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Fast Kinetics of Nucleotide Binding to *Clostridium perfringens* Family II Pyrophosphatase Containing CBS and DRTGG Domains

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Abstract—We earlier described CBS-pyrophosphatase of *Moorella thermoacetica* (*mt*CBS-PPase) as a novel phosphohydrolase that acquired a pair of nucleotide-binding CBS domains during evolution, thus endowing the protein with the capacity to be allosterically regulated by adenine nucleotides (Jämsen, J., Tuominen, H., Salminen, A., Belogurov, G. A., Magretova, N. N., Baykov, A. A., and Lahti, R. (2007) *Biochem. J.*, **408**, 327-333). We herein describe a more evolved type of CBS-pyrophosphatase from *Clostridium perfringens* (*cp*CBS-PPase) that additionally contains a DRTGG domain between the two CBS domains in the regulatory part. *cp*CBS-PPase retained the ability of *mt*CBS-PPase to be inhibited by micromolar concentrations of AMP and ADP and activated by ATP and was additionally activated by diadenosine polyphosphates (AP_nA) with $n > 2$. Stopped-flow measurements using a fluorescent nucleotide analog, 2′(3′)-*O*-(N-methylanthranoyl)-AMP, revealed that *cp*CBS-PPase interconverts through two different conformations with transit times on the millisecond scale upon nucleotide binding. The results suggest that the presence of the DRTGG domain affords greater flexibility to the regulatory part, allowing it to more rapidly undergo conformational changes in response to binding.

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Key words: inorganic pyrophosphatase, CBS domain, DRTGG domain, *Clostridia perfringens*, adenine nucleotides, diadenosine polyphosphate

Inorganic polyphosphates, comprising two to hundreds of phosphate residues, are important metabolites found in all cell types [1]. The shortest polyphosphate, PP_i, is generated in vast amounts as a byproduct of principal biosynthetic reactions, such as protein, RNA, and DNA synthesis, and determines their equilibria. PP_i regulates calcification, cell proliferation, and iron transport, along with many other cellular processes [2]. PP_i hydrolysis is catalyzed by soluble pyrophosphatases (PPases; EC 3.6.1.1) from two non-homologous families, I and II. Family I PPases have been identified in all kingdoms of life [3, 4], while family II PPases are only found in eu- and archaebacteria, often pathogenic ones [5, 6]. With a few exceptions, each bacterial species contains only one type of soluble PPase. While the overall structures of family I and family II PPases are distinct, their active sites are nearly identical, presenting a remarkable example of convergent evolution [7, 8]. Family II PPases differ in that they contain a tightly bound transition metal ion, usually Mn^{2+} or Co^{2+}, but also require Mg^{2+} for maximal activity [9].

A quarter of nearly 500 reported family II PPase sequences identified in bacterial genomes contain a ~125-residue insert comprising two CBS domains (also known as Bateman domain [10]) reported to bind adenine nucleotides in different proteins [11, 12]. The initially characterized CBS-PPase from *Moorella thermoacetica* (*mt*CBS-PPase) resembles common family II PPases that lack CBS domains. Specifically, this enzyme contains all 13 conserved active site residues identified in

Abbreviations: AP_nA, P1,P*n*-diadenosine 5′-polyphosphate with *n* bridging phosphate residues; CBS, cystathionine β-synthase; *cp*CBS-PPase, CBS domain-containing PPase from *Clostridium perfringens*; Mant-AMP, 2′(3′)-*O*-(N-methylanthranoyl)-AMP; *mt*CBS-PPase, CBS domain-containing PPase from *Moorella thermoacetica*; PPase, inorganic pyrophosphatase.

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common family II PPases by X-ray crystallography [7, 8], forms a homodimer that partially dissociates in the absence of divalent metals, and requires transition metal cofactors for activity [13]. However, a striking difference between *mt*CBS-PPase and common family II PPases is that *mt*CBS-PPase activity is highly sensitive to micromolar concentrations of adenine nucleotides, whereby AMP and ADP are inhibitors and ATP is the activator [13].

