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ORIGINAL PAPER

Insights into different dependence of dNTP triphosphohydrolase on metal ion species from intracellular ion concentrations in *Thermus thermophilus*

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Abstract Deoxyribonucleoside triphosphate (dNTP) triphosphohydrolase (dNTPase) from *Thermus thermophilus* HB8 (TTHB8) hydrolyzes wide variety of dNTPs to deoxyribonucleoside and inorganic triphosphate in magnesium-dependent manner. In this paper, we assess the specificity for various metal ions and of the dNTP triphosphohydrolase activity of the dNTPase from TTHB8. Manganese and cobalt ions more effectively induced the activity for dNTPs than magnesium and, unexpectedly, brought about the degradation of single kind of dNTP. Manganese and cobalt concentrations of 10 nM were enough to induce the activity, while magnesium of about 1 mM was required for the induction of the activity. To further evaluate metal ions inherent to dNTPase in TTHB8 cells, we measured intracellular concentrations of major metal ions in TTHB8 cells by inductively coupled plasma emission spectroscopy and compared them with the dependence of metal ion concentration on dNTPase activity. Though cobalt ion was below detectable level, magnesium and manganese ions were detected at sufficient level to induce dNTPase activity. These results suggest that both manganese and magnesium ions are likely to be functional under intracellular condition. In addition, the proposed model of dNTPase activity induced by magnesium and multiple dNTPs was discussed based on the results obtained in this study.

Keywords HD domain · Inductively coupled plasma emission spectroscopy · dGTPase · dNTP triphosphohydrolase · Intracellular ion concentration · *Thermus thermophilus*

Abbreviations

dNTP	Deoxyribonucleoside triphosphate
dNTPase	dNTP triphosphohydrolase
TTHB8	*Thermus thermophilus* HB8
Tt-dNTPase	dNTPase from *T. thermophilus*
Ec-dGTPase	dGTP triphosphohydrolase from *E. coli*
ICP-ES	Inductively coupled plasma emission spectroscopy
CD	Circular dichroism

Introduction

Phosphohydrolase activity of the HD superfamily proteins, which retain highly conserved two His and two Asp residues nearby the catalytic sites, is intimately related to

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divalent metal cations (Aravind and Koonin 1998; Huai et al. 2003; Hogg et al. 2004; Kondo et al. 2007). Among proteins of this superfamily, the specificity of metal ion on the activity of dGTP triphosphohydrolase from *E. coli* (Ec-dGTPase) is magnesium > manganese > cobalt based on the degree of activation (Seto et al. 1988). The guanosine 5′-diphosphate 3′-diphosphate hydrolase from *E. coli* requires two metal ions, manganese and magnesium, for the optimal degradation activity, but the activity is not induced by calcium or iron (Sy 1977). The *E. coli* tRNA nucleotidyltransferase shows different dependence on metal ions for the hydrolysis of various substrates (Yakunin et al. 2004). Also, in the case of the other enzymes, it was reported that the property of metal ion cofactors is critical for substrate discrimination (Sinha and Singh 1980; Bowen and Dupureur 2003; Lukacin et al. 2004). These studies show that metal ion specificity of HD superfamily proteins is diverse and emphasize the importance of the study of metal ion cofactors for the HD superfamily proteins.

Deoxyribonucleoside triphosphate (dNTP) triphosphohydrolase (dNTPase) from *Thermus thermophilus* HB8 (TTHB8) belongs to HD superfamily proteins and shows significant sequence similarity to Ec-dGTPase; however, shows different enzymatic properties from Ec-dGTPase (Kondo et al. 2004). Although both the dNTPase of TTHB8 (Tt-dNTPase) and Ec-dGTPase hydrolyze dNTPs to deoxyribonucleoside and inorganic triphosphate in magnesium-dependent manner, only the former has a complicated mechanism for induction of dNTP triphosphohydrolase activity (Seto et al. 1988; Kondo et al. 2004). The activity of Tt-dNTPase is induced only in the presence of more than two kinds of dNTPs, which include dATP or dTTP, and magnesium (Kondo et al. 2004), whereas the hydrolytic activity for a single kind of dNTP has not been observed even in the presence of magnesium. Ec-dGTPase can hydrolyze a single kinds of dNTP in the presence of magnesium (Seto et al. 1988). Because HD superfamily proteins have a variety of the specificity of metal ions and substrates, the study with these cofactors may reveal further features of Tt-dNTPase different from dGTPase. However, it has not been established whether other metal cations affect the substrate specificity or catalytic property of Tt-dNTPase.

