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Report

γ-Glutamylcysteine, a Glutathione Precursor, Exhibits Higher Thiol Reactivity for Complex Formation with Iron (III) Ions Compared to Glutathione

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Glutathione (GSH), the most abundant intracellular thiol compound, protects various cells from metal toxicities by forming complexes with metal ions through the thiol group. γ-Glutamylcysteine (γ-EC), a glutathione precursor, is anticipated to be a functional thiol compound. However, unlike GSH, the characteristics of γ-EC in metal complex formation are largely unclear. In this study, we analyzed the ability of γ-EC to form complexes with various metal ions. 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) assays demonstrated that the reaction ratios between DTNB and γ-EC and GSH were slightly reduced by adding light metal ions, such as K^+, Mg^{2+}, and Al^{3+}. These results indicated that γ-EC and GSH exhibit low thiol reactivity and weak complex formation with these ions. In contrast, the reaction ratio was reduced in a concentration-dependent manner by the addition of heavy metal ions, such as Ag^+, Cu^{2+}, Zn^{2+}, and Fe^{3+}. Specifically, the reaction ratio in the γ-EC-treated group was significantly reduced by the addition of Fe^{3+} compared to that in the GSH-treated group. These data indicate that, while γ-EC as well as GSH form the complexes with Ag^+, Cu^{2+}, and Zn^{2+}, γ-EC has a stronger interaction with Fe^{3+} than GSH. In the proposed complex model based on the hard and soft acids and bases (HSAB) principle, GSH theoretically forms unstable nine-membered rings with Fe^{3+}, whereas γ-EC can form more stable six-membered rings, resulting in a strong interaction between γ-EC and Fe^{3+}.

Key words γ-glutamylcysteine, glutathione, thiolate complex, heavy metal ion, 5,5'-dithiobis (2-nitrobenzoic acid)

INTRODUCTION

Exposures of organisms to metals, such as cadmium and copper induce toxic effects, including oxidative stress and inflammatory responses.[1)] As a defense response against the toxicities, thiol compounds, such as glutathione (GSH) and metallothionein protect various cells by forming complexes with metal ions through thiol groups.[2)] Therefore, to elucidate the defense response mechanism against metal exposure, it is crucial to understand the characteristics and structures of the complex formation between thiol compounds and metal ions.

Because GSH is the most abundant intracellular thiol compound, it plays a crucial role in intracellular protection against metal exposure and is used as a drug against metal poisoning. GSH strongly forms complexes with heavy metals, such as mercury and copper, with proposed structures for these complexes. In contrast, GSH forms weaker complexes with light metals and several heavy metals, such as magnesium and iron.[2,3)] Consequently, GSH cannot form strong complexes with all metals. Therefore, it is crucial to clarify the characteristics of the complex formation of other thiol compounds with metals.

γ-Glutamylcysteine (γ-EC) is the precursor of GSH in its biosynthetic pathway (Fig. 1A) and also a thiol compound.[4)] However, its function has rarely been analyzed. γ-EC has likely been overlooked in research because — (1) γ-EC is only a precursor of GSH, which plays a major defense function, and (2) the *in vivo* production of γ-EC is a rate-determining step in the biosynthesis of GSH. Notably, numerous angiotensin-converting enzyme (ACE) inhibitors have thiol groups; therefore, we discovered that γ-EC has an ACE-inhibitory function.[5)] These findings indicate that the thiol group of γ-EC is functional. Therefore, γ-EC may form complexes with metal ions, although its characteristics in the metal complex formation are largely unclear.

In this study, we analyzed the ability of γ-EC to form complexes with various metal ions, including light and heavy metals, *in vitro* by assessing the thiol reactivity, and subsequently compared it to the GSH activity.

