

GTP cyclohydrolase I utilizes metal-free GTP as its substrate

Takahiro Suzuki^{1,2}, Hideki Kurita³ and Hiroshi Ichinose^{1,2}

¹Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Japan;

²Division of Molecular Genetics, Institute for Comprehensive Medical Science; ³Department of Hygiene, School of Medicine, Fujita Health University, Aichi, Japan

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 Mg²⁺, as well as by Mn²⁺, Co²⁺ or Zn²⁺, 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 Zn²⁺

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 Zn²⁺ 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 Zn²⁺ 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 (BH₄), and the cellular BH₄ content is regulated mainly by the activity of this enzyme. BH₄ is an essential cofactor for three aromatic amino-acid monooxygenases – phenylalanine, tyrosine, and tryptophan hydroxylases – and for nitric oxide synthase [1]. BH₄ 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 BH₄ affects the amounts of neurotransmitters such as catecholamines, serotonin, melatonin and nitric oxide. The role of BH₄ in the activity of nitric oxide synthase also makes BH₄ 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 BH₄ content [4–8]. At the post-transcriptional level, BH₄ 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 Zn²⁺ [12] and Ca²⁺ [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], Zn²⁺ was shown to be bound to the active centre of the homodecameric GCH enzyme. As for Ca²⁺, mutations of the recombinant rat enzyme in an EF-hand-like motif, which is absent in bacteria, inhibited both the binding of Ca²⁺ to the enzyme and enzyme activity [13]. In addition, inhibition of the enzyme activity by various divalent cations including Mg²⁺ and Zn²⁺ 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 Mg²⁺ inhibited the enzyme activity by reducing the concentration of metal-free GTP by complex formation. Mg–GTP complex and Mg²⁺ 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 Zn²⁺ was

Correspondence to H. Ichinose, Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259, Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan. Fax: + 81 45 924 5807, Tel.: + 81 45 924 5822, E-mail: hichinos@bio.titech.ac.jp

Abbreviations: BH₄, 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 μ L) 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 μ g) 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 spectrophotometry 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 μ L) were added to 90- μ L 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

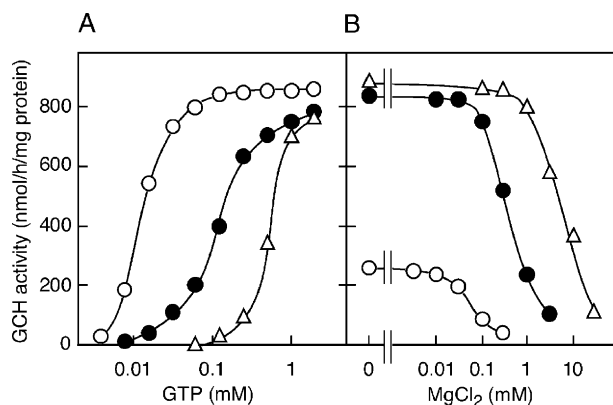


Fig. 1. Effect of Mg^{2+} on the enzyme activity of recombinant human GCH. (A) Purified recombinant enzyme was incubated in the absence (\circ) or presence of 1 mM (\bullet) or 5 mM (Δ) $MgCl_2$ at the indicated concentrations of GTP. (B) Enzyme was incubated at 0.1 (\circ), 1 (\bullet), or 10 (Δ) mM 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 $> 15 \mu M$ 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.