In this paper, we have assessed differential dependence of dNTP triphosphohydrolase activity on various metal ion species in this thermophilic prokaryote. To evaluate metal ions inherent to Tt-dNTPase in TTHB8 cells, we have measured intracellular concentrations of major metal ions in TTHB8 cells by inductively coupled plasma emission spectroscopy (ICP-ES) and compared the intracellular metal ion concentrations with the dependence of metal ion concentration on dNTPase activity.

Materials and methods

Protein expression and purification

Tt-dNTPase was prepared as described previously (Kondo et al. 2004) except for the addition of a further purification step by Resource Q (GE healthcare Biosciences, Piscataway, NJ, USA) prior to gel filtration. Protein bound to the Resource Q column was subsequently eluted with 0.15–0.28 M NaCl containing 50 mM Tris–HCl (pH 8.0).

Phosphohydrolase assay

The initial velocity of phosphohydrolase activity was assayed at 37°C in 50 mM HEPES (pH 7.5), 100 mM KCl, 450 nM Tt-dNTPase as described previously (Kondo et al. 2004), and a substrate and metal ion at the indicated concentrations. Metal ion specificity was assayed in the presence of 2 mM dNTPs (dTTP or mixture of dATP and dTTP, Sigma-Aldrich, St Louis, MO, USA) and 10 mM $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, or $ZnSO_4$ (Wako Pure Chemicals, Tokyo, Japan). The dependence of the activity on metal ion concentrations was assayed in the presence of 2 mM dTTP and 0.15–20 mM $MnCl_2$ or $CoCl_2$. The data points were plotted against free metal concentration calculated by MaxChelator (Patton et al. 2004). The dependence of the activity on substrate concentrations was assayed in the presence of 10 mM $MnCl_2$ and various concentrations of dNTPs, which are in the range to have no inhibitory effect on the activity by depletion of free metal ions. All data points were fitted to the Hill equation (1) for the condition that ratio of product/substrate is under 0.1.

$$k_{\mathrm{app}} = k_{\mathrm{cat}}[S]^n / \left([S]^n + K_{\mathrm{M}}^n\right) \quad (1)$$

where k_{app} is the apparent rate constant (i.e., initial velocity divided by enzyme concentration); k_{cat} is the maximum rate constant (unit of s^{-1}); $[S]$, the initial substrate concentration; and n is the Hill coefficient.

Estimation of the volume of a TTHB8 cell

TTHB8 cells were grown at 70°C in TR medium (Hashimoto et al. 2001) until the cell density reached 2×10^8 cells ml^{-1}, which corresponded to late logarithmic growth phase of TTHB8 (Oshima and Imahori 1974). The cells were harvested by centrifugation at 11,000 *g* for 10 min. On average 2.5 g of TTHB8 cells was obtained from 1.5 l culture of 2×10^8 cells ml^{-1}. Before harvesting, microscopic photographs of the cells in the culture medium were obtained. Examination of the images allowed

the cell length of 451 to be measured along the longitudinal axis. The average length of the TTHB8 cell was calculated by dividing the sum of the length by the number of cells examined. Similarly, the average diameter of the TTHB8 cell was calculated based on electron microscopic examination of 109 cells. Based on these values, the average cell volume was estimated by assuming each cell to have the shape of a cylinder. The statistically calculated average height and the diameter were $1.20 \times 10^{-5} \pm 7.40 \times 10^{-6}$ m/cell and $4.73 \times 10^{-7} \pm 7.18 \times 10^{-8}$ m/cell, respectively. The volume of a TTHB8 cell was estimated from these statistic values to be $2.11 \times 10^{-12} \pm 1.35 \times 10^{-12}$ ml/cell.

