Cystathionine β-Synthase (CBS) Domain-containing Pyrophosphatase as a Target for Diadenosine Polyphosphates in Bacteria*

Received for publication, July 21, 2015, and in revised form, September 11, 2015 Published, JBC Papers in Press, September 23, 2015, DOI 10.1074/jbc.M115.680272

Viktor A. Anashkin1, Anu Salminen1, Heidi K. Tuominen1, Victor N. Orlov2, Reijo Lahti1, and Alexander A. Baykov3,4

From the 1Department of Biochemistry, University of Turku, FIN-20014 Turku, Finland and the 2Belozersky Institute of Physico-Chemical Biology and Department of Chemistry, Lomonosov Moscow State University, Moscow 119899, Russia

Background: Many soluble pyrophosphatases contain two regulatory nucleotide-binding CBS domains with or without an intercalating DRTGG domain.

Results: Linear P1,Pn-diadenosine 5'-polyphosphates (ApnAs, n = 3–6) bind with nanomolar affinity to and activate DRTGG domain-containing pyrophosphatases; Ap3A binds cooperatively.

Conclusion: Nucleotide-regulated pyrophosphatases may represent receptors for ApnAs in bacteria.

Significance: The results suggest a novel regulatory pathway in some bacteria, involving ApnAs as messengers.

Among numerous proteins containing pairs of regulatory cystathionine β-synthase (CBS) domains, family II pyrophosphatases (CBS-PPases) are unique in that they generally contain an additional DRTGG domain between the CBS domains. Adenine nucleotides bind to the CBS domains in CBS-PPases in a positively cooperative manner, resulting in enzyme inhibition (AMP or ADP) or activation (ATP). Here we show that linear P1,Pn-diadenosine 5'-polyphosphates (ApnAs, where n is the number of phosphate residues) bind with nanomolar affinity to DRTGG domain-containing CBS-PPases of Desulfitobacterium hafniense, Clostridium novyi, and Clostridium perfingens and increase their activity up to 30-, 5-, and 7-fold, respectively. ApA, Ap3A, and Ap6A bound noncooperatively and with similarly high affinities to CBS-PPases, whereas Ap3A bound in a positively cooperative manner and with lower affinity, like mononucleotides. All ApnAs abolished kinetic cooperativity (non-Michaelian behavior) of CBS-PPases. The enthalpy change and binding stoichiometry, as determined by isothermal calorimetry, were ~10 kcal/mol nucleotide and 1 mol/mol enzyme dimer for ApA and Ap3A but 5.5 kcal/mol and 2 mol/mol for Ap3A, AMP, ADP, and ATP, suggesting different binding modes for the two nucleotide groups. In contrast, Eggertella lenta and Morerella thermoacetica CBS-PPases, which contain no DRTGG domain, were not affected by ApnAs and showed no enthalpy change, indicating the importance of the DTRGG domain for ApnA binding. These findings suggest that ApnAs can control CBS-PPase activity and hence affect pyrophosphate level and biosynthetic activity in bacteria.

Diadenosine polyphosphates (ApnAs)3 are ubiquitous compounds in which two adenosine moieties are linked through ribose 5'-C by a chain of three to six phosphate residues. First discovered in 1965 as by-products of chemical ATP synthesis (1), ApnAs have subsequently been identified in organisms belonging to all kingdoms of life. Many enzymatic reactions leading to ApnAs are known (2), of which the reaction catalyzed by aminocyl-tRNA synthetase, lysyl-tRNA synthetase in particular (3), is the best known. Escherichia coli lysyl-tRNA synthetase produces ApnAs by a side reaction during lysyl-tRNA synthesis via attack of the terminal phosphate group of ATP and other monoadenosine phosphates on the enzyme-bound aminocyl adenylate intermediate (4). Because ATP prevail in cells, the product of its reaction with aminocyl adenylate, ApnA, is the most prevalent ApnA. Lysyl-tRNA synthetase can additionally convert ApnA to ApnA (5). ApnAs are degraded in the cell by specific and nonspecific enzymes, including ApnA hydrolase and phosphodiesterase (6, 7), which balance the intracellular concentration of ApnAs as a submicromolar level. However, their concentrations in prokaryotes can rise up to 300 μM under stress conditions (8).

Because of its association with stress, Ap4A was originally classified as an intracellular “alarmone” (8–10). An alternative view is that Ap4A formation represents a compensatory mechanism that helps to sustain basic physiology during stress and assist in the return to normal physiology in bacteria (11). In eukaryotes, ApnAs may have a second messenger role (12). Regardless of which theory is true, it is clear that ApnAs participate, in some as yet poorly understood ways, in a number of cellular phenomena associated with stress, such as DNA replication and repair (13) and cell division (14). In eukaryotes,

3 The abbreviations used are: ApnA, 5'-5'-Pn-diadenosine polyphosphate with n phosphate residues; CBS, cystathionine β-synthase; CBS-PPase, CBS domain-containing pyrophosphatase; cnPPase, C. novyi pyrophosphatase; cpPPase, C. perfingens pyrophosphatase; dhPPase, D. hafniense pyrophosphatase; efpPPase, E. lenta pyrophosphatase; mTPPase, M. thermoacetica pyrophosphatase; Ppase, pyrophosphatase; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid; ITC, isothermal titration calorimetry.
Ap₆,As are involved in many other processes, including neurotransmission (15), apoptosis (16), and analgesia (17). Of note, Ap₆A is used in hypoxia therapy in humans (18).

Understanding the roles of Ap₆,As requires knowledge of their target proteins. Using a radioactive photocrosslinking Ap₆A analog, Johnstone and Farr (19) detected 12 Ap₆,As binding proteins in E. coli extract, some of which were identified as heat shock proteins based on their electrophoretic mobilities. Guo et al. (20) and Azhar et al. (21) used pulldown assays with immobilized Ap₆A analogs followed by mass-spectral analysis to identify, respectively, 6 and 13 binding proteins in E. coli. The three protein sets obtained in these studies partially overlapped. Few Ap₆,As protein complexes have been subjected to biophysical and mechanistic studies. Apart from cases where Ap₆,As act as substrates or products of their metabolizing enzymes, the chaperone GroEL binds Ap₆A with a dissociation constant of 10 μM; the complex exhibits increased ATPase and chaperoning activities (11). Human 5’-nucleotidase II is allosterically activated by Ap₆,As (n = 4–6), which bind with dissociation constants of 60–80 μM (22).