The majority of CBS-PPases differ from *mt*CBS-PPase in that they contain an additional DRTGG domain closely associated with CBS domains [14]. In this study, we characterized such a PPase from *Clostridium perfringens* (*cp*CBS-PPase) and showed that it is similar to *mt*CBS-PPase by sensitivity to adenine nucleotides, including diadenosine polyphosphates. However, the rates of the ligand-induced conformational transitions are much faster in *cp*CBS-PPase, suggesting a role for the DRTGG domain in regulatory site performance.

MATERIALS AND METHODS

Reagents. The 2′(3′)-*O*-(N-methylanthranoyl) derivative of AMP (Mant-AMP), 5′-adenosine tetraphosphate (AP_4), and P1,P*n*-diadenosine 5′-polyphosphates (AP_nA, n = 2-6) were from Jena Bioscience. All other nucleotides were obtained from Sigma-Aldrich. AMP was obtained as the free acid, ADP as the potassium salt, and all other nucleotides as sodium salts. Contaminating ADP from stock ATP solutions was removed by incubation with creatine kinase and phosphocreatine [13]. Where required, nucleotides were additionally purified with HPLC using a DEAE-derivatized column and a linear gradient of 0-1 M triethylammonium bicarbonate buffer. The purity and identity of nucleotides were confirmed with TLC or HPLC. The concentrations of stock nucleotide solutions were calibrated by measuring absorbance in the ultraviolet region (ε_{259} = 15,900 and 27,000 $M^{-1}\cdot cm^{-1}$ for monoadenosine and diadenosine derivatives, respectively).

Expression and purification of *cp*CBS-PPase. *Clostridium perfringens* 13 CBS-PPase (CPE2055) was expressed in *E. coli* [14] and purified as described for *mt*CBS-PPase [15], with minor modifications. MOPS-NaOH, pH 7.2, was used as the buffer for all steps of the purification procedure. Pooled fractions after DEAE-Sepharose chromatography were concentrated 10-fold, while fractions resulting from gel filtration were concentrated only 2-3-fold to avoid aggregation. Transition metal forms of *cp*CBS-PPase were isolated by adding 2 mM metal ion to the isolation medium used in all purification steps. All enzyme preparations were >95% homogenous, as verified by SDS-PAGE on an 8-25% gradient gel with the Phast electrophoresis system (GE Healthcare). Protein concentrations were determined with the NanoOrange protein assay [16].

Activity measurements. Activity assays and all other measurements described below were performed at 25°C in 0.1 M MOPS/KOH buffer, pH 7.2, including 0.1 mM $CoCl_2$ and 20 mM $MgCl_2$, except where specified. An aliquot (5-200 μl) of diluted enzyme solution was added to the assay mixture (25 ml total volume) to generate a final enzyme concentration of 0.1-10 μg/ml, followed 1 min later by PP_i or *vice versa*. Formation of P_i was continuously monitored for 2-5 min using an automatic P_i analyzer [17], and the initial rate obtained from the initial slope of the recorder tracing. Rates of phosphate accumulation were converted to rates of substrate consumption under the assumption that PP_i hydrolysis yields two P_i molecules. The results of parallel rate measurements agreed within 10%.

Stopped-flow measurements. These measurements were performed with a fluorescent AMP analog, Mant-AMP, in which an N-methylanthranoyl group is attached through the 2′- or 3′-ribose oxygen [18]. The excitation spectrum of Mant-AMP, containing a peak at 355 nm, and the emission spectrum of protein Trp residues largely overlap, resulting in very efficient energy transfer between Trp and Mant-AMP. This facilitates excitation of Mant-AMP fluorescence at the Trp peak (295 nm), ensuring considerably lower background fluorescence from unbound Mant-AMP compared with excitation at 355 nm. Mant-AMP binding measurements were performed using the Applied Photophysics SX18.MV stopped-flow analyzer (Applied Photophysics, Inc.), as described previously for *mt*CBS-PPase [15], except that the final enzyme concentration used was 1 μM. Fluorescence, excited at 295 nm, was monitored at >400 nm either in a logarithmic time base for 10 sec or with a split time base, where 500 points were collected for the initial 0.1 sec and the remaining 500 points for 0.1-5 sec.

