β-Citryl-l-glutamate Is an Endogenous Iron Chelator That Occurs Naturally in the Developing Brain

Michiko Hamada-Kanazawa, Makiko Kouda, Akira Odani, Kaori Matsuyama, Kiyoka Kanazawa, Tatsuya Hasegawa, Masanori Narahara, and Masaharu Miyake*

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kobe-Gakuen University; 1–1–3 Minatojima, Chuo-ku, Kobe 650–8586, Japan; Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University; Kakuma-machi, Kanazawa 920–1192, Japan; and Department of Environmental Biochemistry, Yamanashi Institute of Environmental Sciences; 3597–1 Kenmarubu, Kamiyoshida, Fujyoshida, Yamanashi 403–0003, Japan. Received November 21, 2009; accepted February 13, 2010; published online February 18, 2010

The compound β-citryl-l-glutamate (β-CG) was initially isolated from developing brains, while it has also been found in high concentrations in testes and eyes. However, its functional roles are unclear. To evaluate its coordination with metal ions, we performed pH titration experiments. The stability constant, log βH+ for M,(β-CG)H, was calculated from pH titration data, which showed that β-CG forms relatively strong complexes with Fe(III), Cu(II), Fe(II) and Zn(II). β-CG was also found able to solubilize Fe more effectively from Fe(OH)2 than from Fe(OH)3. Therefore, we examined the effects of β-CG on Fe-dependent reactive oxygen species (ROS)-generating systems, as well as the potential ROS-scavenging activities of β-CG and metal ion-(β-CG) complexes. β-CG inhibited the Fe-dependent degradation of deoxyribose and Fe-dependent damage to DNA or plasmid DNA in a dose-dependent manner, whereas it had no effect on Cu-mediated DNA damage. In addition, thermodynamic data showed that β-CG in a physiological pH solution is an Fe(II) chelator rather than an Fe(III) chelator.

Taken together, these findings suggest that β-CG is an endogenous low molecular weight Fe chelator.

Key words β-citryl-l-glutamate; iron; chelation; developing brain

We previously isolated a compound containing glutamate and citrate residues from the brains of newborn rats, and identified it as β-citryl-l-glutamate (β-CG).1) This compound appears in high concentrations (300–600 μM) during the period characterized by growth and differentiation of neurons in developing rats, guinea pigs, and chicks, and then decreases with maturation.2–5) In another study, we reported that β-CG was present at a concentration of about 700 μM in the testes of adult rats, predominantly in germinal cells, which accumulate at the stage of spermatogenesis coinciding with the appearance of late spermatocytes and early spermatids. Moreover, β-CG levels were markedly decreased in the testes of rats with seminiferous tubule failure or experimental cryptorchidism.6) Recently, β-CG was also found in tissues from the retina (20–400 μM) and lens (5–600 μM) of bovine eyes, with preferential localization in the equator regions of lens, where epithelial cells terminally differentiate to form lens fibers during lens development.7) Together, these findings suggest that β-CG plays roles in regulating the differentiation of neuronal, germinal, and lens cells. However, its functional roles with those tissues remain unclear.

The tetra-carboxyl nature of β-CG, in which a β-carboxyl residue of citrate and amino-residue of glutamate are linked with an amide bond, indicates that it may coordinate with metal ions. Indeed, microorganisms have developed Fe(III)-chelating compounds known as siderophores, including the citrate siderophores, staphyloferrin A and B.8,9) Staphyloferrin A consists of 1 ornithine and 2 citrate residues, whereas staphyloferrin B contains 1 residue each of 2,3-diaminopropionate, citrate, ethylenediamine, and 2-ketoglutarate. Other carboxylate-type siderophores have also been isolated, including rhizoferrin,10) which consists of 2 citrate and 1 putrescine residues. Additionally, deferoxamine, a hydroxamate-type siderophore, has a high binding affinity for Fe(III), and has been used clinically in humans to remove excess Fe from blood and tissues.11) Mugineic acid was the first phytosiderophore shown to play a role in the uptake and transport of Fe in higher plants,12,13) and its structure has been determined to be that of a polycarboxylic acid.