Sample preparation for ICP-ES

Harvested TTHB8 cells were treated in two ways in order to (1) quantify the level of total metal ion or (2) level of unbound metal ion. In order to quantify the level of total ion, cells (2.5 g) were initially washed three times with 50 mM Tris–HCl (pH 7.5) to remove all traces of growth medium. The cells were then suspended in a mixture of 18 M H_2SO_4 (9 ml) and 13 M HNO_3 (3 ml), 2 ml of 9.8 M H_2O_2 was added very slowly in order to completely degrade the biopolymers in the cell. The resultant solution was incubated at 50°C until no bubble was observed. The final volume of the sample solution was 15.5 ml. The control solution for the total metal-ion level was prepared by mixing an equal volume of the acid with ultrapure water to make the total volume 15.5 ml. Finally, the solution was diluted five times with ultrapure water, filtrated through Millex (Millipore, Bedford, MA, USA) and then submitted for ICP-ES analysis.

Sample preparation for quantification of unbound metal-ions was as follows. Cultured cells (2.5 g) were washed as described above and suspended in 10 ml of ultrapure water. The suspension was treated with ultrasonication for 5 min to disrupt the cells. A 10 ml aliquot of ultra pure water was treated similarly for the control solution. The sonicated solution was diluted to 15.5 ml. After further dilution twice, the solution was subjected to ultrafiltration with VIVASPIN 3,000 (Sartorius, Göttingen, Germany). A cut-off value of 3,000 was selected to remove proteins regardless of chelating ability for metals. The result was unaffected by using a different rotation speed for the ultrafiltration device (ranging from 2,000 to 8,000 rpm).

ICP-ES measurement

The sample solutions were analyzed by CIROS CCD ICP-ES instrument (Rigaku, Tokyo, Japan). If the intensity exceeded the recommended limit of detection, the sample solution was diluted with ultrapure water. Standard solutions of metal ions (Na, Mg, Si, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, Cd, and W from Wako Pure Chemicals; S, P and K from Sigma-Aldrich) were used for analysis by dilution with ultrapure water. The concentration of the standard solutions was adjusted so that accurate quantification could be performed for each metal in the sample.

Based on the intensity data of the standard solutions, a calibration curve was constructed by plotting the intensity against with the concentration (ppb). The concentration (in unit of ppb) of each metal ion in the sample solutions was determined from the calibration curve. The intracellular concentration of each metal ion was calculated from the concentration in the sample and the total volume of 2.5 g cells. The total cell volume was calculated from the average cell volume, cell density and the volume of the culture medium. Standard deviation was calculated based on data from three measurements.

Biophysical properties

Circular dichroism (CD) measurements were carried out as described previously (Kondo et al. 2004), except for the sample solution containing 20 mM HEPES, pH 7.5, 100 mM KCl and 5 μM Tt-dNTPase in the presence or absence of 10 mM $MnCl_2$. Gel filtration analysis was also carried out as described previously (Kondo et al. 2004), except for the elution buffer containing 50 mM HEPES (pH 8.0), 100 mM KCl and additional metal ions.

Results and discussion

Metal ion specificity

The effect of seven divalent metal ions (each at 10 mM) on dNTPase activity for a 2 mM mixture of dATP and dTTP was investigated. Manganese, cobalt and nickel were able to stimulate the activity as well as magnesium, which is the essential metal ion (Kondo et al. 2004), in the following order: manganese > cobalt > magnesium > nickel (Fig. 1a, white bars). No activity was detected in the presence of calcium, iron and zinc (Fig. 1a, white bars). The enzyme activity was lowered in the presence of magnesium and calcium or magnesium and zinc, compared to magnesium alone (data not shown). Therefore, calcium and zinc were likely to have an inhibitory effect on the activity.

Unexpectedly, the activity for a single dTTP was observed in the presence of manganese, and cobalt (Fig. 1a, black bars). The apparent rate constant k_{app}s for

the single dNTPase activity were 3.2 and 0.69 s^{-1} in the presence of 10 mM manganese and cobalt, respectively. These k_{app} values were higher than those for mixed dATP and dTTP in presence of magnesium (0.4 s^{-1}). These results indicate direct involvement of metal ions in the catalytic reaction.