Data analysis. All nonlinear least-squares fittings were performed with the Scientist program (Micromath). The dependence of activity on nucleotide (L) concentration at a fixed substrate concentration was fit to Eq. (1), where A_{+L} and A_{-L} represent activities with and without bound ligand, respectively, and K_d is the apparent dissociation constant of the enzyme–ligand complex.

$$A = A_{+L} + (A_{-L} - A_{+L})/(1 + [L]/K_d) \quad (1)$$

Time courses from stopped-flow measurements were analyzed in terms of Scheme 1, where EN, EN*, and EN** represent three conformational isomers of the enzyme–nucleotide complex. The binding step was too rapid to be resolved in time, whereas conversion of EN into EN* and EN** was described by two transit times, τ_1 and τ_2. The transit times are complex functions of the four rate constants governing the EN ↔ EN* and EN* ↔ EN** equilibria in Scheme 1 [19]. The time courses were fit to Eqs. (2)-(4), where F represents fluorescence inten-

sity at time t, $[E]_0$ is total enzyme concentration, and A_1 and A_2 are the amplitudes for two consecutive relaxation processes.

$$E + N \leftrightarrow EN \leftrightarrow EN^* \leftrightarrow EN^{**}$$

Scheme 1

$$d[EN^*]/dt = ([E]_0 - [EN^{**}] - [EN^*])/\tau_1 - [EN^*]/\tau_2 \quad (2)$$

$$d[EN^{**}]/dt = [EN^*]/\tau_2 \quad (3)$$

$$F = F_0 + A_1[EN^*] + A_2[EN^{**}] \quad (4)$$

The dependences of the transit times on nucleotide concentrations were fit to Eq. (5), whereby $\tau_{i,lim}$ represents the transit time for the ith transit step at infinite nucleotide concentration, [N] is the nucleotide concentration, and K_d is the dissociation constant of the enzyme–nucleotide complex.

$$1/\tau_i = (1/\tau_{i,lim})/(1 + K_d/[N]) \quad (5)$$

RESULTS

Catalytic properties and regulation of *cp*CBS-PPase. *cp*CBS-PPase belongs to family II PPases that depend on both Mg^{2+} and a transition metal cofactors bound to different sites for activity. Transition metal ions are bound slowly, requiring long incubation for metal exchange. Accordingly, different combinations of metal cofactors were examined for their ability to activate *cp*CBS-PPase at the two site types. The enzyme was preincubated with Co^{2+} or Mn^{2+} to saturate the tight binding site and assayed for PPase activity in the presence of similar or different metal ion(s), which interacted with the loose binding site. Our results (shown in Table 1) demonstrated that *cp*CBS-PPase resembles *mt*CBS-PPase [13] in that Co^{2+} was superior to Mn^{2+} at the preincubation stage, whereas Mg^{2+} was optimal for PPase activity in the assay. Based on these findings, in subsequent analyses, the preincubation medium was supplemented with 0.1 mM Co^{2+} whereas the assay medium contained both 0.1 mM Co^{2+} and 20 mM Mg^{2+}. The PPase activity measured under these conditions displayed a K_m value of 50 ± 10 μM in terms of total PP_i concentration at pH 7.2 and was relatively insensitive to pH in the range of 6.2-8.5 at nearly saturating PP_i concentration (0.2 mM).

An array of potential CBS domain ligands was screened to determine effects on PP_i hydrolyzing activities (Table 2). Measurements were conducted at a nearly saturating substrate concentration (0.2 mM). Profound effects on activity were observed with most adenine nucleotides, except AP_4 and AP_2A, which did not appear to influence PP_i hydrolysis. Depending on the nucleotide, the effect was either inhibition or activation. Interestingly, all diadenosine polyphosphates with $n > 2$ acted as activators.