We are interested in the structural similarities between β-CG and citrate siderophores, as well as mugineic acids. Our preliminary examination of the metal ion-coordinating activity of β-CG using cellulose TLC at neutral pH revealed the formation of a complex between β-CG and metal ions, such as Ca(II), Mg(II), Zn(II), Cu(II) and Fe(II) ion, but not Fe(III) ion. The stability constant calculated from pH titration data showed that β-CG forms relatively strong complexes with Fe(III), Cu(II), Fe(II), and Zn(II) ions. Therefore, we examined the effects of β-CG on Fe(II)-dependent reactive oxygen species (ROS)-generating systems, as well as the potential ROS-scavenging activities of metal ion-(β-CG) complexes in the present study. Our results showed that β-CG inhibits Fe(II)-H2O2-induced degradation of deoxyribose, Fe(II)-mediated hydroxyl-radical damage to plasmid DNA, and bleomycin-Fe(II)-dependent damage to DNA in dose-dependent manners. However, β-CG had no effect on Cu(II)-ascorbate mediated DNA damage. In addition, thermodynamic data showed that β-CG in a physiological pH solution is a Fe(II) chelator rather than a Fe(III) chelator. Taken together, our findings suggest that β-CG is an endogenous low molecular weight Fe chelator.

MATERIALS AND METHODS

Materials Superoxide dismutase and deferoxamine mesylate were purchased from Sigma. β-CG was synthesized as described previously.1) Deoxyribose, benzoate and 2-thiobarbituric acid (TBA) were obtained from Nacalai Tesque. All other reagents were purchased from commercial sources at the highest grade available.

* To whom correspondence should be addressed. e-mail: miyake@pharm.kobegakuin.ac.jp © 2010 Pharmaceutical Society of Japan
Preparation of Metal Ion-Chelator Complexes  Metal ion-complexes with chelators were prepared according to the method described by Darr et al. After stirring vigorously for 16 h at room temperature, residual metal ion oxides, including Fe(II)O, Fe(III)2O3, Mn(IV)O2, Ni(II)O, Cu(II)O, and Zn(II)O, were removed by centrifugation. Moreover, the supernatants were clarified by passage through a 0.22-μm filter unit (Millipore, Ultrafree-MC). Solutions were prepared as 2 mM stock solutions and stored at 4 °C until use.

pH Titrations pH titrations were carried out at 25±0.05 °C and I=0.1 M (KCl) in a nitrogen atmosphere, as previously reported. The pH values were determined using an Accumet model 15 pH meter (Fisher Scientific) equipped with a No. 39314 glass electrode and a No. 511105 double junction reference electrode (Beckman). The titrations were continued to basic pH for most samples, however, only to neutral pH for Fe(II) and Fe(III), because precipitation occurred at neutral pH. The stability constant β was defined by Eq. 1 and calculated from the titration data by least-square experiments using the SUPERQUAD program, as follows:

$$pM + qL + rH^{+} \rightarrow M_{p}(L)_{q}H_{r}$$

where M, L, and H refer to free metal, free ligand, and proton, respectively (charges are omitted for clarity). Ligand concentration was determined by the titration. Conversion of pH meter readings to hydrogen ion concentrations was based on the conversion factor $f=10^{-pH}[H^{+}]=0.82$ and $pK_{w}=13.88$.

Determination of Fe-Solubilizing Capacity For quantitative and reproducible evaluation of the Fe(III)-solubilizing capacity of β-CG, a procedure previously described by Taka-gi was used, with a slight modification. An aqueous suspension of Fe(OH)3 was prepared by neutralizing an FeCl3 solution with NaOH. To 200 μl of a sample solution, 10 μl of 0.5 M sodium acetate buffer (pH 5.6) was added, then 40 μl of a 40 mM Fe(OH)3 suspension was added to the buffered solution and the mixture was allowed to incubate at 60 °C for 1 h. Finally, the reaction mixture was centrifuged and 4 μl of 3 M H2SO4 was added to the supernatant. For colorimetric determination of dissolved iron, 10 μl of 8% hydroxylamine hydrosulfide was added to a 200 μl aliquot of the supernatant. Following incubation at 55 °C for 20 min, another 20 μl of sodium acetate buffer (2 M, pH 4.7) and 0.25% o-phenanthroline were added successively. Absorbance at 490 nm was then assayed. To determine the Fe(II)-solubilizing capacity, 40 mM Fe(OH)3 containing 0.5 mM ascorbic acid was used for partial reduction of Fe(OH)3 into Fe(OH)2. The following steps were the same as described for Fe(III).

Ascorbate Oxidation and Benzoate Hydroxylation Assay Ascorbate oxidation and benzoate hydroxylation assays were performed using standard methods. In the ascorbate oxidation experiments, ascorbic acid (0.125 mM) was incubated in phosphate buffer (12.5 mM, pH 7.4) in the presence of Fe(III) (37.5 μM), and the chelator (1—70 μM) at room temperature for 40 min. Absorbance at 265 nm was measured after 10- and 40-min incubation periods, and the decrease between those time points was calculated. The benzoate hydroxylation assay was based on the ability of hydroxyl radicals to hydroxylate benzoate to fluorescent products (dihydroxybenzoates). Benzoic acid (1.35 mM) was incubated at room temperature for 1 h in 13.5 mM phosphate buffer (pH 7.4) with 6.8 mM H2O2, an Fe chelator, and Fe(II)SO4 (40 μM). The reaction was started by the addition of Fe, and kept in the dark, then fluorescence was measured with excitation at 308 nm and emission at 410 nm.

