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# GTP cyclohydrolase I utilizes metal-free GTP as its substrate

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GTP cyclohydrolase I (GCH) is the rate-limiting enzyme for the synthesis of tetrahydrobiopterin and its activity is important in the regulation of monoamine neurotransmitters such as dopamine, norepinephrine and serotonin. We have studied the action of divalent cations on the enzyme activity of purified recombinant human GCH expressed in *Escherichia coli*. First, we showed that the enzyme activity is dependent on the concentration of Mg-free GTP. Inhibition of the enzyme activity by $Mg^{2+}$, as well as by $Mn^{2+}$, $Co^{2+}$ or $Zn^{2+}$, was due to the reduction of the availability of metal-free GTP substrate for the enzyme, when a divalent cation was present at a relatively high concentration with respect to GTP. We next examined the requirement of $Zn^{2+}$ for enzyme activity by the use of a protein refolding assay, because the recombinant enzyme contained approximately one zinc atom per subunit of the decameric protein. Only when $Zn^{2+}$ was present was the activity of the denatured enzyme effectively recovered by incubation with a chaperone protein. These are the first data demonstrating that GCH recognizes Mg-free GTP and requires $Zn^{2+}$ for its catalytic activity. We suggest that the cellular concentration of divalent cations can modulate GCH activity, and thus tetrahydrobiopterin biosynthesis as well.

*Keywords*: GTP cyclohydrolase I; magnesium; recombinant protein; tetrahydrobiopterin; zinc.

Metal ions are essential for many physiological functions of the brain. They may also induce or aggravate numerous neurodegenerative processes. Thus, it is important to understand the roles of metal ions in normal and pathological brain functions.

GTP cyclohydrolase I (GCH) is the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin ($BH_4$), and the cellular $BH_4$ content is regulated mainly by the activity of this enzyme. $BH_4$ is an essential cofactor for three aromatic amino-acid monooxygenases – phenylalanine, tyrosine, and tryptophan hydroxylases – and for nitric oxide synthase [1]. $BH_4$ deficiency caused not only a decrease in the activity of these enzymes but also a decrease in the protein levels of tyrosine hydroxylase and nitric oxide synthase [2,3]. Therefore, the availability of $BH_4$ affects the amounts of neurotransmitters such as catecholamines, serotonin, melatonin and nitric oxide. The role of $BH_4$ in the activity of nitric oxide synthase also makes $BH_4$ an important factor for the immune system and endothelial cell function.

Various hormones and cytokines are known to induce the expression of the GCH gene in neural, lymphocytic and endothelial cells, and in different cell lines, resulting in an increased $BH_4$ content [4–8]. At the post-transcriptional level, $BH_4$ was shown to inhibit, and phenylalanine to stimulate, GCH activity through interaction with GFRP, a GTP cyclohydrolase I feedback regulatory protein [9]. GCH, which is a homodecameric protein, shows positive cooperativity against the GTP substrate [10] and phenylalanine changes the substrate velocity curve from sigmoidal to hyperbolic [11].

Recent biophysical studies suggest a stimulatory effect of $Zn^{2+}$ [12] and $Ca^{2+}$ [13] on GCH activity. By crystallographic analysis using purified *Escherichia coli* enzyme [14], an N-terminally truncated form of the recombinant human enzyme [12], and a stimulatory complex of rat GCH and GFRP induced by phenylalanine [15], $Zn^{2+}$ was shown to be bound to the active centre of the homodecameric GCH enzyme. As for $Ca^{2+}$, mutations of the recombinant rat enzyme in an EF-hand-like motif, which is absent in bacteria, inhibited both the binding of $Ca^{2+}$ to the enzyme and enzyme activity [13]. In addition, inhibition of the enzyme activity by various divalent cations including $Mg^{2+}$ and $Zn^{2+}$ was reported, based on experiments using crude preparations from mammalian and bacterial tissues [16] and the enzyme purified from rat liver [10].

In the present study, we examined the effect of various divalent cations on purified recombinant human GCH expressed in *E. coli* to clarify the molecular mechanism of action of divalent cations on the GCH enzymatic activity. We showed that GCH activity was totally dependent on metal-free GTP and that $Mg^{2+}$ inhibited the enzyme activity by reducing the concentration of metal-free GTP by complex formation. Mg–GTP complex and $Mg^{2+}$ had little effect on the GCH activity at the concentrations tested here. Also, by performing a protein refolding assay for GCH, we demonstrated that a stoichiometric amount of $Zn^{2+}$ was

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*Abbreviations*: $BH_4$, tetrahydrobiopterin; GCH, GTP cyclohydrolase I; GdnHCl, guanidine hydrochloride; NOS, nitric oxide synthase.