Inorganic pyrophosphatases (PPases; EC 3.6.1.1), the major PP₁-,metabolizing enzymes in all types of organisms, belong to three nonhomologous families (23). Family II PPases, found in bacteria and archaea, are homodimeric Mn²⁺- or Co²⁺-metalloenzymes that additionally require Mg²⁺ for catalysis (24). A quarter of the more than 500 putative family II PPase sequences contain a regulatory insert comprising a pair of cystathionine β-synthase (CBS) domains (Bateman module (25)) within one of the two catalytic domains. Regulatory CBS domains are found in proteins in all kingdoms of life and generally bind adenine nucleotides as regulating molecules (26–28); mutations in CBS domains of human proteins are associated with hereditary diseases (29, 30). Interestingly, only in CBS-PPases (but not all of them), are the CBS domains intercalated by another (DRTGG) domain. CBS-PPases are activated by ATP and inhibited by AMP and ADP (31, 32). Both catalysis and regulation involve marked positive cooperativity, which is Mg²⁺-dependent (32).

The structure of the isolated dimeric regulatory insert of Clostridium perfringens PPase (cpPPase) obtained for crystals grown in the presence of 0.25 mM Ap₆A contains an Ap₆A molecule bound by two CBS domain pairs at the subunit interface (33), raising the possibility that Ap₆A may be a physiological ligand of CBS-PPases. Preliminary activity measurements (33, 34) suggested that Ap₆A activates cpPPase. Here we show that all Ap₆,As bind with nanomolar affinities to three DRTGG domain-containing CBS-PPases and modulate their catalytic activity and cooperative behavior. Our data thus identify a new type of ligand for CBS domains and an important target of Ap₆,As in the protein world.

**Experimental Procedures**

**Enzymes and Reagents**—Genes for CBS-PPases from De- sulfitobacterium hafniense (dhPPase), Clostridium novyi (cnPPase), C. perfringens (cpPPase), Eggerthella lenta (elPPase), and Moorella thermoacetica (mtPPase) were expressed in E. coli, and the produced CBS-PPases were purified as described previously (32–34). Inactive aggregates were separated from soluble active proteins during size exclusion chromatography. The final products were at least 95% pure as estimated by SDS-PAGE using a Phast system with 8–25% gradient gels (GE Healthcare). Protein concentrations were determined with a Nanodrop spectrophotometer (Thermo Scientific) using Aₙ₅₄₀ of 0.478 for dhPPase, 0.548 for cnPPase, 0.426 for cpPPase, 0.493 for elPPase, and 0.48 for mtPPase, as calculated from their amino acid compositions with ProtParam. Molar concentrations were calculated based on subunit molecular masses of 60.4, 63.6, 60.8, 52.5, and 48.1 kDa, respectively. All enzyme concentrations are given in terms of the dimer.

P₃, P₄-diadenosine 5’-polynucleotides (Ap₆,As) with n = 3–5 were from Sigma; Ap₆A was from Jena Bioscience. All Ap₆,As were ≥97% pure, and Ap₆A was essentially free of other Ap₆,As, according to the manufacturer analyses (HPLC). The concentrations of stock nucleotide solutions were calibrated by measuring absorbance in the ultraviolet region (ε₂₈₀ = 31,800 M⁻¹·cm⁻¹ for dinucleotides and 15,900 M⁻¹·cm⁻¹ for mononucleotides).

**Kinetic Assays**—The activity assay medium contained 5 mM MgCl₂, 140 μM PP₁ (yielding 50 μM MgPP₃ complex) and 0.1 M TES-KOH (pH 7.2), except where specified otherwise. In measurements done at higher Mg²⁺ concentrations, buffer concentration was decreased appropriately to maintain constant ionic strength. The reaction was initiated by adding enzyme, and P₁ accumulation caused by MgPP₃ hydrolysis was continuously recorded for 2–3 min at 25 °C using an automated P₁ analyzer (35). Initial velocities of Pπi hydrolysis were typically estimated graphically from the slopes of the tangents to the initial portion of hydrolysis time courses recorded with the P₁ analyzer.

**Isothermal Calorimetry**—A VP-ITC calorimeter (MicroCal Ltd.) was used. Enzyme and nucleotide solutions were made in 0.1 M MOPS-KOH (pH 7.2) buffer containing 2 mM MgCl₂, 0.1 mM CoCl₂, and 150 mM KCl. Titrations were performed at 25 °C by successive 10-μl injections of 0.1–10 mM mononucleotide or 33 μM dinucleotide solution into 2 ml of CBS-PPase solution (2.5–5 μM in terms of the dimer); the interval between injections was 5 min. All samples were degassed before the experiment. Binding isotherms were corrected by subtracting the ligand dilution isotherms, determined by titrating nucleotide solutions into the buffer.

**Calculations and Data Analysis**—The values of the apparent dissociation constants for the magnesium complexes of PP₁ used to maintain required free Mg²⁺ ion and MgPP₃, complex concentrations at pH 7.2 were 112 μM for MgPP₃ and 2.84 mM for Mg₃PP₃ (36). Nonlinear least square fittings were performed using the program Scientist (Micromath). The dependence of hydrolysis rate on nucleotide concentration ([N]) was fit to Equation 1,

\[ v = \frac{v_N + (v_0 + v_N)K_{N2}/2[N] + v_NK_{N3}[N]^3}{1 + K_{N2}[N]} + K_{N3}[N]^3 \]  

(Eq. 1)

where v₁ and v₂ are activities of free and nucleotide-saturated enzyme, respectively, and K₁, K₂, and K₃ are the macroscopic dissociation constants describing successive binding of nucleotide to two regulatory sites per enzyme molecule. Cooperative kinetics of substrate (MgPP₃) hydrolysis were analyzed with Equation 2,
Nucleotide-regulated Soluble Pyrophosphatases

![Graph showing concentration dependences of the effects of Ap₃Ps on the activity of three CBS-PPases measured at fixed concentrations of substrate (50 μM MgPP) and Mg²⁺ (5 mM). The lines show the best fits of Equations 1 or 4 (see text for details). Activity without nucleotides (220, 350, and 800 s⁻¹ for dhPPase, cpNPPase and cpPPase, respectively) was taken as unity. dh, dhPPase; cn, cpNPPase; cp, cpPPase.](image)

\[ v = k_{cat}[E]_0(1 + 0.5K_m/[S])/(1 + K_m/[S] + K_mK_m/[S]^2) \]  
(Eq. 2)

which assumes different Michaelis constants \((K_{m1} \text{ and } K_{m2})\) and equal \(k_{cat}\) values for the two active sites in the dimer. \([E]_0\) and \([S]\) are total enzyme and substrate concentrations, respectively. The corresponding binding schemes and details of the fitting procedure were described previously (32).