Free Radical-Induced Lipid Peroxidation of Rat Brain Homogenate Lipid peroxidation in the brain homogenates prepared from Wistar rats was induced by an Fe(II)-ascorbate system, using a previously reported method. The reaction mixture contained a final volume of 0.74 ml, and included 0.3 mg of rat tissue protein, ascorbic acid (0.135 mM), chelators (1—80 μM), and 15 mM phosphate buffer, pH 7.0. Peroxidation was started by adding Fe(II)SO4 (8.6 μM). The tubes were incubated at 37 °C for 20 min followed by the addition of 1 ml of 1% (w/v) TBA and 1 ml of 2.8% (w/v) trichloroacetic acid (TCA). The tubes were heated at 100 °C for 10 min to develop the malondialdehyde (MDA)-TBA chromogen and then centrifuged at 4 °C for 10 min. The fluorescence in the supernatant was measured using a spectrofluorometric method, with excitation at 515 nm and emission at 553 nm.

Assay of Fe(II)-H2O2-Induced Damage to Deoxyribose Fe(II)-H2O2-induced damage to deoxyribose was determined essentially as described previously, with a slight modification. Briefly, deoxyribose (1.23 mM), phosphate buffer (15.4 mM, pH 7.4), H2O2 (77 μM), and the chelator were mixed, then the reaction was started by the addition of Fe(II)SO4 (46.2 μM). Samples were incubated in a final volume of 0.65 ml for 15 min at 37 °C. After incubation, 1 ml of 1% (w/v) TBA was added to each sample followed by 1 ml of 2.8% (w/v) TCA. The following steps were the same as described for the lipid peroxidation experiment.

Fe(II)- or Cu(II)-Induced DNA Degradation DNA degradation in the bleomycin-Fe(II)-DNA system and Cu(II)-ascorbate induced DNA degradation were determined according to methods described respectively, with a minor modification. For determination-DNA degradation in the bleomycin-Fe(II)-DNA experiment, the reaction mixture contained the following reagents, added in the order stated: calf thymus DNA (0.2 mg/ml), bleomycin sulphate (0.2 mg/ml), Fe(II)SO4 (120 μM), Tris–HCl buffer (10 mM, pH 7.4) and the chelators (1—100 μM). The reaction was started by adding Fe(II)SO4 or bleomycin. The tubes were incubated in a final volume of 0.5 ml at 37 °C for 1 h, followed by the addition of 1 ml of 1% (w/v) TBA and 1 ml of 2.8% (w/v) TCA. The tubes were heated at 100 °C for 10 min to develop the MDA-TBA chromogen, which was measured spectrometrically by excitation at 532 nm with emission at 553 nm.

As for DNA degradation in the Cu(II)-ascorbate-DNA experiment, calf thymus DNA (0.2 mg/ml) was incubated with CuCl2 (90 μM), ascorbic acid (0.36 mM), and chelators (1—80 μM) in 10 mM phosphate buffer (pH 7.4). Total reaction volumes of 0.55 ml were incubated at 37 °C for 2 h, followed by the addition of 1 ml of 1% (w/v) TBA and 1 ml of 2.8% (w/v) TCA. The following steps were the same as described for the bleomycin-Fe(II)-DNA experiment.

Measurement of DNA Integrity Using the Plasmid DNA in the Presence of Cu(II) or Fe(II) Previous studies have shown that some chelators can prevent Fe(II)-dependent...
hydroxyl radical-mediated strand breaks in plasmid DNA.

The effects of the chelators on Cu(II)-ascorbate- and Fe(II)-H₂O₂-induced plasmid DNA damage were examined as previously described using the plasmid pEGFP-N1 (BD Biosciences Clontech). For the Cu(II)-ascorbate experiment, the following reagents were added in the stated order: sterile phosphate buffered saline (PBS) (10 mM), chelators (10—100 μM), Cu(II)Cl₂ (90 μM), ascorbic acid (0.363 mM), then incubated in a final volume of 55 μL at room temperature for 30 min. In the Fe(II)-H₂O₂ experiment, the following reagents were added in the stated order: sterile PBS (10 μL), plasmid DNA (10 μg), chelators (10—100 μM), Fe(II)SO₄ (60 μM), and H₂O₂ (0.15 mM), then incubated in a final volume of 60 μL at room temperature for 30 min. These samples were loaded onto a 0.5% agarose gel containing 2 μg/mL ethidium bromide and gels was electrophoresed at 100 V for 30 min. After migration, the DNA was visualized using UV transillumination device. The DNA bands were quantified by scanning the gel pictures with NIH image.