The dependence of the activity on the metal ion concentrations was measured with the active metal ions. Optimal activity for a single dNTP was observed in the presence of 3–8 mM of free manganese or 0.2 mM of free cobalt (Fig. 1b). Higher concentrations of manganese and cobalt showed an inhibitory effect (Fig. 1b). By contrast, the optimal activity for dTTP and dATP was observed at 16–36 mM free magnesium (Fig. 1b). Interestingly, upon incubation in the presence of cobalt the reaction mixture became cloudy, suggesting that cobalt-induced aggregation of Tt-dNTPase.

Whether any structural change occurred upon binding of metal ion was verified by CD spectra and gel filtration analyses. The secondary structure of Tt-dNTPase did not change drastically upon addition of manganese (Fig. 2a). CD spectrum in the presence of magnesium also had showed similar profile (Kondo et al. 2004). The gel filtration profile was identical in the absence or presence of manganese, which gave a single peak with an apparent molecular mass corresponding to about 210 kDa. However, addition of more than 2 mM of cobalt caused disassembly of the oligomer, leading to the appearance of the second peak corresponding to about 59 kDa (Fig. 2b). As mentioned earlier, addition of cobalt resulted in the gradual precipitation of the protein, implying that binding of this metal ion can affect the quaternary structure of Tt-dNTPase.

Intracellular metal ion level in TTHB8

Although Tt-dNTPase showed different kinetics in the presence of magnesium and manganese, we could not evaluate which metal cation was authentic in vivo. No

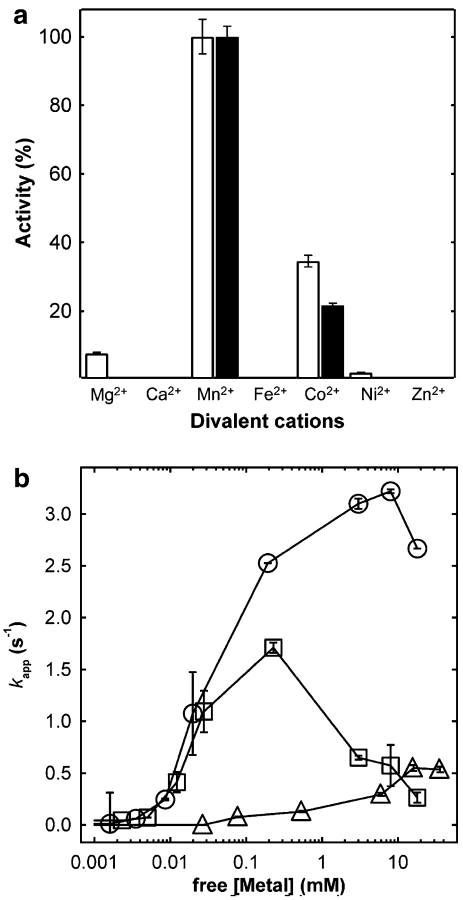


Fig. 1 Effects of divalent metal ions on dNTPase activity. **a** Metal ion specificity. *White* and *black bars* represent the activity in the presence of dATP and dTTP and in the presence of dTTP, respectively. Maximum activity under the respective conditions was standardized to 100%. **b** Dependence of the activity for dTTP on the concentration of manganese (*circles*), cobalt (*squares*) and magnesium in the presence of dATP (*triangles*)