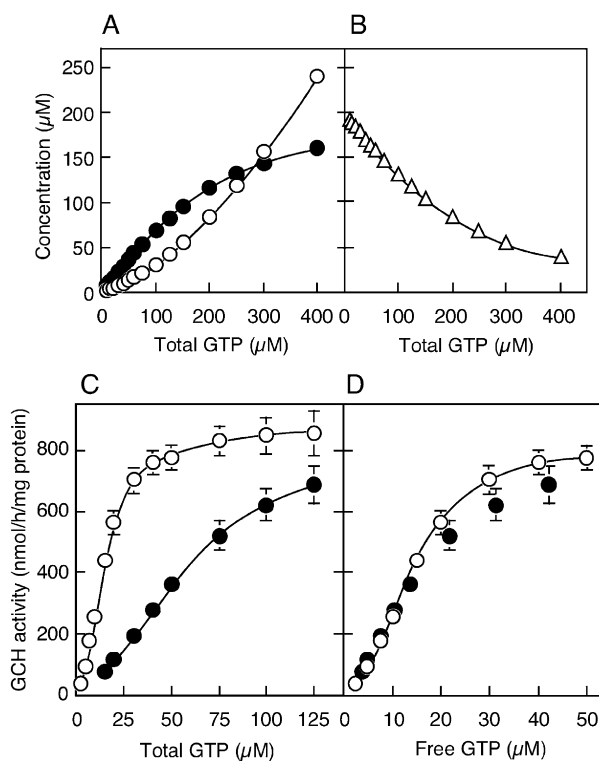


Fig. 2. GTP dose–response curves of the human GCH activity in the presence and absence of Mg^{2+} . (A and B) Concentrations of Mg-free GTP (\circ), Mg–GTP complex (\bullet), and Mg^{2+} (Δ) at the indicated total GTP concentrations in the presence of 200 μM $MgCl_2$ were plotted. The concentrations 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 (\bullet) and in the absence of $MgCl_2$ over a range of total GTP of 2.5–125 μM (\circ). 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 \pm 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

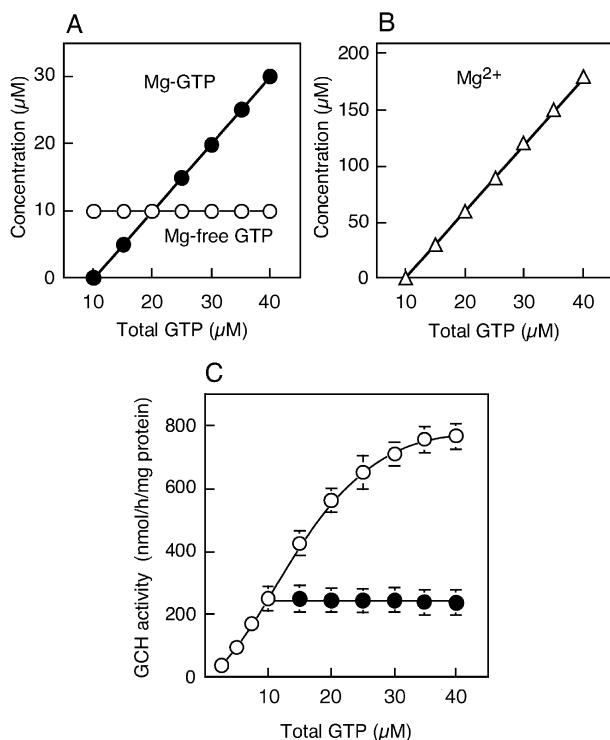


Fig. 3. Enzyme activity at a constant concentration of Mg-free GTP.

To fix the concentration of Mg-free GTP at 10 μM constant, we adjusted the concentrations of MgCl_2 in the reaction mixture to 35, 70, 105, 140, 175, and 210 μM at the total GTP concentrations of 15, 20, 25, 30, 35, and 40 μM , respectively. Under these conditions at a constant 10 μM Mg-free GTP (A; \circ), the concentrations of Mg-GTP complex (A; \bullet) and Mg^{2+} (B; \triangle) were plotted. (C) GCH activity was measured at a constant 10 μM Mg-free GTP when the concentrations of Mg-GTP complex and Mg^{2+} were increased as described above (\bullet). GCH activity without Mg^{2+} was also measured (\circ). Results represent the mean \pm SD from three independent experiments.