Untreated plasmids appeared on gels as major supercoiled (SC) DNA bands. When the plasmid was treated with Fe(II) or Cu(II), the double-strand SC DNA was nicked one by one or Cu(II), the double-strand SC DNA was nicked one by one due to the steric requirement of the amide bond. The donor molecule. It was possible to build a molecular model showing such coordination for the citrate moiety, whereas it was impossible to build such a model for the glutamate moiety due to the steric requirement of the amide bond. The donor

### Table 1. Stability Constants (log βₚₜₜₜ) for M₃(β-CG),H₄, at 25 °C and I=0.1

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<th>Metal ions</th>
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<th>Citrate</th>
<th>Ref.</th>
<th>Glutamate</th>
<th>Ref.</th>
<th>Deferoxamine</th>
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<td>13.988</td>
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<td>16.27</td>
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<td>014</td>
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<td>25)</td>
<td>1.9</td>
<td>26)</td>
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<td>29)</td>
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a) [M₃(β-CG)]H₄[MP][L][H⁺].  b) Standard deviations (n=4—8).
change from the carboxylate $O^-$ of citrate to the amide $O$ of $\beta$-CG resulted in loss of negative charge and forced decrement of the stability constant.

At acidic-neutral pH values, the Fe(II), Fe(III), and Cu(II) systems revealed deprotonation species (Fig. 2A). Notably, the Fe(III) complex showed easy formation of the deprotonated species 11-1 and 11-2. The crystal structure of the Fe(III)(citrate) complex makes it possible to predict coordination of deprotonated alcohol $O$ in the Fe(III)($\beta$-CG) complex (Fig. 1). Thus, the unusual species found in the Fe(III)(citrate) complex makes it possible to predict coordination of deprotonated alcohol $O$ in the Fe(III)($\beta$-CG) complex.

Other cases of deprotonation of $pK_a$ 6.6 (Fe(II)) and 5.3 (Fe(III)) were attributed to deprotonation of the coordinated water molecule, and were greater than the values for the metal ions at 5.7 (Fe(II)) and 2.7 (Fe(III)) (Academic Software 2001). In addition, the deprotonation $pK_a$ value for the Cu(II)($\beta$-CG) complex (5.3) was lower than that for coordinated $H_2O$ deprotonation ($pK_a$ = 6.7), while the solution exhibited a deep blue color at neutral pH. These findings suggest deprotonation of the amide moiety and coordination of the deprotonated $N^-$ of the amide moiety in the Cu(II)($\beta$-CG) complex.

The species distribution curves presented in Fig. 2A show that Fe(II)($\beta$-CG) is the main species from pH 5 to 7 in the Fe(II)($\beta$-CG) system, while Fe(II)($\beta$-CG)(OH) predominates above pH 6.5. In the Fe(III)($\beta$-CG) system, Fe(III)($\beta$-CG)(OH) appears from pH 3 to 5, while Fe(III)($\beta$-CG)(OH)$_2$ is the main species from pH 4 to 7. In the Cu(II)($\beta$-CG) system, Cu(II)($\beta$-CG) is the main species from pH 4 to 5.5, while Cu(II)($\beta$-CG)(OH) appears from pH 4.5 and predominates above pH 6.

Citrate itself is known to be one of the siderophores in bacteria and plants. However, it is not formed by bacteria, though it can function as an exogenous siderophore in Escherichia coli, which possesses a transporter system specific for Fe(III)(citrate). Deferoxamine has a high binding affinity for Fe(III), and has been used clinically in humans to remove excess iron in the treatment of $\beta$-thalassemia. Log $\beta_{1,10}$ values for deferoxamine with Fe(II), Fe(III), Zn(II), and Cu(II) were obtained from previous reports and are also shown in Table 1. Furthermore, the distributions of the complex species of $\beta$-CG, citrate, and deferoxamine with Fe(III) and Fe(II) as a function of pH were calculated from our data as well as those shown in previous reports and are presented in Fig. 2B.