Figure 1 shows the concentration dependences of the effects of four adenine nucleotides on *cp*CBS-PPase activity measured at a fixed PP_i concentration (0.2 mM). These data allowed estimation of the residual activity of the enzyme–nucleotide complex (A_{+L}/A_{-L}) (with Eq. (1)) and the apparent nucleotide-binding constant (K_d). The estimated kinetic parameters are presented in Table

Table 1. Different metal ions as cofactors of *cp*CBS-PPase in PP_i hydrolysis

Metal ion(s)		Activity, sec^{-1}
Preincubation	Assay	
Co	–	2.8 ± 0.2
Co	Co	10 ± 0.5
Co	Mg	180 ± 20
Co	Co, Mg	200 ± 15
Co	Mn	11 ± 1
Co	Ca	4.5 ± 0.5
Mn	–	0.25 ± 0.05
Mn	Mn	0.45 ± 0.05
Mn	Mg	40 ± 5
Mn	Co	0.25 ± 0.05
Mn	Co, Mg	85 ± 15
Mn	Ca	0.40 ± 0.05

Note: Enzyme (10 μM) was preincubated for 24 h with 2 mM metal ion (Co^{2+} or Mn^{2+}), diluted 1000-fold, and assayed with 0.2 mM PP_i in the absence of metal ions or presence of 0.1 mM transition metal or Ca^{2+} ion ± 20 mM Mg^{2+}.

Table 2. Effects of nucleotides and related compounds (100 μM) on *cp*CBS-PPase activity measured with 0.2 mM PP_i

Compound	Activity, %
–	100
AMP	28 ± 5
ADP	35 ± 3
ATP	210 ± 10
AP_4	105 ± 6
AP_2A	104 ± 4
AP_3A	260 ± 20
AP_4A	250 ± 10
AP_5A	250 ± 30
AP_6A	250 ± 20

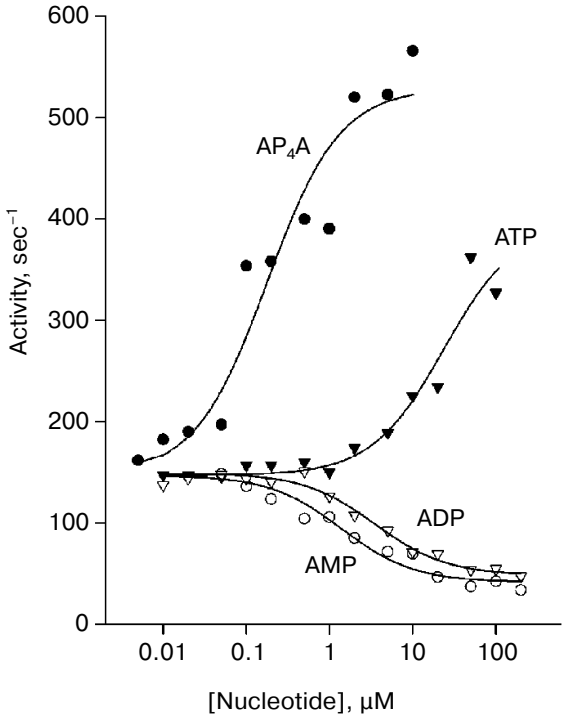

Fig. 1. Nucleotide modulation of *cp*CBS-PPase activity in PP_i hydrolysis measured at a fixed PP_i concentration (0.2 mM).

3. Neither nucleotide seems to abrogate *cp*CBS-PPase activity. The K_d value was the lowest for AP_4A and increased with the length of the phosphate chain for the monoadenosine polyphosphates.