The dependences of \(K_{N1}, K_{N2}, K_{m1}, \text{ and } K_{m2}\) on Mg²⁺ (M) concentration were fit to Equation 3,

\[ K_i = (K_{i0} + [M]/K_m)/(1 + (K_{i0} + [M])/(K_{i0}/K_m)) \]  
(Eq. 3)

where \((K_{i0}, \text{ and } K_{i0})_x\) are the limiting values of the respective \(K_N\) or \(K_m\) at 0 and infinite Mg²⁺ concentrations, and \(K_m\) is the metal binding constant.

Alternatively, rate dependences on substrate and nucleotide concentrations were fit to a Hill-type equation 4,

\[ v = v_o + (v_i - v_o)/(1 + K_i/L)^h \]  
(Eq. 4)

where \(L\) is \(S\) or \(N\), \(v_i\) is the rate at infinite \([L]\), and \(h\) is the Hill coefficient. The value of \(v_o\) was set to 0 when \(L\) was substrate, and the value of \(h\) was set to unity for noncooperative binding.

Isothermal titration calorimetry (ITC) data were analyzed with a MicroCal ITC subroutine in Origin 7.0 software using a single-binding site model. Thermodynamic parameters were calculated from the standard relationship, \(\Delta G = RT\ln K_N = \Delta H - T\Delta S\).

Results

Effects of Ap₃Ps on CBS-PPases at a Fixed Mg²⁺ Concentration—Fig. 1 shows the concentration dependences of the effects of four Ap₃Ps with \(n = 3-6\) on the activities of three CBS-PPases measured at fixed substrate (MgPP) and Mg²⁺ concentrations (50 μM and 5 mM, respectively). Nanomolar concentrations of Ap₃Ps caused marked activation in all cases, except that Ap₃A was effective with cpPPase at micromolar concentrations.

Analyses of the dependences shown in Fig. 1 and of similar dependences measured at different substrate concentrations (1 and 300 μM) were initially done using Equation 4. The value of the Hill coefficient was indistinguishable from unity (1 ± 0.05) at all substrate concentrations for Ap₃Ps with \(n = 4-6\). In contrast, Ap₃A bound cooperatively (\(h = 1.4-1.7\)) at all substrate concentrations. Accordingly, the data for Ap₃A were analyzed with Equations 1 and 4 in their general forms, whereas Equation 4 with \(h = 1\) was used for the other Ap₃Ps. The parameter values derived from this analysis are summarized in Tables 1 and 2.

The values of the activation factor \((v_i/v_o)\) and their trends with changing polyphosphate length and substrate concentration were similar for the three enzymes. The value of \(v_i/v_o\) was greater at low than at high substrate concentrations. In the presence of 300 μM substrate, which is in excess of the respective Michaelis constants (32), \(v_i/v_o\) approached a value of ~2 in all cases.

The apparent binding affinities of the nucleotides could be compared on the basis of the average binding constant \((K_{N1}K_{N2}/K_N)\) for Ap₃A and respective \(K_N\) values for the other dinucleotides. As Tables 1 and 2 make clear, the binding affinity estimated at 50 μM substrate was markedly lower for Ap₃A compared with other dinucleotides for all CBS-PPases. Increasing \(n\) did not affect dhPPase affinity, but it did slightly increase cpNPPase affinity and decrease cpPPase affinity. Increasing substrate concentration had opposite effects on the affinity of Ap₃A and Ap₄A for dhPPase and cpPPase (increased) and cpNPPase (decreased). The note, cpPPase exhibited much lower affinity for all dinucleotides compared with other CBS-PPases.

Surprisingly, neither dinucleotide at a concentration up to 10 μM affected activities of elPase or mtPase measured with 50 μM substrate. These CBS-PPases differ from those described above by having no DRTGG domain in their regulatory regions, which are formed by only two CBS domains. Moreover, 10 μM Ap₃A did not affect the concentration dependence of ADP inhibition of elPase or mtPase (data not shown), indicating that the dinucleotide is unable to interact with the ADP-binding site.

Dependence of CBS-PPase Activity on Mg²⁺ Concentration—Given that cooperativity in CBS-PPases is Mg²⁺-dependent (32), measurements analogous to those illustrated in Fig. 1 were conducted for two representative dinucleotides, Ap₃A and Ap₄A, over a 0.05–20 mM Mg²⁺ concentration range; substrate concentration was fixed at 50 μM. The results of these experiments (Fig. 2) indicated that Ap₃A bound with positive cooperativity and Ap₄A bound noncooperatively to all CBS-PPases at all Mg²⁺ concentrations. In only one case (dhPPase with Ap₃A), the degree of cooperativity, as characterized by the values of \(h\) and the ratio \(K_{N2}/K_{N1}\), showed a pronounced dependence on [Mg²⁺] because of the opposite effects of Mg²⁺ on \(K_{N1}\) and \(K_{N2}\) (Fig. 2). In all other cases, \(K_{N1}\) and \(K_{N2}\) changed in the same direction to approximately the same degree and, consequently, without a marked effect on cooperativity. Of note, the ratio \(K_{N2}/K_{N1}\) equals 4 in the case of noncooperative binding and is less than 4 for positively cooperative binding (37).

In most cases (except for dhPPase with Ap₃A), Mg²⁺ modulated dinucleotide binding, with the direction of the effect depending on both the nature of the nucleotide and the CBS-
effect on Ap3A and Ap4A demonstrated no or only small variations with
edly suppressed the kinetic cooperativity in
Menten Parameters—
were similar for both steps of Ap3A binding and Ap4A binding
parameter values summarized in Table 3. The values of
binding could be described by Equation 3, yielding the
\[ \frac{V}{V_0} = \frac{K_{N1}}{K_{N2}} \]
\[ K_{N1} \]
\[ K_{N2} \]
\[ 4K_{N1}/K_{N2} \]
\[ h \]