*Enzyme*: GTP cyclohydrolase I (EC 3.5.4.16).

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essential for the enzyme activity. Our data thus suggest that physiological and pathological changes in the levels of divalent cations including $Mg^{2+}$ and $Zn^{2+}$ may affect GCH activity and $BH_4$ levels *in vivo*.

## Experimental procedures

### Purification of recombinant human GCH

Recombinant human GCH was expressed in *E. coli* and purified as described previously [17]. We used this purified recombinant human enzyme for analysis of the action of divalent cations. Protein concentrations were determined by the method of Bradford [18], with bovine γ-globulin used as a standard.

### Measurement of GCH activity

GCH activity was assayed as described previously [17]. The typical incubation mixture (total volume, 100 μL) contained 20 mM Tris/HCl (pH 7.5), 100 mM KCl, 1 $mg{\cdot}mL^{-1}$ BSA, and GTP as a substrate. The recombinant protein (10 μg) was incubated with various concentrations of GTP and divalent cations at 37 °C for 30 min.

### Calculation of the concentrations of metal–GTP complex and metal-free GTP

Concentrations of metal-containing GTP, metal-free GTP, and GTP-free divalent cations in the reaction mixture for the measurement of the enzyme activity were determined by using the MAXCHELATOR program (WINMAXC ver.2.10 and SLIDERS ver.2.00, http://www.stanford.edu/~cpatton/maxc.html) [19]. Stability constants and enthalpy changes for metal–nucleotide complexes were obtained by referring to NIST Critically Selected Stability Constants of Metal Complexes: Version 6.0 (http://www.nist.gov/srd/nist46.htm). For calculation of concentrations of metal–GTP complex and metal-free GTP, we used stability constants and enthalpy changes of metal–ATP or proton–ATP complex as a substitute for those of the metal–GTP complex, because there were no data for stability constants and enthalpy changes of the Mg–, Zn–, Co– or Mn–GTP complexes in $K^+$ salt as a background electrolyte; however, stability constants of GTP with respect to $Mg^{2+}$ in $Na^+$ salt as a background electrolyte and stability constants and enthalpy changes of GTP with respect to $H^+$ in $K^+$ salt as a background electrolyte were very similar to those of ATP in the database, and apparent stability constants of GTP with respect to $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ were almost the same as those of ATP given in a previous report [20]. Based on the condition of the incubation mixture for the enzyme activity described as above, parameters used in the calculation program were 37 °C, pH 7.5, and 0.110 ionic strength. Calculated values were considered to be accurate in a chelator-buffering range, which is within one order of magnitude of the $K_d$ value for a metal–chelator complex.

### Atomic absorption spectrophotometry

Zinc and calcium contents of the purified recombinant human GCH protein were determined by atomic absorption spectrophometry using a polarized Zeeman atomic absorption spectrometer, type Z-8100 (Hitachi, Tokyo, Japan).

### Refolding assay

For the protein refolding assay in the presence of GroE, which is a chaperone protein, we referred to previous reports [21–24]. For denaturation, GCH was incubated on ice with 4 M guanidine hydrochloride (GdnHCl) for 30 min. The solution of denatured GCH was then diluted 100-fold with refolding buffer containing 50 mM Tris/HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ATP, and a 2.5-fold molar excess of GroE. Equal molar amounts of GroES and GroEL (Takara Bio, Japan) were mixed for preparing the GroE complex. For refolding, the mixture was incubated at 25 °C for 60 min. Spontaneous refolding was performed in the absence of GroE.

For the experiment involving $Zn^{2+}$ addition after refolding, the sample refolded in the presence of EGTA or $Zn^{2+}$ was desalted by filtration through a spin-column (Micro Bio-spin 6, Bio-Rad). For elimination of $Mg^{2+}$ and ATP, which are essential for the refolding reaction, as well as that of $Zn^{2+}$ or EGTA, from the refolded samples, the spin-column was equilibrated with a solution containing 50 mM Tris/HCl pH 7.5, 50 mM KCl, and 1 mM dithiothreitol. After desalting, ions or chelators were added to aliquots of the filtered samples and preincubation was carried out at 25 °C for 5 min. Finally, aliquots of the samples (10 μL) were added to 90-μL volumes of the assay mixture for measurement of GCH activity, which was performed as described above.