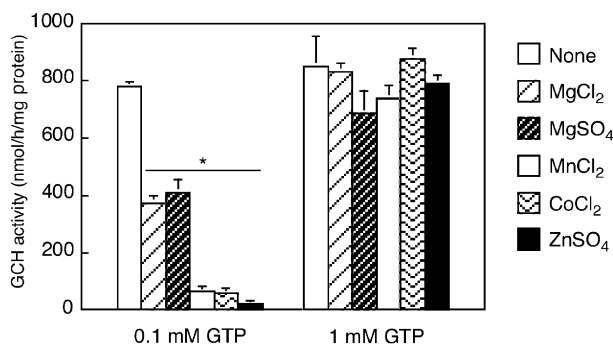


Fig. 4. Effect of various divalent cations on the enzyme activity of recombinant human GCH. Enzyme activity was measured at the GTP concentration of 0.1 or 1 mM in the presence of 0.5 mM MgCl_2 , MgSO_4 , MnCl_2 , CoCl_2 , or ZnSO_4 . Results represent the mean \pm SD of three independent experiments. P values were calculated based on the value for the vehicle only: * $P < 0.001$.

of various cations: the substrate-velocity curve for metal-free GTP was in the plateau phase around 500 μM (Fig. 1).

Zn^{2+} bound to the purified recombinant human GCH

In contrast to the inhibitory effect of Zn^{2+} on enzyme activity when the ion was in molar excess over the GTP substrate, as was shown in Fig. 4, a recent crystallographic study showed that Zn^{2+} bound to the active centre of the bacterial and human GCH enzymes, with the conclusion that Zn^{2+} participated in the catalytic reaction [12]. Besides Zn^{2+} , Ca^{2+} at nanomolar concentrations was suggested to activate the enzyme [13]. We performed atomic absorption spectrophotometry using the purified recombinant human enzyme to examine whether GCH contained metal ions. By amino acid composition analysis, the concentration of the subunit of the GCH enzyme in the solution examined was calculated to be $14.4 \pm 0.7 \mu\text{M}$. The concentration of zinc in the solution was estimated to be $16.2 \pm 1.4 \mu\text{M}$ by atomic absorption spectrophotometry, whereas that in the buffer control was not detectable ($< 0.3 \mu\text{M}$). The data indicate that the purified recombinant enzyme bound ≈ 1 zinc per subunit. On the other hand, calcium was not detectable in the enzyme solution ($< 0.5 \mu\text{M}$).

Requirement of Zn^{2+} for the GCH enzymatic activity

We next examined whether or not Zn^{2+} was essential for the enzyme activity of the human recombinant GCH. Zn^{2+} seemed to bind tightly to the enzyme protein, because preincubation of the recombinant enzyme with Zn^{2+} chelating agents such as EDTA, EGTA or N,N,N',N' -tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) at 1 mM and 25 $^\circ\text{C}$ for 20 min had little effect on enzyme activity (data not shown). To examine the effect of Zn^{2+} on enzyme activity, we established a procedure for the refolding of GCH protein by using a chaperone protein, GroE. In this experiment, we measured enzyme activity at a concentration of GTP high enough, i.e. 1 mM, to give the V_{max} value, thus cancelling the inhibition by the complex formation of metal-free GTP substrate with Mg^{2+} at 0.5 mM and Zn^{2+} at $< 30 \mu\text{M}$, which were carried over from the refolding reaction. The recombinant enzyme incubated at 4 $^\circ\text{C}$ in 4 M GdnHCl was rapidly inactivated (within 5 min). The enzyme activity of the sample denatured for 30 min was $\approx 2\%$ of that of the nondenatured one (Fig. 5A). The denatured sample diluted with the refolding buffer was incubated at 25 $^\circ\text{C}$ with or without Zn^{2+} or EGTA in the presence or absence of GroE, before measuring the GCH enzyme activity (Fig. 5B). In the presence of GroE, the enzyme activity recovered to a greater extent than in its absence. It was further elevated by the addition 10 μM ZnSO_4 to the refolding mixture, whereas it was inhibited by the addition of EGTA (Fig. 5B). The activity of the enzyme refolded in the presence of ZnSO_4 was $67.5 \pm 12.3\%$ of that of the nondenatured enzyme (Fig. 5B). Far less enzyme activity was recovered with 10 μM ZnSO_4 in the absence of GroE (Fig. 5B). When the refolding reaction was carried out at 4 $^\circ\text{C}$ instead of 25 $^\circ\text{C}$, or without ATP or Mg^{2+} at 25 $^\circ\text{C}$, the enzyme activity was not recovered (data not shown). In contrast with Zn^{2+} , Ca^{2+} at 10 μM in the refolding reaction did not have any effect on the enzyme activity (data not shown).