**TABLE 1**
Kinetic parameters for activation of three CBS-PPases by Ap3A in the presence of 5 mM Mg2+

| Enzyme   | [MgPP] | \( V_0/V_0^* \) | \( K_{N1} \) | \( K_{N2} \) | \( 4K_{N1}/K_{N2} \) |
|----------|--------|-----------------|--------------|--------------|----------------------|
| dhPPase  | 1      | 32 ± 6          | 460 ± 80     | 100 ± 20     | 213 ± 5              |
|          | 50     | 2.54 ± 0.01     | 82 ± 6       | 30 ± 2       | 7.5 ± 0.4            |
|          | 300    | 1.78 ± 0.03     | 12 ± 4       | 4 ± 1        | 7.1 ± 0.4            |
| cpPPase  | 1      | 5.1 ± 0.1       | 19 ± 1       | 12 ± 1       | 14.8 ± 0.3           |
|          | 50     | 2.67 ± 0.05     | 41 ± 8       | 22 ± 4       | 30 ± 1               |
|          | 300    | 1.77 ± 0.01     | 55 ± 9       | 22 ± 4       | 35 ± 0.9             |

\[ dhPPase \]
\[ Ap3A \]
\[ 100 \]
\[ 50 \]
\[ 300 \]
\[ cpPPase \]
\[ 1 \]
\[ 50 \]
\[ 300 \]
\[ \text{cpPPase} \text{ Ap3A} \text{ Ap4A} \]
\[ \text{cpPPase} \text{ Ap3A} \text{ Ap4A} \]
\[ \text{cpPPase} \text{ Ap3A} \text{ Ap4A} \]

**TABLE 2**
Kinetic parameters for activation of three CBS-PPases by diadenosine
polyphosphates with \( n = 4 \) – 6 in the presence of 5 mM Mg2+
The value of the Hill coefficient was indistinguishable from unity in all cases.

| Enzyme/dinucleotide | [MgPP] | \( V_0/V_0^* \) | \( K_{N1} \) |
|---------------------|--------|-----------------|--------------|
| dhPPase             | Ap3A   | 18 ± 1          | 121 ± 0.3    |
|                     | 50     | 3.0 ± 0.1       | 4.9 ± 0.2    |
|                     | 300    | 1.9 ± 0.02      | 4.3 ± 0.2    |
|                     | Ap4A   | 50               | 5.2 ± 0.06   |
|                     | 50     | 2.58 ± 0.04     | 4.4 ± 0.3    |
| cpPPase             | Ap3A   | 6.0 ± 0.3       | 3.9 ± 0.1    |
|                     | 50     | 3.14 ± 0.05     | 7.0 ± 0.2    |
|                     | 300    | 1.51 ± 0.01     | 1.65 ± 0.7   |
|                     | Ap4A   | 50               | 3.03 ± 0.08  |
|                     | 50     | 2.08 ± 0.03     | 3.3 ± 0.2    |

PPase origin (Figs. 2 and 3). Mg2+ generally stimulated Ap3A
binding, except for dhPPase, where it exerted the opposite
effect on \( K_{N1} \) (Fig. 2). Mg2+ exhibited a full range of effects on
Ap3A binding (Fig. 2): stimulation (cpPPase), suppression
(cpPPase), and no effect (dhPPase). The effect of Mg2+ on dinu-
cleotide binding could be described by Equation 3, yielding
the parameter values summarized in Table 3. The values of
\( K_{N1} \) governing the Mg2+ effects were in the millimolar range and
were similar for both steps of Ap3A binding and Ap3A binding
for a given CBS-PPase.

The degree of activation \( (V_0/V_0^*) \) of dhPPase and cpPPase by
Ap3A and Ap4A demonstrated no or only small variations with
Mg2+ concentration (Fig. 2). In contrast, activation of cpPPase
showed a bell-shaped dependence (Ap3A) or markedly
decreased (Ap4A) with increasing Mg2+ concentration.

Analysis of CBS-PPase Activation in Terms of Michaelis-
Menten Parameters—As previously reported, the rate of MgPP,
hydrolysis by CBS-PPases does not obey Michaelis-Menten
kinetics, requiring the use of a more complex equation with
two Michaelis constants (32). Their ratio, \( K_{N1}/K_{N2} \), was less than 4,
and the Hill coefficient was greater than 1, indicating positive
kinetic cooperativity.

Surprisingly, Ap3A and Ap4A completely abolished or mar-
edly suppressed the kinetic cooperativity in dhPPase, cpPPase
and cpPPase, as indicated by a Hill coefficient with a value close
to 1 (Table 4 and Fig. 3). That the \( h \) value is greater than 1 for
cpPPase in the presence of Ap3A may reflect incomplete satu-
ration of this enzyme by the dinucleotide, which binds much
more weakly to cpPPase compared with the other CBS-PPases,
especially at low substrate concentrations (Table 1).

The kinetics of activation by Ap3A was investigated over a
range of Mg2+ concentrations (Fig. 3). The results showed that
10 \( \mu M \) activator increased \( k_{cat} \) decreased the Michaelis con-
stant, and abolished kinetic cooperativity. Again the largest
effects were observed with cpPPase, which was therefore
explored in greater detail.

The effects of four Ap3A on the Mg2+ concentration de-
pendence of \( k_{cat} \) for cpPPase were qualitatively similar (Fig. 4).
Mg2+ induced a transition from low to high activity over a
narrow range of concentrations, requiring a term with \( [Mg2+] \)
in the corresponding equation (see Fig. 3 legend) (32). All four
activators increased the limiting value of \( k_{cat} \) at infinite \( [Mg2+] \)
(\( k_{cat,M} \)) and decreased the Mg2+ binding constant (\( K_{M} \)) - 2-fold
(Table 5). Most surprisingly, Ap3A binding conferred catalytic
activity to the otherwise inactive cpPPase at low \( [Mg2^+] \) (see
Fig. 5 legend) (32). The activity of Ap3A-activated cpPPase
in these conditions approached its maximum activity observed at
high \( [Mg2^+] \) in the absence of Ap3A (Fig. 4).

Fig. 5 illustrates the concentration dependence of cpPPase
activation by Ap3A in the presence of 0.5 mM Mg2+, analyzed in
terms of \( k_{cat} \) and \( K_{M} \) values. The value of \( k_{cat} \) increased \(~7.5-
fold\) (from 240 \pm 100 to 1800 \pm 100 s\(^{-1}\)), \( K_{M} \) decreased \(~18-
fold\) (from 70 \pm 10 to 4 \pm 1 \( \mu M \)), and \( K_{M} \) changed insigni-
ificantly with increasing Ap3A concentration from 0 to 5 \( \mu M \).
The Ap3A binding constant estimated from \( k_{cat} \) and \( K_{M} \) de-
cencies was 0.04 \pm 0.01 and 1.7 \pm 1.0 \( \mu M \), respectively. Because
\( k_{cat} \) and \( K_{M} \) dependences report on Ap3A binding to substrate-
free enzyme and enzyme-substrate complex, respectively, a
likely implication is that Ap3A and the first bound substrate
molecule mutually stabilize binding of each other to cpPPase
20 – 40-fold.