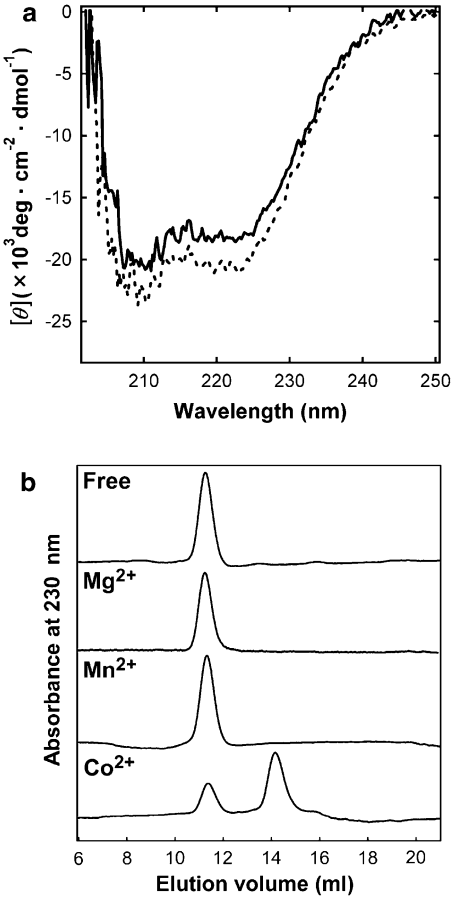


Fig. 2 Biophysical properties in the presence of metal ions. **a** CD spectra. *Solid line* and *dotted lines* indicate spectra of metal-free and Mn^{2+}-bound Tt-dNTPase, respectively. **b** Size exclusion chromatography. The sample containing 5 μM protein and 10 mM metal ions (or 2 mM cobalt ion) as chloride salts was eluted in 50 mM HEPES (pH 7.5), 100 mM KCl and 10 mM metal chloride (or 2 mM cobalt chloride)

metal ion was detected in Tt-dNTPase overexpressed in *E. coli* (data not shown) and we have not succeeded in purification of native Tt-dNTPase from TTHB8 cells. In this regard, it is crucial to determine the intracellular concentration of metal ions in TTHB8 cells. Thus, we measured the intracellular level of all kinds of metal ions as we could. Although manganese, cobalt and magnesium were the focus of our interest, information about intracellular ions should be highly significant from the perspective of evaluating the activity of other metal-dependent enzymes in vivo. ICP-ES is a sensitive technique for the specific detection of any metal ion species simultaneously, including magnesium and manganese. The samples were prepared to measure the precise level of the total metal ions and unbound metal ions. Acid treatment was performed to recover metal ions bound to molecules such as protein or nucleic acid. Although the plasma emission of V, Cr, Co and W were also measured, these spectra could not be detected under this condition. Table 1 shows the results of the estimated concentrations of measurable metal ions in the acid-treated or sonic-treated samples. The former values reflect the total metal ion level in vivo, whereas the latter corresponds to the biopolymer-unbound metal ion content. The levels of Mg, Ca, Mn, Fe, Ni, Cu and Zn were dramatically higher in the acid-treated samples than those in the sonic-treated one. These results indicate that the metal ions are likely to function by incorporation into biopolymers.

The results in Table 1 are the first characterization of intracellular concentration of metal ions in TTHB8. Thus, the feature of the metal ion concentration was evaluated. Though the level can vary with osmotic strength of the extracellular growth medium, this result can be the standard for the comparison of metal ion metabolism in TTHB8 with that in the other organisms. Compared the level of total metal ion (Table 1, acid treatment) in TTHB8 with that by ICP measurement in *E. coli*, the level of Mg, K, Cu, Zn and Mo has similar digits in both bacteria, while that of Ca, Mn, Fe and Ni are abundant by one or more order of magnitude in TTHB8 (Outten and O'Halloran 2001). V, Co and W are not detected in TTHB8 though in *E. coli* V has been detected (Outten and O'Halloran 2001). Focus on major metal ion, the level of K in TTHB8 is slightly lower compared with reported values, 100–200 mM, for other cells (Outten and O'Halloran 2001; Silver 1996). Dominy et al. reported that high ionic strength stabilizes the mesophilic protein, but destabilizes the thermophilic or hyper-thermophilic proteins (Dominy et al. 2002). Therefore, the slightly lower K level in TTHB8 might be optimal for the maintenance of protein stability. High-level composition of some multivalent metal ions such as Ca, Mn and Fe may be required for the increase of thermostability of proteins. For instance, Class II xylose isomerases can gain higher thermostability in the presence of manganese, cobalt or magnesium than in the absence of metal ions (Epting et al. 2005). Also cation-dependent activity and stability of phosphatases (Sinha et al. 1981; Singh and Sinha 1982), including their high temperature catalysis (Singh 2007) have been reported in an obligate thermophile, *Thermoactinomyces vulgaris*. It appears that the ion composition of TTHB8 reflects the characteristics of thermophilic bacterium.