In the Fe(III)(chelator) system, Fe(III)(Cit) is presented at a concentration of approximately 80% total Fe(III) at pH 4. Moreover, the species decreases gradually to pH 6, while the speciation diagram is dominated by Fe(III)(Def) above pH 6. In the Fe(II)(chelator) system, Fe(II)(Cit) is the main species from pH 5 to 7.5. However, Fe(II)($\beta$-CG)(OH) appears from pH 6 and reaches a concentration of approximately 30% total Fe(II) at pH 7.5. In addition, Fe(II)($\beta$-CG) is presented at a concentration ranging from 10—15% total Fe(II) between pH 5 and pH 7, whereas Fe(II)(Def) appears for the first time above pH 7.6.

**Determination of Fe-Solubilizing Capacity** Due to the oxidation of Fe(II) ion to Fe(III) ion, as well as the poor aqueous solubility of Fe(III) ion in the presence of oxygen and at neutral pH, free Fe ions are not found in mammalian cells. Fe is sequestered as insoluble Fe hydroxides or in complexes with Fe binding proteins, such as transferrin and ferritin. Siderophores including deferoxamine and mugineic acids solubilize Fe(III) from Fe(III) hydroxide in the environment. Thus, the Fe(III) or Fe(II)-solubilizing capacities of $\beta$-CG, citrate, and glutamate from Fe(OH)$_3$ were determined in the presence or absence of 0.5 mM ascorbic acid (Fig. 3). $\beta$-CG was able to solubilize Fe more effectively from Fe(OH)$_2$ than from Fe(OH)$_3$, though a loss of negative charge decreased the Fe(II)-solubilizing activity. This is in contrast to the results with citrate, which solubilized Fe from both hydrated Fe oxides in a similar manner, and those with glutamate, which had only a weak effect. Both citrate and glutamate were slightly more effective at solubilizing Fe(OH)$_2$ than Fe(OH)$_3$.

**Effects of $\beta$-CG on Fe(II)-H$_2$O$_2$-Induced Damage to Deoxyribose** The addition of Fe(II) salts to a solution of deoxyribose in the presence of H$_2$O$_2$ caused rapid degradation of deoxyribose, forming a product that reacted with TBA to form a chromagen. As shown in Fig. 4, both $\beta$-CG and deferoxamine inhibited the degradation of deoxyribose in a dose-dependent manner. The inhibitory effects of deferoxamine observed here are in good agreement with previously reported results. In contrast to deferoxamine, ethylenediaminetetraacetic acid (EDTA) caused slight activation at a low concentration, while it had little effect at higher concentrations.

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**Fig. 1.** Possible Structures of the M($\beta$-CG)$_n$H$_r$ Complexes (M=Fe(III), Fe(II), Cu(II), Zn(II), and Mg(II))

Figures (110—11-2) show the $pK_a$ values for the M($\beta$-CG)$_n$H$_r$ complexes as presented in Table 1.
centrations. Findings regarding the effects of EDTA on the Fe-dependent hydroxyl radical-generating system differ, with some reporting stimulation and others no effect on inhibition. EDTA was used as a positive control, because it is not large enough to surround the Fe atom, though it is nominally hexadentate, and allows occupation of the sixth coordination site by H₂O or ascorbate. This results in redox cycling, which generates free radicals. Therefore, EDTA was ineffective in preventing damage, though it is capable of chelating Fe ion. Citrate showed lower inhibitory effects than b-CG, even at higher concentrations, while glutamate had no detectable effect.

Ascorbate Oxidation and Benzoate Hydroxylation

The effects of β-CG, deferoxamine, and EDTA on the reducibility of Fe(III) were determined by ascorbic acid (a superoxide anion measurement system). β-CG had no effect on Fe(III) at concentrations up to 60 μM, however the presence of deferoxamine imposed a slight block on the level of oxidation (data not shown). In contrast, EDTA treatment at 10 μM increased the value to about 5-fold greater than that of the control, which agreed well with a previous report.

In addition, β-CG and deferoxamine had scant effects on benzoate hydroxylation (a hydroxyl radical measurement system) (data not shown), whereas EDTA at 50 μM increased to 4-fold over that of the control, which agreed well with a previous report.

Fig. 2. Species Distribution as a Function of pH in the Ternary M(β-CG)H Complex, Where M, L, and H Refer to Free Metal, Free Ligand, and Proton, Respectively

(A) Species distribution as a function of pH in the ternary M(β-CG)H system under anaerobic conditions in an aqueous solution at 25 °C and I=0.1 (KCl). The percentages for each species were calculated from $p_{\text{K}_w}$ values, which were evaluated from pH titration data by least-square experiments using the SUPERQUAD program. The concentrations of Fe(II), Fe(III), Cu(II), and β-CG were each 1 mM. (B) Species distribution as a function of pH in the ternary Fe(β-CG, citrate, deferoxamine)H system at 25 °C and I=0.1 (KCl). The concentrations of Fe(II), Fe(III), β-CG, citrate, and deferoxamine were each 1 mM.