We next examined the amount of Zn^{2+} required for the stimulatory effect in the refolding assay. The presence of Zn^{2+} during the refolding procedure elicited a

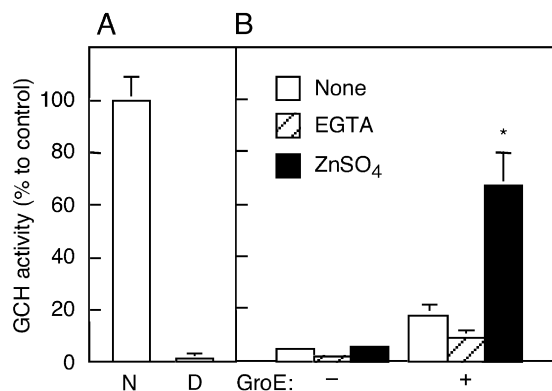


Fig. 5. GCH enzymatic activity was activated by addition of Zn²⁺ during protein refolding. (A) Enzyme activity of the denatured (D) or nondenatured (N) enzyme was measured. For the preparation of denatured samples, the enzyme was incubated on ice with 4 M GdnHCl for 30 min, and the mixture was then diluted with the refolding buffer (final concentration of GdnHCl, 40 mM). For preparation of the nondenatured control enzyme was diluted with refolding buffer containing 40 mM GdnHCl. (B) Enzyme activity of the refolded enzyme was measured. The denatured sample containing 10 μ M ZnCl₂, 10 μ M EGTA, or vehicle only was incubated at 25 °C for 60 min in the refolding buffer in the presence or absence of GroE. Results represent the mean \pm SD of three independent experiments. *P* values were calculated based on the value for the vehicle only: **P* < 0.001.

dose-dependent increase in the GCH enzyme activity, and the maximum effect was achieved at \approx 10–30 μ M (Fig. 6A). The apparent EC₅₀ value of Zn²⁺ was estimated to be 235 nM at 70 nM GCH subunit (Fig. 6B).

Effect of Zn²⁺ addition on the GCH enzyme activity after refolding

To clarify whether the presence of Zn²⁺ during refolding was required for the recovery of enzyme activity, we

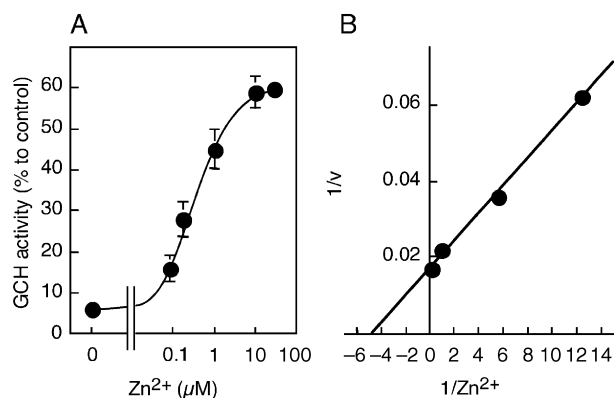


Fig. 6. Dose dependency of the stimulatory effect of Zn²⁺ on enzyme activity during refolding. We performed the refolding assay for the recombinant GCH in the presence of GroE at various Zn²⁺ concentrations. The Zn²⁺ concentration in the presence of 10 μ M EGTA was presumed to be zero and that following the addition of the vehicle only was calculated to be 81 nM Zn²⁺ based on the data from the atomic absorption spectrometry. (A) The concentration of Zn²⁺ was plotted on a logarithmic scale. (B) Double reciprocal plot of (A). Results represent the mean \pm SD of three independent experiments.

Table 1. Effect of the addition of Zn²⁺ ions after refolding on GCH activity. The denatured samples refolded in the presence of EGTA or Zn²⁺ following the desalting were prepared as described in Experimental procedures. The activities were determined in the presence of 1 mM GTP. The activity of the enzyme refolded in the presence of ZnSO₄ and then preincubated in vehicle only (none) was taken as 100%. Results represent the mean \pm SD of three independent experiments. *P* values were calculated based on the value obtained by preincubation with vehicle only.