Table 1 Intracellular concentration of metal ions in TTHB8

Metal	TR medium (mM)	Intracellular concentration (mM)	
		Acid treatment	Sonic treatment
Na	20	42.6 ± 1.8	21.6 ± 0.94
Mg	0.17	35.0 ± 2.7	1.53 ± 0.044
Si	0.19	0.343 ± 0.074	0.321 ± 0.0093
P	1.5	221 ± 8.2	32.1 ± 0.91
K	4.4	72.1 ± 2.9	43.5 ± 1.4
Ca	0.29	6.74 ± 0.57	0.130 ± 0.011
Mn	0.00014	0.163 ± 0.0057	0.000906 ± 0.000079
Fe	0.0034	1.77 ± 0.058	0.0034 ± 0.00042
Co	<0.000017[a]	<0.000624[a]	<0.000624[a]
Ni	<0.00016[a]	0.150 ± 0.000057	<0.00587[a]
Cu	<0.000031[a]	0.0548 ± 0.0038	<0.00113[a]
Zn	0.0050	0.584 ± 0.041	0.00255 ± 0.0010
Mo	<0.000058[a]	0.011 ± 0.0038	0.00504 ± 0.0019
Cd	<0.000018[a]	0.00657 ± 0.0011	<0.00066[a]

[a] Detections were under detectable level

Metal ion cofactor under physiological condition

We evaluated which metal cation was effective for Tt-dNTPase activity in vivo based on intracellular ion concentration and dependence of the activity on ion concentration in vitro. The analysis revealed the total concentrations of magnesium and manganese are 35 and 0.163 mM in the TTHB8 cells (Table 1, acid treatment), respectively. These concentrations were sufficient to cause induction of Tt-dNTPase activity (Fig. 1b). The analysis also revealed that free magnesium ion (1.53 mM) is just adequate to induce Tt-dNTPase, whereas free manganese ion was far too low to induce activity (Table 1, sonic treatment). Thus, in conclusion, the likely metal ion cofactor of Tt-dNTPase is magnesium under standard growth conditions. Manganese is likely to function as a cofactor of Tt-dNTPase only under extreme environmental conditions where the concentration of manganese in the cell is extremely high. Meanwhile several extremophiles are known to accumulate very high intracellular Mn^{2+} (Daly et al. 2004), suggesting that vigorous dNTP

hydrolysis is required to the survival under extreme conditions. Metal ion dependence of activity and stability of phosphatases has already been reported (Sinha et al. 1981; Singh and Sinha 1982; Bhatnagar and Singh 2004; Singh 2007).

Steady-state kinetics in the presence of manganese

Apart from significance in vivo discussed above, the fact that Tt-dNTPase hydrolyzed a single dNTP depending on manganese is interesting in terms of the activation mechanism of the enzyme. Thus, the dependence of the activity on dNTP concentration was assayed in the presence of manganese. The enzyme exhibited a hyperbolic dependence on dATP and dTTP concentrations, whereas a sigmoidal dependence on dGTP and dCTP concentrations was observed (Fig. 3). Therefore, the Hill equation was applied to these data, which was achieved by curve fitting to Eq. (1) (see "Materials and methods"). The K_M values of dATP and dTTP were much lower than for dGTP and dCTP (Table 2). These results are consistent with the order of the specificity of the activatory dNTPs in the presence of magnesium (Kondo et al. 2004). By contrast, the n values of dGTP and dCTP were significantly greater than those for dATP and dTTP, although the value for each dNTP except for dATP was >1. These results suggest that in the presence of manganese more than two molecules of dNTPs bind to Tt-dNTPase with positive cooperativity.