Fig. 3. Fe-Solubilizing Capacities of β-CG, Citrate and Glutamate

The Fe(III) and Fe(II)-solubilizing capacities of β-CG, citrate, and glutamate from Fe(OH)₃ were determined in the presence or absence of 0.5 mM ascorbic acid. Suspensions of 6.4 mM Fe(OH)₃ and 6.4 mM Fe(OH)₃ containing 0.5 mM ascorbic acid were incubated with β-CG, citrate, or glutamate for 1 h at 60 °C, then centrifuged at 12000 rpm. We used 8% hydroxylamine hydrochloride for reduction of dissolved iron in the supernatants. After incubation for 20 min at 55 °C, 0.25% o-phenanthroline was added to the solution and absorption was measured at 490 nm. Data are expressed as the mean±S.E.M. (bar). Asterisks indicate significant differences between the Fe(OH)₂ and Fe(OH)₃ samples ($^*$p<0.001 and **p<0.05 by Student’s t test; n=3—8).
Effect of β-CG on Free Radical-Induced Lipid Peroxidation of Rat Brain Homogenate  
Incubation of a rat brain homogenate with Fe(II)-ascorbate at pH 7.4 caused lipid peroxidation. β-CG had no effect, even at concentrations of up to 100 μM (data not shown), whereas both EDTA and deferoxamine inhibited lipid peroxidation in a dose-dependent manner at concentrations up to 20 μM, consistent with a previous report.16)

Effects of Chelators on Fe(II)-Induced Plasmid DNA Damage, and Bleomycin-Fe(II)-Dependent Degradation of DNA  
We subsequently examined the abilities of the chelators to prevent Fe(II)-mediated hydroxyl-radical damage to plasmid DNA (Fig. 5A). Untreated plasmid appeared on gels as a major supercoiled (SC) DNA band. When the plasmid was treated with Fe(II) and H₂O₂, SC DNA was mostly converted to linear (L) DNA, with a small amount of open circular (OC) DNA (Fig. 5Aa). β-CG, deferoxamine and EDTA showed dose-dependent protective effects toward SC DNA in the presence of Fe(II) (Fig. 5Ab). Although EDTA was redox active in the ascorbate oxidation and benzoate hydroxylation assays, this chelator was protective toward SC DNA, because of its ability to sequester Fe from the Fe-DNA complex.36) These findings agreed with previously well-characterized protective effects of EDTA against Fe-mediated DNA damage.19,36)

Neither β-CG nor deferoxamine inhibited bleomycin-Fe(II)-dependent damage to DNA when the reaction was initiated by adding FeSO₄, whereas EDTA had an inhibitory effect in a dose-dependent manner (Fig. 5Ba). When the reaction was initiated by adding bleomycin, both β-CG and deferoxamine showed slight stimulatory effects at low concentrations, while dose-dependent inhibition was seen at high concentrations (Fig. 5Bb). In contrast, 10 μM of EDTA strongly inhibited the damage to DNA and then showed constant inhibition. These results suggest that unlike EDTA, neither β-CG nor deferoxamine is able to remove iron from the Fe(II)-bleomycin complex, given that the DNA-damaging activity of bleomycin was previously shown to be dependent on the chelation of Fe(II) ions.

Effects of Chelators on Cu(II)-Induced Plasmid DNA Damage, and Bleomycin-Cu(II)-Ascorbate Induced Degradation of DNA  
Both DNA and the telomeric sequence are also susceptible to Cu(II)-mediated reactive oxygen species (ROS) damage.18) We examined the effects of β-CG, deferoxamine, and EDTA on Cu(II)-induced oxidative damage via Fe(II) and H₂O₂. The degradation of deoxyribose by 30 μM of Fe(II) and 50 μM of H₂O₂ in the presence of various chelators was determined using a TBA test. Absorption was measured using a spectro-fluorometric method, with excitation at 515 nm and emission at 553 nm. Data are expressed as the mean±S.E.M. (bar). *p<0.001 and **p<0.05 vs. control by Student’s t test (n=3—15).

Fig. 4. Degradation of Deoxyribose in the Presence of Fe(II) and H₂O₂  
The degradation of deoxyribose by 30 μM of Fe(II) and 50 μM of H₂O₂ in the presence of various chelators was determined using a TBA test. Absorption was measured using a spectro-fluorometric method, with excitation at 515 nm and emission at 553 nm. Data are expressed as the mean±S.E.M. (bar). *p<0.001 and **p<0.05 vs. control by Student’s t test (n=3—15).