Preincubation	GCH activity (% of control)	
	Refolding with ZnSO ₄	Refolding with EGTA
None	100.0 \pm 0.5	21.3 \pm 0.9
ZnSO ₄	103.4 \pm 1.8	64.5 \pm 1.8*
EGTA	101.4 \pm 0.6	17.8 \pm 1.6

**P* < 0.001.

examined the effect of Zn²⁺ addition on the GCH enzyme after refolding. The addition of 10 μ M ZnSO₄ after refolding elevated the enzyme activity of the sample refolded with EGTA; however, it was less effective than the addition of Zn²⁺ during refolding (Table 1). EGTA had no significant effect on the enzyme activity of the samples after refolding (Table 1) or on the activity of the nondenatured enzyme (data not shown). The dose dependency of the effect of Zn²⁺ after refolding was similar to that during refolding (data not shown).

Discussion

In the present study, we demonstrated that GCH utilizes metal-free GTP as the substrate for the enzyme reaction. Inhibition of the GCH activity by divalent cations such as Mg²⁺ and Zn²⁺ was due to a reduction in the concentration of metal-free GTP substrate by complex formation. We also showed that Zn²⁺ at a micromolar level was required for the enzyme activity by discriminating it from the inhibitory action of Zn²⁺. Our data are the first to show the requirement of Zn²⁺ for the enzyme activity of the wild-type enzyme.

Many nucleotide-hydrolyzing enzymes such as G proteins and kinases recognize Mg–GTP or Mg–ATP complex as their substrate. In contrast with these enzymes, GCH activity is dependent on the concentration of Mg-free GTP. Our data showed that the Mg–GTP complex and Mg²⁺ affected the enzyme activity very little at the concentrations tested here. The structure of the active centre for the *E. coli* GCH binding to dGTP also supports our findings, because Mg²⁺ assistance for binding to the GTP substrate was neither realized nor necessary [14].

However, there remains a possibility that the Mg–GTP complex at higher concentrations competitively antagonizes the Mg-free GTP substrate, because the enzyme activity was slightly decreased in the presence of 200 μ M MgCl₂ at > 15 μ M Mg-free GTP (Fig. 2D). When the concentration of total GTP was increased to 75, 100, 125 μ M in the presence of 200 μ M MgCl₂, the concentration of the Mg–GTP complex was calculated to be 53.1, 68.5, and 82.5 μ M, respectively; and that of Mg-free GTP to be 21.8,

31.4 and 42.4 μM , respectively (Fig. 2D). As the Mg-GTP complex at $< 30 \mu\text{M}$ had little effect on enzyme activity at 10 μM Mg-free GTP (Fig. 3), there is a possibility that $> 50 \mu\text{M}$ Mg-GTP complex can be a competitive inhibitor or a low-affinity substrate for the GCH enzyme.

Other divalent cations such as Mn^{2+} , Co^{2+} , and Zn^{2+} also inhibited enzyme activity when present at a molar excess over the GTP substrate by a reduction in the concentration of metal-free GTP substrate by complex formation. Our data suggest that the inhibition by various divalent cations of bacterial and mammalian GCH enzymes shown previously [16] would also be due to a reduction in the availability of the metal-free GTP substrate by formation of complexes with the divalent cations.

Because Mg^{2+} is relatively abundant in cells it is considered to be the main cation affecting the concentration of metal-free GTP substrate for GCH *in vivo* under physiological conditions. A change in the intracellular concentration or distribution of Mg^{2+} may thus affect the enzyme activity of GCH. Interestingly, 6-pyruvoyltetrahydropterin synthase, which is the second enzyme in the pathway for BH_4 biosynthesis and which acts on the *D-erythro-7, 8-dihydroneopterin* triphosphate produced by GCH, requires Mg^{2+} for its activity [25,26] in spite of the inhibitory effect of Mg^{2+} on GCH. Our data suggest that there may be an optimal range of Mg^{2+} concentration for BH_4 biosynthesis *in vivo*.