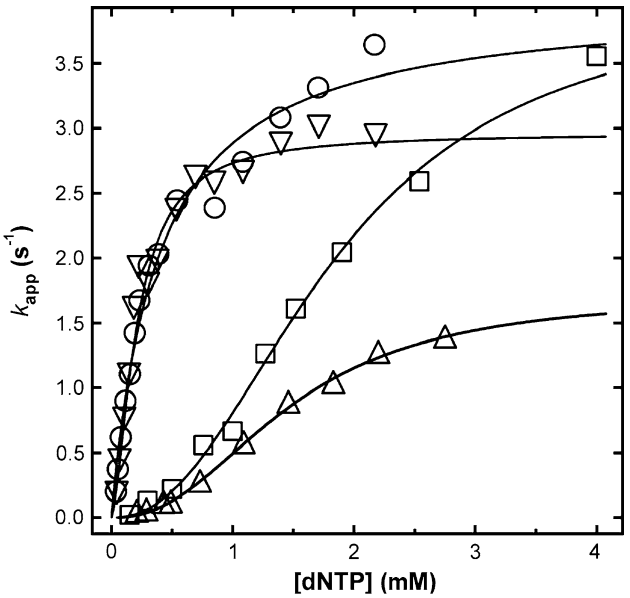


Fig. 3 The dNTPase activity in the presence of manganese. Reaction mixtures contains 50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM $MnCl_2$ and various concentrations of single dNTP. The data points were fitted to the Hill equation. The activity for dATP, dTTP, dGTP and dCTP are represented as *circles*, *inversed triangles*, *triangles* and *squares*, respectively. The *lines* indicate the theoretical curves

This also supports the hypothesis that Tt-dNTPase retains several dNTP-binding sites (Kondo et al. 2004). On the other hand, the k_{cat} values are more than tenfold greater than for those in the presence of magnesium, suggesting that manganese is markedly more efficient in the catalytic step compared to magnesium.

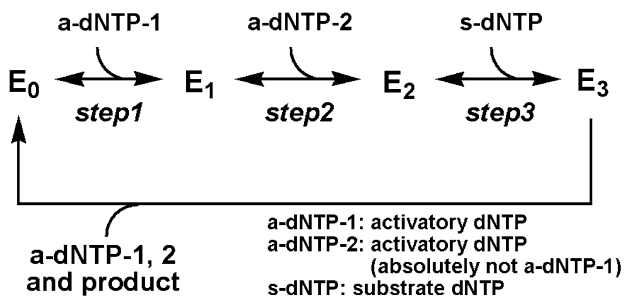
In the previous paper, we proposed the model of magnesium- and dNTPs-induced activity, on which the activation procedure proceeds by at least three sequential steps of dNTP binding (Scheme 1) (Kondo et al. 2004).

In this model, we suggested that in recognizing the first dNTP (Scheme 1, step 1), dATP and dTTP are highly specific for Tt-dNTPase, whereas recognition of the second dNTP (Scheme 1, step 2) specifically excludes the dNTP bound in the first step. Then binding of the two sorts of dNTP allows the enzyme to hydrolyze a dNTP with broad specificity (Scheme 1, step 3).

Along with this model, it can be considered that Tt-dNTPase utilized any dNTP as the second effector in the presence of manganese. This means that the enzyme acquired broad specificity for an activatory dNTP on step 2 in Scheme 1. We had no evidence that manganese affected the conformation of Tt-dNTPase. However, it should be mentioned here that the oligomeric state of Tt-dNTPase was affected by cobalt ion (Fig. 2b), which induced the activity for a single kind of dNTPs as well as manganese ion (Fig. 1a, black bars). These results suggest the possibility that the metal ions, which are able to induce the activity for a single kind of dNTP, could affect the quaternary conformation and might induce a quaternary conformational change necessary for the activation.

Table 2 Kinetic parameters in the presence of manganese on the Hill equation

Substrate	k_{cat} (s^{-1})	K_M (μM)	n	k_{cat}/K_M (s^{-1} M^{-1})
dATP	4.0 ± 0.40	380 ± 99	1.0 ± 0.1	1.5×10^4
dTTP	3.0 ± 0.12	210 ± 18	1.6 ± 0.2	1.4×10^4
dGTP	1.7 ± 0.08	1,500 ± 78	2.3 ± 0.1	1.1×10^3
dCTP	4.0 ± 0.15	1,800 ± 92	2.2 ± 0.2	2.2×10^3



Scheme 1

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References

Aravind L, Koonin EV (1998) The HD domain defines a new superfamily of metal-dependent phosphohydrolases. Trends Biochem Sci 23:469–472

Bhatnagar K, Singh VP (2004) Ca^{2+} dependence and inhibitory effects of trifluoperazine on plasma membrane ATPase of *Thermoactinomyces vulgaris*. Curr Microbiol 49:28–31