Fig. 5. Collapse of Higher-Order Structure of Plasmid DNA via Fe(II) (A) and Bleomycin-Fe(II) Mediated Degradation of DNA (B)  
(A) Plasmid DNA was incubated with 60 μM of Fe(II) in the presence of 0.15 mM of H₂O₂ for 30 min at room temperature. After incubation, plasmid DNA was resolved on a 0.5% TAE agarose gel and visualized with ethidium bromide. The reaction was initiated by the addition of plasmid DNA to a reaction mixture. (a) Image of a typical agarose gel showing bands of supercoiled (SC), open circular (OC), and linear (L) forms with 50 μM of the chelators. Def: deferoxamine. (b) Densitometric analysis of the results in (a), showing the percentage of DNA in its SC form. SC DNA (% of control) was calculated, with the value without Fe(II) considered to be 100% and the value without a chelator as 0% (blank). (B) Damage to DNA was catalyzed by 0.1 mg/ml of bleomycin together with 60 μM of Fe(II) and determined using a TBA test. MDA-TBA chromogen levels were measured using a spectrometric method (A₅₃₂). (a) Reaction started by adding FeSO₄. (b) Reaction started by adding bleomycin. Data are expressed as the mean±S.E.M. (bar). *p<0.001 and **p<0.05 vs. control by Student’s t test (n=3—6).
plasmid DNA double-strand breaks in the presence of ascorbic acid (Fig. 6). Neither β-CG nor deferoxamine had an effect, even at concentrations up to 100 μM, whereas EDTA showed dose-dependent inhibition up to 30 μM (Fig. 6A). In addition, β-CG and deferoxamine had no effects on Cu(II)-ascorbate induced degradation of DNA, at concentrations up to 75 μM, whereas EDTA showed gradual inhibition up to 50 μM (Fig. 6B).

**Effects of Metal Ion-(β-CG) Complexes on Superoxide Quenching Activities** Superoxides generated in *vivo* are involved in the pathologies of many diseases, thus, superoxide dismutases (SODs) are thought to have potential uses as pharmaceutical agents. Many low-molecular SOD-mimicking compounds, mainly Cu and Mn complexes, have been synthesized as functional models of the native enzymes.\(^{37}\) A novel Fe-containing superoxide quenching mimic, Fe(II)-ethylenediamine derivative [Fe(II) TPEN], was reported by Nagano *et al.*\(^{38}\) Thus, we examined the ROS-scavenging activities of metal ion-(β-CG) complexes. Metal ion-complexes with β-CG, EDTA, and deferoxamine were separately prepared by mixing Fe(II)O\(_2\), Fe(IIIO\(_2\)), Mn(IV)O\(_2\), and Ni(II)O\(_2\) suspensions with specific chelators (see Materials and Methods). Fe-complexes with β-CG and EDTA showed no superoxide quenching activity at physiological pH levels (data not shown). Furthermore, deferoxamine, in Mn-complexes had strong superoxide quenching activities, whereas that in Fe(II), Fe(III), and Ni(II) complexes showed weak activities. The superoxide quenching activity of Mn(IV) (deferoxamine) was similar to that noted in a previous report.\(^{39}\)

**DISCUSSION**

β-CG has been found in high concentrations in developing brain, testis, lens, and retina tissues, each of which is also characterized by a high level of anaerobic metabolism and susceptible to oxidative damage, such as hypoxia-ischemia, hyperthermia, and UV insult. However, the functional roles of β-CG in those tissues remain unclear. Based on the structural similarities between β-CG and siderophores as well as munigicic acids, we speculated that β-CG has a metal ion-coordinating activity. The present pH titration data indicate that β-CG forms a complex with divalent metals, such as Fe and Cu. A change from one carboxylate of the citrate molecule to the amide of the β-CG molecule results in loss of the negative charge and a decrease in the stability constant. Thus, it can be speculated that the glutamate moiety or amide bond formed in the β-CG molecule leads to acquisition of different metal ion-coordinating properties that citrate alone do not have. Indeed, β-CG in a physiological pH solution was shown to be an Fe(II) chelator rather than an Fe(III) chelator in the present study (Figs. 2A, B). In addition, we found that β-CG was able to solubilize Fe more effectively from Fe(OH)\(_3\) than from Fe(OH)\(_2\). However, citrate was shown to solubilize Fe from both hydrated Fe oxides in a similar manner, while glutamate had only a weak effect (Fig. 3). Our preliminary examination of the metal ion-solubilizing activity of β-CG from solid water-insoluble Fe(IIIO) revealed that β-CG was able to solubilize Fe(II) more effectively from Fe(IIIO), after shaking for about 20 h at room temperature, whereas citrate and glutamate alone did not solubilize it (data not shown). β-CG also showed relatively more affinity to Fe(II) (Table 1 and Fig. 2), as compared with muvineic acids and siderophores, which are nearly specific for Fe(III).\(^{12,34}\) The bulk of chelatable Fe in a cytoplasmic pool is comprised of Fe(II) ion, because of the reductive environment of the cells.\(^{40}\) Moreover, β-CG fractions isolated by gel-filtration from newborn rat brains, testes, and a whole eye were also found to contain substantial amounts of Ca, Zn, Fe, and Cu (data not shown). β-CG inhibited the Fe(II)-dependent degradation of deoxyribose and Fe(II)-dependent damage to DNA or plasmid DNA in a dose-dependent manner, whereas it had no effect on Cu(II)-mediated DNA damage (Figs. 4—6). Together, these findings show that β-CG is an endogenous low molecular weight Fe chelator.