### Statistics

ANOVA followed by Bonferroni/Dunn's multiple comparison test was used for statistical evaluation of differences in the enzyme activity. $P < 0.05$ was accepted as statistically significant.

## Results

### Interaction of $Mg^{2+}$ with the GTP substrate in solution is responsible for decrease in the GCH activity

GCH has enzyme activity in the absence of $Mg^{2+}$, whereas many other nucleotide hydrolyzing enzymes such as G proteins and kinases recognize Mg–GTP or Mg–ATP as the substrate. We first examined the effect of $Mg^{2+}$ on the kinetics of enzyme activity of the purified recombinant human GCH. As shown in Fig. 1A, the dose–response curve for the GTP substrate was shifted to the right in the presence of 1 mM $MgCl_2$ and, to a much greater extent in the presence of 5 mM $MgCl_2$, whereas the enzyme activities at the high GTP concentrations remained unchanged. If $Mg^{2+}$ acted directly on the enzyme we would expect the dose dependency of inhibition by $MgCl_2$ to be constant at various GTP concentrations. However, as shown in Fig. 1B, dose dependency for inhibition shifted to lower concentrations of $MgCl_2$ as the concentration of the GTP substrate was decreased. These results suggest that formation of the GTP–$Mg^{2+}$ complex was responsible for the

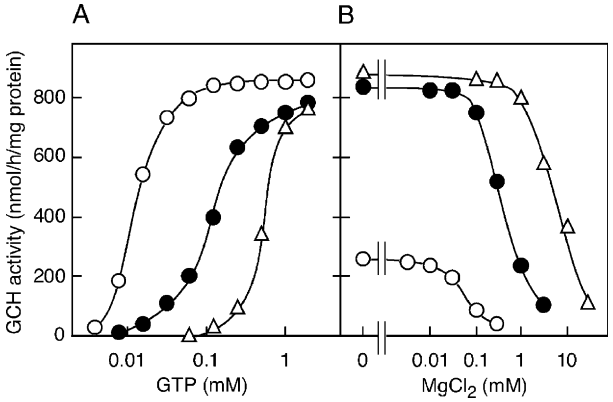


**Fig. 1. Effect of $Mg^{2+}$ on the enzyme activity of recombinant human GCH.** (A) Purified recombinant enzyme was incubated in the absence (○) or presence of 1 mM (●) or 5 mM (△) $MgCl_2$ at the indicated concentrations of GTP. (B) Enzyme was incubated at 0.1 (○), 1 (●), or 10 (△) mM GTP in the presence of the indicated concentrations of $MgCl_2$. Each figure is representative of two independent experiments.

shift in the GTP dose–response curve at higher $Mg^{2+}$ concentrations.

## GCH recognizes Mg-free GTP

We next examined the dependency of the enzyme activity on Mg-free GTP. We assumed that the concentration of total GTP in the absence of $MgCl_2$ was equal to that of metal-free GTP, because 1 mM EDTA did not affect the dose–response curve for the GTP substrate (data not shown). We calculated the metal-free GTP concentrations in the presence of 200 μM $MgCl_2$. The concentrations of metal-free GTP at 15, 20, 30, 40, 50, 75, 100 and 125 μM total GTP were reduced in the presence of 200 μM total $Mg^{2+}$ to 3.63, 4.92, 7.60, 10.4, 13.5, 21.8, 31.4 and 42.4 μM, respectively, in the presence of 200 μM $MgCl_2$ (Fig. 2A). We measured GCH activity under these conditions, and plotted it against total GTP (Fig. 2C) or Mg-free GTP (Fig. 2D). Although the enzyme activity was significantly decreased by the addition of $MgCl_2$ (Fig. 2C), the dependency of the enzyme activity on Mg-free GTP was similar in the presence and absence of $MgCl_2$ (Fig. 2D). The enzyme activity was, however, slightly decreased at > 15 μM Mg-free GTP in the presence of $MgCl_2$ compared with the values in the absence of $MgCl_2$ (Fig. 2D).