Thermodynamics and Stoichiometry of Nucleotide Binding
CBS-PPases—Using ITC allowed the direct measurement of
changes in free energy (\( \Delta G \)), enthalpy (\( \Delta H \)), and entropic free
energy (\( \Delta S \)) components of nucleotide binding to CBS-
PPases. A typical titration profile is shown in Fig. 6A. The
results of similar titrations performed with different CBS-
PPases and nucleotides are summarized in Fig. 6B and Table 6.
One important result was that titrations of the DRTGG
domain-lacking elPPase or mtPPase with up to 10 \( \mu M \) Ap3A or
Ap₃A produced no ITC signal, consistent with the inability of the dinucleotides to activate these CBS-PPases and modulate their inhibition by ADP. Because the lack of effect on activity did not rule out the possibility of a “silent” binding, the ITC data, which report on a different aspect of the binding reaction, provided an important support for the lack of complex formation between the DRTGG domain-lacking CBS-PPases and Ap₃A. This interpretation was supported by parallel measurements employing AMP, ADP, and ATP (Table 6), which produced similar enthalpy changes in the cases, where previous measurements (32) revealed effects on activity, but no or reduced enthalpy change (εPPase with AMP and cnPPase with ADP, respectively), where no effect on activity was observed (32). Together, these findings suggest that modulation of activity and heat production are coupled phenomena and that the DRTGG domain is required for tight binding of diadenosine polyphosphates, but not monoadenosine phosphates, to CBS-PPases. The inability of εPPase to bind AMP is not associated with the absence of the DRTGG domain because another DRTGG domain-lacking CBS-PPase, mtPPase, is inhibited by AMP and hence binds it (31).

Another important finding was that Δ_H, as calculated per mole of nucleotide, was nearly two times greater for Ap₄A and Ap₅A than for Ap₃A and the mononucleotides in the titrations with the DRTGG domain-containing CBS-PPases. This effect correlated with a 2-fold lower binding stoichiometry for Ap₄A and Ap₅A compared with that for mononucleotides and Ap₃A. Because of the very tight binding, K_N and, accordingly, TΔS values could not be estimated with adequate precision in most Ap₃A titrations. Where K_N (and hence ΔG) values were available, the free energy change of nucleotide binding was dominated by ΔH, with a significant contribution from TΔS, likely because of a hydrophobic effect. The ΔG values derived from ITC measurements are in a fair agreement with those obtained from nucleotide effects on activity (see Ref. 32 for mononucleo-
otides and Table 1 for Ap3A). It should be noted that ITC measurements can hardly distinguish positive binding cooperativity and yield an average ΔH value for all binding sites.

**Discussion**

CBS domains, found in many proteins, are known for their ability to bind adenine nucleotides and in this way regulate activities of their carrier proteins. The list of regulating adenine nucleotides includes AMP, ADP, ATP, S-adenosyl methionine, NADH, and analogs of AMP and ATP (27). Examples of less common CBS domain ligands include Mg2+ (38), DNA, and RNA (39, 40). We earlier reported that crystals of the isolated dimeric regulatory region of cpPPase grown in the presence of Ap3A contains one Ap3A molecule per dimer bridging two

**TABLE 3**

| Enzyme | K_{N1} | K_{N1,M} | K_{N2} | K_{N2,M} | K_{N} | K_{N,M} | K_{m} |
|---|---|---|---|---|---|---|---|
| dhPPase | 65 ± 2 | 68 ± 3 | 0.2 ± 0.3 | 72 ± 2 | 53 ± 1 | 1.5 ± 0.4 | 5.5 ± 0.5 |
| cpPPase | 390 ± 10 | 42 ± 6 | 3.2 ± 0.7 | 110 ± 10 | 14 ± 4 | 5 ± 3 | 4.3 ± 0.3 |

**TABLE 4**

| Enzyme | k_{cat,0} | k_{cat,0,M} | k_{cat,0} | k_{cat,0,M} | k_{cat,0} | k_{cat,0,M} | k_{cat,0} | k_{cat,0,M} |
|---|---|---|---|---|---|---|---|---|
| dhPPase | 65 ± 2 | 68 ± 3 | 0.2 ± 0.3 | 72 ± 2 | 53 ± 1 | 1.5 ± 0.4 | 5.5 ± 0.5 | NA |
| cpPPase | 390 ± 10 | 42 ± 6 | 3.2 ± 0.7 | 110 ± 10 | 14 ± 4 | 5 ± 3 | 4.3 ± 0.3 | 8.6 ± 0.5 |

* NA, not attendant.
pairs of CBS domains, whereas each CBS domain pair binds an AMP molecule (33). We also found that Ap3A induces a significant opening of the interface compared with the AMP-bound form. The results reported above extend these earlier findings by showing that (a) Apn,n As with \( n = 3–6 \) bind three CBS-PPases with nanomolar affinity and activate them \textit{in vitro}; (b) Ap3A binding is only observed in CBS-PPases that have an intercalating DRTGG domain in the regulatory region; and (c) unlike common adenine nucleotides, long chain Apn,n As (\( n > 3 \)) abolish or markedly reduce kinetic cooperativity (non-Michaelian behavior) in CBS-PPases. The unique features of Apn,n A complexes of CBS-PPases compared with those of their complexes with mononucleotides and complexes of other CBS proteins with their regulating ligands are described below. Notably, Apn,n As have not been reported as ligands for any other CBS protein.

Based on their binding properties, Apn,n As can be divided into two groups. Ap3A bound to CBS-PPases cooperatively and with lower affinity, as characterized by either \( K_{n,1} \) and \( K_{n,2} \) or their average value \( \sqrt{K_{n,1} K_{n,2}} \) (Table 1). The other dinucleotides \( (n = 4–6) \) bound noncooperatively and with a higher affinity that did not depend significantly on the \( n \) value (Table 2). The affinities of Apn,n As with \( n = 4–6 \) for CBS-PPases surpassed that of adenine mononucleotides (32) by 2–3 orders of magnitude. Such high affinities are unprecedented among other CBS proteins, which generally bind their nucleotide ligands in the millimolar range. The difference in the binding affinities of the two Apn,n A groups was most pronounced with Ap4A, amounting to 3 orders of magnitude. As previously demonstrated (33), Ap3A interacts through both of its adenine moieties with two CBS domain pairs of different subunits in cpPPase. Such an arrangement is also likely with Ap2A and Ap4A, consistent with their similar \( \Delta H \) values and binding stoichiometries, determined from ITC measurements (Table 6). In contrast, \( \Delta H \) for Ap3A was half that of Ap2A and Ap4A, and the binding stoichiometry was 2-fold higher, similar to values for mononucleotides (Table 6). These observations likely indicate that Ap3A predominantly binds CBS-PPases through only one adenine moiety.