Fast kinetics of nucleotide binding. The kinetics of nucleotide binding to the CBS domain of *cp*CBS-PPase was determined with Mant-AMP, as established previously with *mt*CBS-PPase [15]. This AMP analog resulted in a considerably larger signal compared with AMP. Upon mixing of Mant-AMP with *cp*CBS-PPase, a fluorescence increase was observed due to fluorescence resonance energy transfer from Trp to Mant (Fig. 2a). The time course of the fluorescence change represents a two-step consecutive reaction (Scheme 1), as indicated by residual analysis in Fig. 2 (b and c). The number of transitions was thus one fewer and the transitions were markedly faster compared with *mt*CBS-PPase [15]. The limiting values of the transit times derived from Fig. 2a were 2.5 and 10 msec. Another difference was that the inverse transit times of the two steps increased in a saturable manner with the Mant-AMP concentration (Fig. 2a, inset), rather than decreased as for *mt*CBS-PPase [15]. Despite these differences, *cp*CBS-PPase appears to follow the mechanism proposed earlier for *mt*CBS-PPase [15], which implies that both transitions refer to conformational changes in the enzyme–nucleotide complex. The possibility that the transit time, τ_1, refers to the nucleotide-binding step does not seem likely, since $1/\tau_1$ would increase to infinity in this case. The K_d values for nucleotide binding derived from the $1/\tau_1$ and $1/\tau_2$ dependences in Fig. 2 inset were identical within the error of measurements – 22 ± 4 μM.

DISCUSSION

*cp*CBS-PPase is inhibited by AMP and ADP and activated by ATP and diadenosine polyphosphates. Such differential regulation in which the nucleotide structure determines the type of regulatory effect on activity, i.e. inhibition or activation, is rather common among CBS proteins and facilitates their sensitivity to the cell energy level [11, 12]. We explained this property in terms of "internal inhibition" by CBS domains [13]. This explanation was based on the findings that homologous PPases lacking CBS domains in their structures are more active by two to three orders of magnitude compared with their CBS domain-containing counterparts, and most mutations in the CBS domain activate *mt*CBS-PPase [20]. By altering the CBS domain structure, the ligands can either suppress or further enhance "internal inhibition", resulting in a

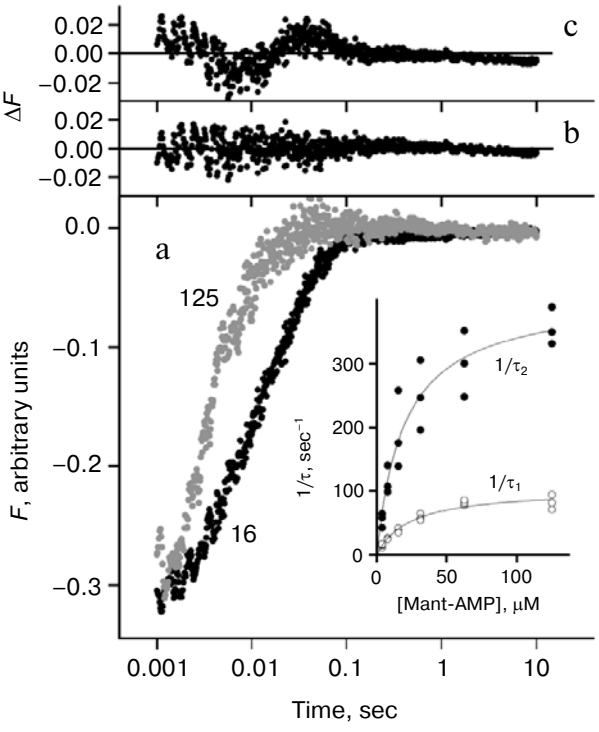

Fig. 2. Rapid kinetic data on Mant-AMP binding. a) Fluorescence stopped-flow kinetic traces upon mixing 2 μM *cp*CBS-PPase and 16 μM (black points) or 125 μM (gray points) Mant-AMP. The abscissa shows time after mixing. Inset, dependence of the reciprocals of the transit times describing Mant-AMP binding to *cp*CBS-PPase on nucleotide concentration. The lines depict the best fit for Eq. (5). b, c) Distribution of residuals of the fits for two-step or one-step transition, respectively, with 16 μM Mant-AMP.