Next we measured enzyme activity at a constant concentration of Mg-free GTP and increasing concentrations of Mg–GTP complex and $Mg^{2+}$. When the concentration of Mg-free GTP was fixed at 10 μM, the concentrations of $MgCl_2$ in the reaction mixture were 35, 70, 105, 140, 175 and 210 μM at the total GTP concentrations of 15, 20, 25, 30, 35 and 40 μM, respectively (Fig. 3A and B). As shown in Fig. 3C, the GCH activity was almost unchanged when the concentrations of Mg-free GTP remained constant at 10 μM in the range 10–40 μM total GTP. These data suggest that the GCH activity was dependent on the concentration of Mg-free GTP and that neither Mg–GTP complex nor $Mg^{2+}$ affected the enzyme activity under the conditions examined.

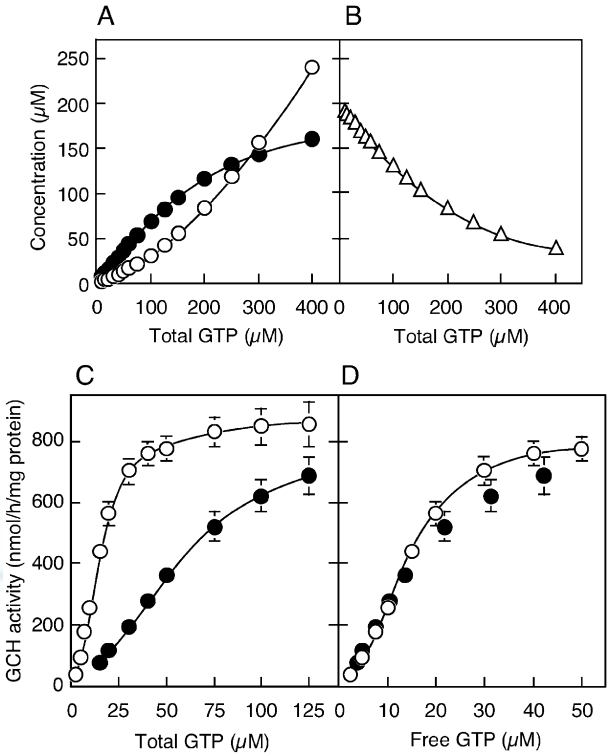


**Fig. 2. GTP dose–response curves of the human GCH activity in the presence and absence of $Mg^{2+}$.** (A and B) Concentrations of Mg-free GTP (○), Mg–GTP complex (●), and $Mg^{2+}$ (△) at the indicated total GTP concentrations in the presence of 200 μM $MgCl_2$ were plotted. The concentrations were calculated as described in Experimental procedures. (C and D) Enzyme activity of the recombinant human enzyme was measured in the presence of 200 μM $MgCl_2$ over a range of total GTP of 15–125 μM (●) and in the absence of $MgCl_2$ over a range of total GTP of 2.5–125 μM (○). Concentrations of total and free GTP in the reaction mixture are plotted on the X-axis of (C) and (D), respectively. Results represent the mean ± SD of three independent experiments.

Various divalent cations at 0.5 mM inhibited enzyme activity when the GTP concentration was 0.1 mM (Fig. 4). Both $MgCl_2$ and $MgSO_4$ inhibited enzyme activity to a similar extent (Fig. 4), confirming that the inhibitory effect was caused by the $Mg^{2+}$ ion. $MnCl_2$, $CoCl_2$, and $ZnSO_4$ inhibited the enzyme activity to a greater degree than $MgCl_2$ and $MgSO_4$ (Fig. 4). In contrast with the inhibition shown at 0.1 mM total GTP, we did not observe any inhibitory effect by any of the divalent cations examined at a higher concentration of the substrate, 1 mM total GTP (Fig. 4). The concentration of metal-free GTP in the presence of 0.5 mM $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Zn^{2+}$ at 0.1 mM total GTP was estimated to be 12.7, 3.49, 4.89, and 1.46 μM, respectively. Nonetheless, the enzyme activities under these conditions showed good accordance with the metal-free GTP dose dependency (data not shown). At 1 mM GTP, metal-free GTP in the presence of 0.5 mM $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Zn^{2+}$ was estimated to be 561, 561, 516, 519, and 510 μM, respectively. These data explain why there was no significant difference in the enzyme activity at 1 mM GTP in the presence