The binding affinities of Apn,n As showed a complex dependence on substrate and metal cofactor concentrations. At a constant Mg\(^{2+}\) concentration, substrate increased the binding affinities of dhPPase and cpPPase for all Apn,n As but exerted an opposite effect on cnPPase (Tables 1 and 2). Accordingly, Ap3A (Table 4) and Ap4A (Fig. 3) decreased the average Michaelis constant \( \sqrt{K_{m,1} K_{m,2}} \) for cpPPase measured in the presence of 5 mM Mg\(^{2+}\) was quite modest, but keeping in mind the bell-shaped dependence of \( \sqrt{K_{m,1} K_{m,2}} \) on [Mg\(^{2+}\)] for this enzyme in the absence of adenine

### Table 6: Thermodynamic parameters for nucleotide complexes of CBS-PPases obtained by isothermal calorimetry

| Enzyme/nucleotide | \( K_{n} \) | \( n \) | \( \Delta H \) | \( -T \Delta S \) |
|-------------------|-------------|----------|-------------|----------------|
| dhPPase | AMP 0.8 ± 0.3 | 0.79 ± 0.05 | −5.6 ± 0.5 | 2.7 ± 0.6 |
| ADP 1.0 ± 0.2 | 0.85 ± 0.04 | −5.9 ± 0.4 | 2.4 ± 0.5 |
| ATP 1.2 ± 0.1 | 0.80 ± 0.02 | −5.8 ± 0.2 | 2.4 ± 0.2 |
| Ap3A 0.12 ± 0.05 | 0.97 ± 0.02 | −5.3 ± 0.2 | 4.0 ± 0.3 |
| Ap4A 0.41 ± 0.01 | −10.4 ± 0.3 | |
| Ap6A 0.41 ± 0.01 | −10.3 ± 0.3 | |
nucleotides (Fig. 3), one would expect, by analogy, greater effects of Ap₃A at low [Mg²⁺].

However, the most striking effect of Ap₃A on substrate binding was abolition of kinetic cooperativity. This effect was observed with both Ap₃A and Ap₄A, representing the two dinucleotide groups and might be explained by two different mechanisms. First, the effectors may disrupt the communication between active sites, allowing them to function independently. Alternatively, the dinucleotides may induce asymmetry in the enzyme dimer such that only one active site operates in the dimer (ultimate negative cooperativity). Determining the three-dimensional structure of the enzyme with bound dinucleotide would make it possible to discriminate between these alternative explanations.

Mg²⁺ effects on nucleotide binding also varied depending on the enzyme (Fig. 2) and differed from those observed with adenine mononucleotides (32). With Ap₃A, values of $K_{N1}$ and $K_{N2}$ for dbPPase changed in different directions, decreasing the degree of cooperativity at low [Mg²⁺] (Fig. 2A). No bound Mg²⁺ ion was observed in the structure of the regulatory region of cpPase (33), suggesting that the modulatory Mg²⁺ resides in the active site. Notable in this regard, three Mg²⁺ ions per active site participate in catalysis among homologous nonregulated family II PPases (24, 41). The effects of Mg²⁺ on nucleotide binding may, in part, be a consequence of its effects on substrate binding, because these measurements were carried out at a nonsaturating substrate concentration (50 μM).

Both Ap₃A and Ap₄A activated CBS-PPases under the conditions tested because of favorable changes in both $k_{cat}$ and the average Michaelis constant ($\sqrt{K_{m1}K_{m2}}$) (Table 4 and Fig. 3). Accordingly, the degree of activation was greater at low substrate concentrations (Table 1) and varied from severalfold to several ten-fold. The largest effects were observed with cpPase. Based on its $k_{cat}$ and $K_m$ values (Fig. 3), this enzyme is predicted to be activated by Ap₃A in the presence of 1 mM Mg²⁺ by a factor of ~51 and ~19 at substrate concentrations of 1 and 10 μM, respectively. At low [Mg²⁺], the activating effect of Ap₃A is dominated by $k_{cat}$, especially with cpPase (Fig. 4 and Table 5). In this enzyme, $k_{cat}$ is strongly Mg²⁺-dependent and Ap₃A markedly released this dependence by allowing catalysis in the enzyme with a vacant Mg²⁺ site and by somewhat increasing its affinity for Mg²⁺ (Table 5). In this respect, Ap₃A partially substitute for Mg²⁺ as an enzyme activator.

Qualitatively similar activating effects on CBS-PPases were previously observed with ATP (31, 32), although ATP effects were smaller in size and required much higher effector concentrations. A further difference is that ATP bound cooperatively, like Ap₃A. The effects of ATP and Ap₃A are thus similar in many aspects. As noted above, activator binding induces significant opening of the CBS domain interface (33). Such opening can be achieved upon binding of a single molecule of Ap₄A or a longer dinucleotide that binds to both subunits of CBS-PPase through two adenine moieties. Structure modeling of the cpPase regulatory region indicated that the polyphosphate chain of Ap₃A is too short for this binding mode. In this case, and with ATP, interface opening apparently results from repulsion between two molecules of the effector bound to different subunits.

The requirement for an intercalating DRTGG domain for Ap₃A binding to CBS domains provides another interpretive challenge. In the structure of the regulatory region and the modeled structure of the whole cpPase, both the DRTGG domain and CBS domain pairs participate in forming the subunit interface (33). DRTGG domain-containing CBS-PPases apparently have a larger binding cavity for the regulating ligands or increased flexibility of the CBS domains at the

**FIGURE 7.** Aligned amino acid sequences of the two CBS domains of the characterized CBS-PPases. Amino acid residues making contacts with Ap₃A or AMP in the crystal structures of cpPase (33) are shown in boxes. Consensus residues based on 180 CBS-PPase sequences are indicated in the two bottom lines. Residue numbering is for full-length cpPase. Consensus residues for different levels of identity are indicated below the set of sequences.
expense of their smaller contribution to the subunit contact area, allowing them to accommodate more bulky Ap₆A molecules. This interpretation is supported by data showing that the DRTGG domain-deficient elP₆Ase (32) and mtPP₆Ase (31) bind ATP with an affinity 1–2 orders of magnitude lower than that of the less bulky AMP and ADP. In contrast, no such discrimination is observed in DRTGG domain-containing CBS-PPases (32). Notably, the primary structures of the CBS domains in DRTGG domain-deficient CBS-PPases (Fig. 7) do not contain specific mutations that would disallow their binding of Ap₆A. Despite a generally low degree of residue conservation in CBS domains, all residues involved in nucleotide binding are found in at least one of the DRTGG domain-deficient CBS-PPases. Based on these considerations, Ap₆A are not expected to bind with comparable affinity to the numerous other CBS proteins that lack a DRTGG or other intercalating domain.