Our data showed that Zn^{2+} is essential for the enzyme activity, in agreement with a suggestion from a previous crystallographic study [12]. Zn^{2+} was proposed to be involved in the catalytic reaction [12]; this proposition is supported by the results of mutation analysis of the *E. coli* enzyme [14] and most recently by the observation that mutational replacement of residues predicted to form the Zn^{2+} binding centre caused catalytic inactivation and reduced the capacity of the *E. coli* enzyme to bind zinc [27]. Although an apo-enzyme for Zn^{2+} was not generated by incubating with Zn^{2+} chelating agents such as EDTA and TPEN, we succeeded in showing the stimulatory effect of Zn^{2+} on the enzyme activity by using a protein refolding assay. Because the intracellular concentration of Zn^{2+} is relatively low, it is reasonable that Zn^{2+} dose-dependent elevation of the enzyme activity occurred in the range of $\approx 0.1\text{--}10 \mu\text{M}$ during the refolding procedure (Fig. 5) and during the preincubation after refolding with EGTA (data not shown). These data suggest that intracellular Zn^{2+} concentrations would be high enough to bind to the GCH enzyme and low enough to avoid the decrease in the enzyme activity by reduction of the intracellular metal-free GTP substrate, which was previously estimated to be $\approx 150 \mu\text{M}$ [28].

Because the zinc concentration in the diluted sample was calculated to be $81 \pm 7 \text{ nM}$, the slight inhibition of enzyme activity detected after addition of 10 μM EGTA to the refolding solution (Fig. 5B) can be attributed to the chelating of Zn^{2+} (based on the Zn^{2+} dose-response curve). We also demonstrated that the addition of Zn^{2+} after refolding was effective for the sample refolded in the presence of EGTA. However, the apo-enzyme is probably unstable, because the addition of Zn^{2+} after refolding was less effective than that during refolding (Table 1). Auerbach *et al.* previously suggested that the absence of Zn^{2+} in the

active centre of the GCH enzyme caused enzymatic inactivation by disulfide formation between the cysteine residues [14] which normally form the Zn^{2+} binding site.

Zn^{2+} would appear to have important roles in nitric oxide production *in vivo*, because Zn^{2+} also binds to nitric oxide synthase (NOS) [29], which utilizes BH_4 and is coincided with GCH by cytokine-mediated stimulation. Zn^{2+} was reported to be required for the stability of NOS, but not for the catalytic reaction itself [30–32]. In contrast with NOS, our data demonstrate that Zn^{2+} is essential for the activity of the GCH enzyme. In addition to GCH, 6-pyruvoyltetrahydropterin synthase also binds Zn^{2+} [33]. Our present study emphasizes the importance of Zn^{2+} in nitric oxide production.

Ca^{2+} was not detectable in the enzyme solution containing $14.7 \pm 0.7 \mu\text{M}$ GCH protein subunit. The presence of Ca^{2+} at micromolar concentrations in the refolding procedure as well as in the GCH enzyme reaction mixture had no effect on enzyme activity (data not shown). Therefore, Ca^{2+} at a micromolar concentration was demonstrated to be neither essential nor stimulatory for enzyme activity, and the inhibitory effect of EGTA during refolding on enzyme activity seemed to be independent of Ca^{2+} chelation. However, there is a possibility that Ca^{2+} might affect enzyme activity under certain conditions that were not examined in this study, because Ca^{2+} was previously shown to bind to rat GCH protein at very low concentrations, e.g. at the nanomolar level [13], which could have been present in any of the assay solutions used in this study. In order to clarify the effect of Ca^{2+} on enzyme activity, we need to conduct further experiments.

Under pathological and physiological conditions, changes in the concentrations of various divalent cations including Mg^{2+} and Zn^{2+} *in vivo* may affect GCH enzymatic activity, thus resulting in changes in the BH_4 level. There are many diseases known to involve alterations in metal metabolism, such as acrodermatitis enteropathica, in which there is a severe zinc deficiency. We suggest that part of the symptoms of these diseases may be caused by altered levels of GCH activity and BH_4 content. Further investigation into the relationship between divalent cations and GCH enzyme activity *in vivo* should be conducted in the future.

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