Bowen LM, Dupureur CM (2003) Investigation of restriction enzyme cofactor requirements: a relationship between metal ion properties and sequence specificity. Biochemistry 42:12643–12653

Daly MJ, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Venkateswaran A, Hess M, Omelchenko MV, Kostandarithes HM, Makarova KS, Wackett LP, Fredrickson JK, Ghosal D (2004) Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science 306:1025–1028

Dominy BN, Perl D, Schmid FX, Brooks CL III (2002) The effects of ionic strength on protein stability: the cold shock protein family. J Mol Biol 319:541–554

Epting KL, Vieille C, Zeikus JG, Kelly RM (2005) Influence of divalent cations on the structural thermostability and thermal inactivation kinetics of class II xylose isomerases. FEBS J 272:1454–1464

Hashimoto Y, Yano T, Kuramitsu S, Kagamiyama H (2001) Disruption of *Thermus thermophilus* genes by homologous recombination using a thermostable kanamycin-resistant marker. FEBS Lett 506:231–234

Hogg T, Mechold U, Malke H, Cashel M, Hilgenfeld R (2004) Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p) ppGpp metabolism during the stringent response. Cell 117:57–68

Huai Q, Colicelli J, Ke H (2003) The crystal structure of AMP-bound PDE4 suggests a mechanism for phosphodiesterase catalysis. Biochemistry 42:13220–13226

Kondo N, Kuramitsu S, Masui R (2004) Biochemical characterization of TT1383 from *Thermus thermophilus* identifies a novel dNTP triphosphohydrolase activity stimulated by dATP and dTTP. J Biochem 136:221–231

Kondo N, Nakagawa N, Ebihara A, Chen L, Liu Z-J, Wang B-C, Yokoyama S, Kuramitsu S, Masui R (2007) Structure of dNTP-inducible dNTP triphosphohydrolase: insight into broad specificity for dNTPs and triphosphohydrolase-type hydrolysis. Acta Crystallogr Sect D Biol Crystallogr 63:230–239

Lukacin R, Matern U, Specker S, Vogt T (2004) Cations modulate the substrate specificity of bifunctional class I *O*-methyltransferase from *Ammi majus*. FEBS Lett 577:367–370

Oshima T, Imahori K (1974) Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonporulating thermophilic bacterium from a Japanese thermal spa. Int J Syst Bacteriol 24:102–112

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492

Patton C, Thompson S, Epel D (2004) Some precautions in using chelators to buffers metals in biological solutions. Cell Calcium 35:427–431

Seto D, Bhatnagar SK, Bessman MJ (1988) The purification and properties of deoxyguanosine triphosphate triphosphohydrolase from *Escherichia coli*. J Biol Chem 263:1494–1499

Silver S (1996) Transport of inorganic cations. *Escherichia coli* and *Salmonella*. American Society for Microbiology, N. W. Washington, DC

Singh VP (2007) Mg^{2+} decreases Arrhenius energies of activation for high temperature catalysis of phosphatases in *Thermoactinomyces vulgaris*. Curr Microbiol 55:179–184

Singh VP, Sinha U (1982) Thermostability & turnover of phosphatases in the obligate thermophile *Thermoactinomyces vulgaris*. Indian J Exp Biol 20:26–30

Sinha U, Singh VP (1980) Phosphate utilization and constitutive synthesis of phosphatases in *Thermoactinomyces vulgaris* Tsilinsky. Biochem J 190:457–460

Sinha U, Singh VP, Srivastava S (1981) Cation-dependent activity and stability of phosphatases in *Thermoactinomyces vulgaris*. Indian J Exp Biol 19:453–457

Sy J (1977) In vitro degradation of guanosine 5′-diphosphate, 3′-diphosphate. Proc Natl Acad Sci USA 74:5529–5533

Yakunin AF, Proudfoot M, Kuznetsova E, Savchenko A, Brown G, Arrowsmith CH, Edwards AM (2004) The HD domain of the *Escherichia coli* tRNA nucleotidyltransferase has 2′, 3′-cyclic phosphodiesterase, 2′-nucleotidase, and phosphatase activities. J Biol Chem 279:36819–36827