Fe is required by numerous essential proteins, such as heme-containing proteins, electron transport chain and microsomal electron transport proteins, and [Fe–S] enzymes, including ribonucleotide reductase, tyrosine hydroxylase, and aconitase. In living cells, Fe enters cells by transferrin (Tf) endocytosis and is released from Tf in the acidic endosome. Fe is reduced to Fe(II) by ferric reductase and transported to the cytosol, where Fe(II) becomes part of the labile or chelatable Fe pool (LIP) and is sensed by cytosolic Fe regulatory proteins (IRP1/e-aconitase and IRP2).\(^{41,42}\) Physiologically, the contents of LIP represent only 3—5% of total cellular Fe. However it is useful as a marker of not only total cell Fe con-
tent, but also for determination of the redox state of cells.\textsuperscript{41} LIP may represent Fe ions attached to phosphate esters (such as ATP, ADP, or GTP), or organic acids (such as citrate), and perhaps to the polar head groups of membrane lipids, or DNA. However, the exact chemical nature of this pool is not clear.\textsuperscript{41,42} The present findings demonstrated the Fe chelating activity of β-CG and inhibition of Fe-dependent ROS generation. In addition, our preliminary experiments revealed that the Fe(β-CG) complex plays a role as an Fe-carrier for mitochondrial aconitase, whereas no Fe complex with citrate, glutamate, or deferoxamine displayed such an effect (data not shown). Moreover, we previously reported that β-CG is mainly localized in the cytosol, with part in the mitochondria,\textsuperscript{5} while the β-CG-specific hydrolysing enzyme was found largely in the mitochondria and microsomal fraction of the testis.\textsuperscript{43,44} Taken together, we speculate that β-CG may contribute to the regulation of redox state as a ligand in LIP, while the Fe(β-CG) complex transfers Fe to aconitase in mitochondria.

During development of the vertebrate nervous system, up to 50\% or more of neurons normally die soon after they form synaptic connections with their target cells.\textsuperscript{45} Under normal physiological conditions, cell death with apoptotic characteristics helps to remove excess or unwanted cells. It has been suggested that developing brain tissues retain part of this developmental cell death program, which may be activated following exposure to hypoxia-ischemia, irradiation, or excitotoxic substances such as glutamate.\textsuperscript{46} Indeed, many of the key elements of apoptosis, such as caspase-3, have been demonstrated to be strongly up-regulated in developing brains.\textsuperscript{47} Neural cells require constant and timely access to Fe, especially during times of peak energy demand, such as mitochondriogenesis, synaptogenesis, and myelination. Indeed both Fe uptake and Fe concentration in the brain are highest during postnatal development at a time period that coincides with peaks in brain growth and myelinogenesis.\textsuperscript{48–50} However, free Fe participates in reactions that produce toxic by-products, the most well known of which is the Fenton reaction, in which reduced metal species, such as Fe(II), react with H$_2$O$_2$ to form hydroxyl radical and OH$^-$ and promote oxidative damage. In previous studies, β-CG was found in high concentrations during the period characterized by growth and differentiation of neurons, and with higher anaerobic metabolism in developing rats, while it decreased with maturation and was found at lower levels in adult rat brains.\textsuperscript{3,4} Oxidative stress is an early feature in the process of brain injury after ischemia in both adult and developing brains. However, the developing brain is more susceptible to oxidative stress and more prone to activate apoptotic mechanisms after injury. In conclusion, cellular Fe concentration must be stringently regulated in the developing brain, because of its poorly developed scavenging systems in spite of high Fe-mediated oxidative damage. β-CG or the resulting Fe(β-CG) complex may contribute to the regulation of redox state in the cytosol or mitochondria as an endogenous Fe chelator.

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