM KCl, 0.5 mM Mn^{2+}. The arrows on top mark the retention times of the respective nucleotides based on standard. Peaks marked by asterisks are candidate products resulting from ligation of cyclic nucleotides.

arising from formation of ligation product(s), as reported recently elsewhere [27]. Nevertheless, it was clearly noted that, after incubation at 80 °C, most 2′,3′-cAMP was broken down into 3′-AMP but not 2′-AMP (Fig. 4A), while no breakdown products of 3′,5′-cAMP (3′-AMP or 5′-AMP) were observed (Fig. 4B). We also detected little or no hydrolysis of 3′,5′-cAMP by the six PDEs (Table 1). These results are consistent with the notion that 2′,3′-cAMP is energetically less stable than 3′,5′-cAMP, with the P–O2′ bond having lower activation energy than the P–O3′ bond. To understand why the P–O2′ bond is weaker, we surveyed crystal structures of various 2′,3′-cyclic nucleotides and found that its bond length is longer by 0.011–0.07 Å (Table 2) [28–31]. Here, it is conceivable that, due to the low activation energy of the P–O2′ bond in 2′,3′-cAMP, the energy barrier for reaching transition state is low, and a metal activated water/hydroxide group in a given PDE active site can launch a nucleophilic attack on the phosphorus atom as long as the 2′,3′-cAMP molecule can access the active site with proximity.

The mechanistic insights of 2′,3′-cAMP hydrolysis we gained here led us to analyze existing reports of proteins hydrolyzing 2′,3′-cAMP, other than the undisputed CNPase family. We found that our conclusion can guide proper interpretation of results of other proteins (Table 3). For the five enzymes that generate 3′-AMP as the sole or predominant product, only *in vitro* enzymatic characterization data was available, four of them contain the metallo-beta-lactamas PDE fold [32]. The activity of the PhnP protein, for instance, was discovered by large-scale substrate screening [7]. Although the authors discussed how the *in vitro* 2′,3′-cAMP hydrolysis might be related to signaling pathways *in vivo*, the reactivity could be a non-specific property of PDEs in general and biologically irrelevant. In comparison, for the two enzymes where involvement in RNA modification is obvious, both can generate 2′-AMP. The CthPnkP protein, a homolog of the λ-phage T4 polynucleotide kinase 3′-phosphatase, hydrolyzed 2′,3′-cAMP into adenosine with 2′-AMP being an intermediate [33], according to the results of its phosphatase inactive mutant CthPnkP-H198D [34]. The tRNA-NT protein, an *E.* coli tRNA nucleotidyltransferase that adds a CCA tri-nucleotide to the carboxy-terminus of a tRNA for maturation, has a remarkable metal independent activity that generates 2′-AMP [35], which is the same as CNPase and very likely to be its physiological function.

Taken together, our results and analysis suggest that 2′,3′-cAMP can be used as a probe to screen for enzymes that work on 2′,3′-cyclic phosphodiester structure under physiological context when 2′-AMP is the product, or merely PDEs in general when 3′-AMP is the product (Fig. 2H). This implies that the numerous reports on

Table 2
The P–O2′ and P–O3′ bond distance of various 2′,3′-cyclic nucleotides.

Nucleotide	CSD refcode[a] or PDB id[b]	P–O2′ (Å)	P–O3′ (Å)	Δ(P–O) (Å)	Ref.
2′,3′-cAMP	–[c]	1.623	1.612	0.011	[24]
2′,3′-cGMP	–[c]	1.617	1.604	0.013	[24]
2′,3′-cCMP	CYCYPH10[a]	1.622	1.597	0.025	[25]
2′,3′-cGPS	1GSP[b]	1.70	1.63	0.07	[26]
Uridine 2′,3′-Vanadate[d]	1JH7[b]	1.75	1.71	0.04	[27]

[a] Cambridge Structure Database refcode id.
[b] PDB code id.
[c] Id not found.
[d] Uridine 2′,3′-vanadate is an analog of 2′,3′-UMP where the phosphorus atom is replaced by vanadium. The corresponding bonds should be V–O2′ and V–O3′, respectively.

Table 3
Steady-state kinetic parameters for proteins that does not hydrolyze 3′,5′-cAMP but hydrolyze 2′,3′-cAMP.

Enzyme	PDE fold	Product(s)	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} μM^{-1})	Ref.
CvfA	HD	3′-AMP	55	17	3.21	[7]
DR1281	Metallo-beta-lactamse[a]	3′-AMP	22.8 ± 0.6	0.55 ± 0.05	41.5 ± 3.9	[5]
PhnP	Same as above	3′-AMP	103 ± 4	0.11 ± 0.01	936 ± 92	[6]
YfcE-C74H	Same as above	3′-AMP	2.6 ± 0.5	0.045 ± 0.015	57 ± 22	[8]
Rv0805	Same as above	3′-AMP > 2′-AMP[b]	150 ± 20	1.6 ± 0.4	94 ± 27	[8]
CthPnkP	Same as above	Adenosine	18 ± 1.4	536 ± 24	30 ± 3	[29]
tRNA-NT	HD	2′-AMP or mixture[c]	460 ± 30	0.49 ± 0.04	940 ± 100	[30]

[a] These metallo-beta-lactamse fold proteins are also referred to as longing to calcineurin-type phosphatase superfamily.
[b] Rv0805 generates a mixture of 3′-AMP and 2′-AMP, with 3′-AMP being the predominant species.
[c] tRNA-NT generates 2′-AMP in the absence of divalent metal ions, but a mixture of 2′-AMP and 3′-AMP in the presence.

2′,3′-cyclic-nucleotide 2′-phosphodiesterase activity (EC 3.1.4.16, see http://www.brenda-enzymes.org/php/result_flat.php4?ecno=3.1.4.16) could be attributed to non-specific activity of some PDE(s). The real functions of these proteins remain to be elucidated. Another implication of our results is that the scarcity of reports of 2′,3′-cyclic mononucleotides isolation from biological systems may be due to their susceptibility to hydrolysis by non-specific PDEs. Treatment with divalent metal chelating agents instead of a specific class of PDE inhibitor will facilitate the isolation process. Finally, we suggest the use of 2′,3′-AMP as a tool to characterize PDE and other phosphohydrolyase, the advantages of which, in comparison to the widely used bis-pNPP, is discussed in Supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.107.

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