Ap₆A binding is expected to significantly change CBS-PP₆Ase activity in vivo, particularly under low energy conditions, when the concentration of the alternative activator, ATP, is low. Although basal intracellular levels of Ap₆A are 4 orders of magnitude lower than those of adenine mononucleotides, Ap₆A concentrations can rise by 2 orders of magnitude under stress conditions (42, 43). Also taking into consideration their extraordinarily high affinity, Ap₆A could be expected to compete with mononucleotides for CBS-PP₆Ase binding in these circumstances. An increase in CBS-PP₆Ase activity is expected to decrease the concentration of PP, and thus release PP₁-mediated inhibition of numerous biosynthetic reactions in which PP₆A is produced as a by-product (44). That the affinity of CBS-PP₆Ases for Ap₆A markedly surpasses that of all known Ap₆A-binding proteins suggests that this enzyme is a dominant target through which Ap₆A fulfill their stress response-related functions in bacteria.

Author Contributions—V. A. A. designed, performed and analyzed the experiments, and contributed to writing the manuscript. A. S. designed and constructed vectors, expressed and purified proteins, and performed bioinformatics analyses. H. K. T. performed ITC experiments with dhPP₆Ase. V. N. O. supervised ITC experiments and data analysis. R. L. designed and supervised the experiments. A. A. B. designed and analyzed experiments and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgment—We thank Dr. P. Semenyuk for help with ITC measurements.

References
1. Reiss, J. R., and Moffatt, J. G. (1965) Dismutation reactions of nucleoside polyphosphates: III. the synthesis of α,β-dinucleoside 5’-polyphosphates. J. Org. Chem. 30, 3381–3387
2. Fraga, H., and Fontes, R. (2011) Enzymatic synthesis of mono and dinucleoside polyphosphates. Biochim. Biophys. Acta 1810, 1195–1204
3. Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., and Randerath, K. (1966) Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-sRNA synthetase. Biochem. Biophys. Res. Commun. 24, 91–97
4. Goerlich, O., Foeckler, R., and Holler, E. (1982) Mechanism of synthesis of adenosine(5’)-tetraphosphate(5’)-adenosine (AppppA) by aminocyl-tRNA synthetases. Eur. J. Biochem. 126, 135–142
5. Wright, M., Boonyalai, N., Tanner, J. A., Hindley, A. D., and Miller, A. D. (2006) The duality of LysU, a catalyst for both Ap₆A and Ap₆A₆ formation. FEBS J. 273, 3534–3544
6. Guranowski, A. (2000) Specific and nonspecific enzymes involved in the catabolism of mononucleoside and dinucleoside polyphosphates. Pharmacol. Ther. 87, 117–139
7. Jiang, Y.-L., Zhang, J.-W., Yu, W.-L., Cheng, W., Zhang, C.-C., Frolet, C., Di Giulmi, A.-M., Vernet, T., Zhou, C.-Z., and Chen, Y. (2011) Structural and enzymatic characterization of the streptococcal ATP/diadenosine polyphosphate and phosphohydrolase Spr1479/Saph. J. Biol. Chem. 286, 35906–35914
8. Lee, P. C., Bochner, B. R., and Ames, B. N. (1983) AppppA, heat shock stress, and cell oxidation. Proc. Natl. Acad. Sci. U.S.A. 80, 7496–7500
9. Bochner, B. R., Zylczak, M., and Georgopoulos, C. (1986) Escherichia coli DnaK protein possesses a 5’-nucleotidase activity that is inhibited by AppppA. J. Bacteriol. 168, 931–935
10. Varshavsky, A. (1983) Diadenosine 5’,5’-P₁,P₄-tetraphosphate: a pleiotropically acting alarmone. Cell 34, 711–717
11. Tanner, J. A., Wright, M., Christie, E. M., Preuss, M. K., and Miller, A. D. (2006) Investigation into the interactions between diadenosine 5’,5’-P₁,P₄-tetraphosphate and two proteins: molecular chaperone GroEL and cAMP receptor protein. Biochemistry 45, 3095–3106
12. Tshori, S., Razin, E., and Nechushtan, H. (2013) Amino-acyl tRNA synthetases generate dinucleotide polyphosphates as second messengers: functional implications. Top. Curr. Chem. 344, 10–12
13. Sillero, M. A., De Diego, A., Osorio, H., and Sillero, A. (2002) Diadenosine polyphosphates stimulate the primer independent synthesis of poly(A) catalyzed by yeast poly(A) polymerase. Eur. J. Biochem. 269, 5323–5329
14. Nishimura, A., Moriya, S., Ukai, H., Nagai, K., Wachi, M., and Yamada, Y. (1997) Diadenosine 5’,5’-P₁,P₄-tetraphosphate (Ap₆A) controls the timing of cell division in Escherichia coli. Genes Cells 2, 401–413
15. Gómez-Villafuertes, R., Pintor, J., Gualix, J., and Miras-Portugal, M. T. (2004) GABA modulates presynaptic signalling mediated by diadenosines on rat synaptic terminals. J. Pharmacol. Exp. Ther. 308, 1148–1157
16. Varlantian, A. A., Suzuki, H., and Poletaev, A. I. (2003) The involvement of diadenosine 5’,5’-P₁,P₄-tetraphosphate in cell cycle arrest and regulation of apoptosis. Biochem. Pharmacol. 65, 227–235
17. Giraldex, L., Díaz-Hernández, M., Gómez-Villafuertes, R., Pintor, J., Castro, E., and Miras-Portugal, M. T. (2001) Adenosine triphosphate and diadenosine pentaphosphate induce [Ca²⁺], increase in rat basal ganglia aminergic terminals. J. Neurosci. Res. 64, 174–182
18. Conant, A. R., Theologou, T., Dihms, W. C., and Simpson, A. W. (2008) Diadenosine polyphosphates are selective vasoconstrictors in human coronary artery bypass grafts cells. Vascul. Pharmacol. 48, 157–164
19. Johnstone, D. B., and Farr, S. B. (1991) AppppA binds to several proteins in Escherichia coli, including the heat shock and oxidative stress proteins DnaK, GroEL, C45 and C40. EMBO J. 10, 3897–3904
20. Guo, W., Azhar, M. A., Xu, Y., Wright, M., Kamal, A., and Miller, A. D. (2011) Identification and identification of diadenosine 5’,5’-P₁,P₄-tetraphosphate binding proteins using magnetic bio-panning. Bioorg. Med. Chem. Lett. 21, 7175–7179
21. Azhar, M. A., Wright, M., Kamal, A., Nagy, I., and Miller, A. D. (2014) Biotin-c10-AppCH₂ppA is an effective new chemical proteomics probe for diadenosine polyphosphate binding proteins. Bioorg. Med. Chem. Lett. 24, 2928–2933
22. Marques, A. F., Teixeira, N. A., Gambaretto, C., Sillero, A., and Sillero, M. A. (1998) IMP-GMP 5’,5’,5’,5’-tetraphosphate: a pleiotropic activator of protein synthesis. J. Neurosci. Res. 51, 24511–24518
23. Aragon, A. R., Teixeira, N. A., Gambaretto, C., Sillero, A., and Sillero, M. A. (2003) IMP-GMP 5’,5’,5’,5’-tetraphosphate: a pleiotropic activator of protein synthesis. J. Neurosci. Res. 51, 24511–24518
24. Marques, A. F., Teixeira, N. A., Gambaretto, C., Sillero, A., and Sillero, M. A. (1998) IMP-GMP 5’,5’,5’,5’-tetraphosphate: a pleiotropic activator of protein synthesis. J. Neurosci. Res. 51, 24511–24518
25. Batten, A. (1997) The structure of a domain common to archaeabacteria and the homocystinuria disease protein. Trends Biochem. Sci. 22, 12–13
26. Erev‐Orebea, J., Oyendarte, I., and Martínez‐Cruz, L. A. (2013) CBS domains: ligand binding sites and conformational variability. Arch. Biochem.
Nucleotide-regulated Soluble Pyrophosphatases