Table 3. Parameters describing the concentration profiles of the effects of adenosine nucleotides on 0.2 mM PP_i hydrolysis

Nucleotide	A_{+L}/A_{-L}	K_d, μM
AMP	0.28 ± 0.03	1.4 ± 0.4
ADP	0.32 ± 0.05	3.6 ± 1.3
ATP	2.7 ± 0.2	24 ± 9
AP_4A	3.6 ± 0.4	0.18 ± 0.05

more active or less active form compared with the basal "internally inhibited" state. This theory is consistent with crystallographic data showing significant opening of the CBS domain interface in the activator (AP_4A) complex compared with that in the inhibitor (AMP) complex [14]. In this context, ATP may trigger similar opening of the domain interface due to steric clashes and electrostatic repulsion between the bulky phosphate parts of bound ATP molecules. A similar logic applies to AP_nA activation.

*cp*CBS-PPase differs from previously characterized *mt*CBS-PPase [13] and other CBS proteins in that it contains a DRTGG domain between two CBS domains of the regulatory insert. Intercalation of the DRTGG domains is observed in most other CBS-PPase sequences, but not other CBS proteins. Moreover, to our knowledge, no other CBS proteins contain any foreign domains between the CBS domains. While DRTGG domains have been identified in many other proteins, their specific functions remain to be elucidated.

The DRTGG domains only contact each other in the dimeric *cp*CBS-PPase structure and do not interact with the rest of the protein [14]. This architecture is expected to provide greater flexibility to the regulatory insert. This prediction is supported by rapid kinetics data on nucleotide binding. The conformational changes induced by Mant-AMP binding occur in two steps in *cp*CBS-PPase, compared to three steps in *mt*CBS-PPase [15]. Importantly, the conformational changes occur considerably faster in *cp*CBS-PPase. The half-times for the two steps in *cp*CBS-PPase are 10 and 2.5 msec, whereas those for the three steps in *mt*CBS-PPase are 25 msec, 12 sec and 156 sec (2.5- to 60,000-fold difference). The observed greater flexibility of the regulatory part allows *cp*CBS-PPase to respond faster to changes in cell energy status (i.e. the distribution of adenine nucleotides). It may also explain the ability of *cp*CBS-PPase to bind diadenosine polyphosphates with varied polyphosphate chain length.

*cp*CBS-PPase additionally differs from *mt*CBS-PPase [15] in that both steps of the conformational transition do not occur unless the binding site with the K_d of 22 μM is occupied by the nucleotide. A likely explanation is that this K_d value refers to the initial binding step, which precedes the conformational transition and occurs at a rate not resolvable with the stopped-flow instrument. It is tempting to further speculate that the two transition steps refer to different subunits within the enzyme dimer. This binding model implies that the two nucleotide binding sites present in the enzyme dimer (one per Bateman domain) are equivalent at the initial binding step (display equal K_d values) but undergo subsequent conformational change in a cooperative manner (at different rates). This explanation is consistent with the binding cooperativity for nucleotides directly observed in *mt*CBS-PPase [15]. Further studies are, however, needed to test the validity of this simple model.

Experiments with diadenosine polyphosphates were performed based on the initial observation that two AMP molecules bound per dimeric regulatory fragment of *cp*CBS-PPase are in close proximity to each other in the crystal structure. Crystallization with AP_4A revealed a structure in which one AP_4A molecule bridged the *cp*CBS-PPase subunits across the dimer interface [14]. Here we showed that diadenosine polyphosphates of different lengths act as activators of *cp*CBS-PPase. These compounds have not been previously reported as regulators of CBS proteins, and thus extend the panel of potential ligands in cells. Normal intracellular levels of diadenosine polyphosphates are on the order of several μM, but can increase by two orders of magnitude under certain conditions [21, 22]. Since AP_4A is effective at 100-fold lower concentrations compared with monoadenosine phosphates (Table 3), and its analogs may bind even more strongly, diadenosine polyphosphates are likely to participate in the regulation of CBS-PPase and other CBS proteins as part of their functions in bacteria, which are related to stress response [21, 22], cell division [23], and cell invasion [24].

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