27. Baykov, A. A., Tuominen, H. K., and Lahti, R. (2011) The CBS domain: a protein module with an emerging prominent role in regulation. ACS Chem. Biol. 6, 1156–1163
28. Kemp, B. E. (2004) Bateman domains and adenosine derivatives form a binding contract. J. Clin. Invest. 113, 182–184
29. Ignoul, S., and Eggermont, J. (2005) CBS domains: structure, function, and pathology in human proteins. Am. J. Physiol. Cell Physiol. 289, C1369–C1378
30. Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J. Clin. Invest. 113, 274–284
31. Jämsen, J., Tuominen, H., Salminen, A., Belogurov, G. A., Magretova, N. N., Baykov, A. A., and Lahti, R. (2007) A CBS domain-containing pyrophosphatase of Moorella thermoacetica is regulated by adenine nucleotides. Biochem. J. 408, 327–333
32. Salminen, A., Anashkin, V. A., Lahti, M., Tuominen, H. K., Lahti, R., Baykov, A. A. (2014) Cystathionine β-synthase (CBS) domains confer multiple forms of Mg$^{2+}$-dependent cooperativity to family II pyrophosphatases. J. Biol. Chem. 289, 22865–22876
33. Tuominen, H., Salminen, A., Oksanen, E., Jämsen, J., Heikkilä, O., Lehtio, L., Magretova, N. N., Goldman, A., Baykov, A. A., and Lahti, R. (2010) Crystal structures of the CBS and DRTGG domains of the regulatory region of Clostridium perfringens pyrophosphatase complexed with the inhibitor, AMP, and activator, diadenosine tetraphosphate. J. Mol. Biol. 398, 400–413
34. Jämsen, J., Baykov, A. A., and Lahti, R. (2012) Fast kinetics of nucleotide binding to Clostridium perfringens family II pyrophosphatase containing CBS and DRTGG domains Biochemistry 77, 165–170
35. Baykov, A. A., and Avaeva, S. M. (1981) A simple and sensitive apparatus for continuous monitoring of orthophosphate in the presence of acid-labile compounds. Anal. Biochem. 116, 1–4
36. Baykov, A. A., Bakuleva, N. P., and Rea, P. A. (1993) Steady-state kinetics of substrate hydrolysis by vacuolar H$^+$-pyrophosphatase. A simple three-state model. Eur. J. Biochem. 217, 755–762
37. Bisswanger, H. (2008) Enzyme Kinetics: Principles and Methods, 2nd Ed., pp. 14–17, Wiley-VCH Verlag, Weinheim, Germany
38. Hattori, M., Tanaka, Y., Fukai, S., Ishitani, R., and Nureki, O. (2007) Crystal structure of the MgtE Mg$^{2+}$ transporter. Nature 448, 1072–1075
39. McLean, J. E., Hamaguchi, N., Belenky, P., Mortimer, S. E., Stanton, M., and Hedstrom, L. (2004) Inosine 5’-monophosphate dehydrogenase binds nucleic acids in vitro and in vivo. Biochem. J. 379, 243–251
40. Águado-Llera, D., Oyenarte, I., Martínez-Cruz, L. A., and Neira, J. L. (2010) The CBS domain protein MJ0729 of M. jannaschii binds DNA. FEBS Lett. 584, 4485–4489
41. Fabrichniiy, I. P., Lehtio, L., Tammenkoski, M., Zyryanov, A. B., Oksanen, E., Baykov, A. A., Lahti, R., and Goldman, A. (2007) A trimeral site and ground-state substrate distortion mark the active site of family II inorganic pyrophosphatase. J. Biol. Chem. 282, 1422–1431
42. Garrison, P. N., and Barnes, L. D. (1992) Determination of dinucleoside polyphosphates, in Ap4A and Other Dinucleoside Polyphosphates (McLennan, A. G., ed) pp. 29–61, CRC Press, Boca Raton, FL
43. Plateau, P., and Blanquet, S. (1994) Dinucleoside oligophosphates in micro-organisms. Adv. Microb. Physiol. 36, 81–109
44. Heinonen, J. K. (2001) Biological Role of Inorganic Pyrophosphate, pp. 123–188, Kluwer Academic Publishers, London