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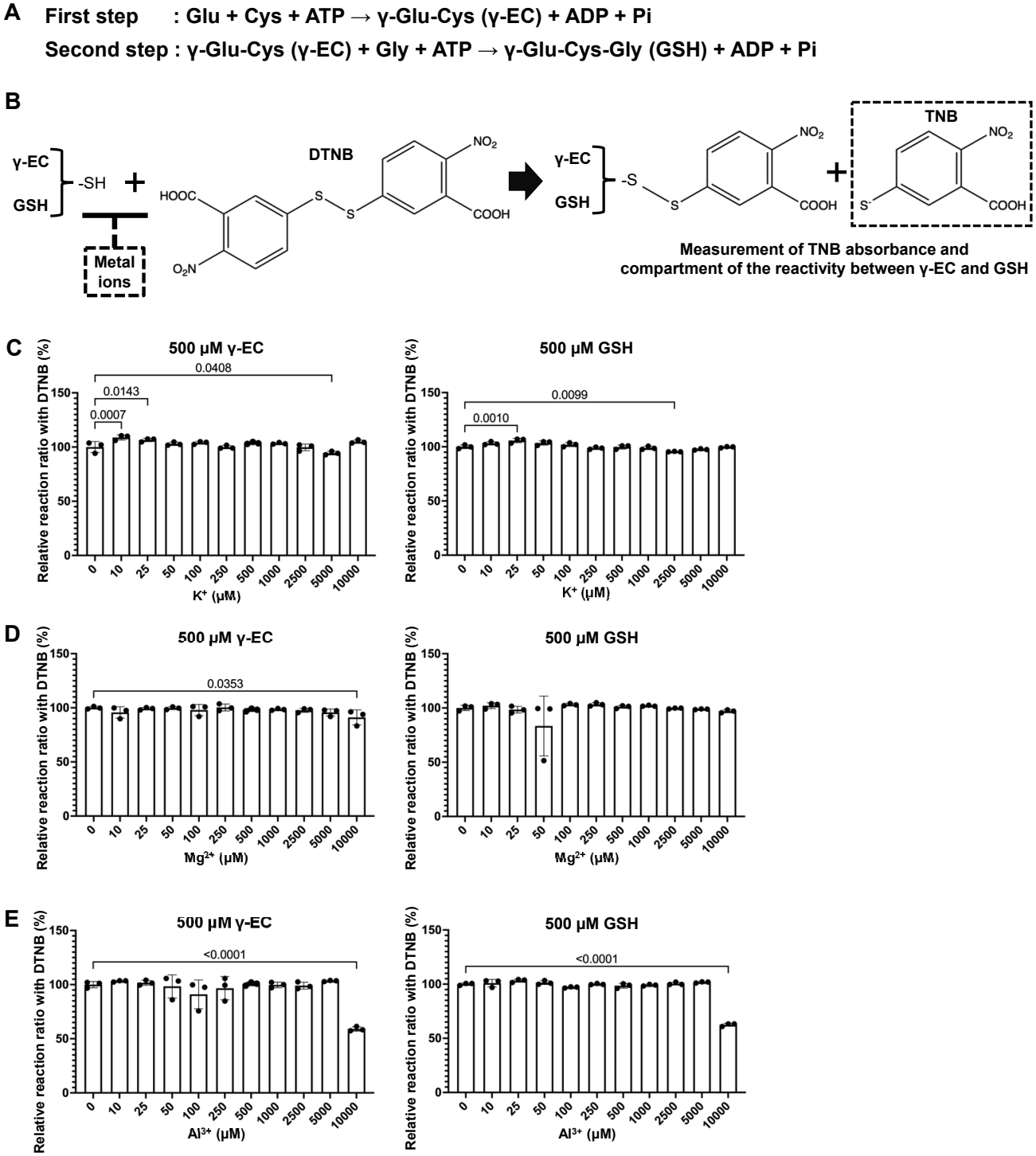

Fig. 1. The Thiol Reactivities of γ-Glutamylcysteine (γ-EC) and Glutathione (GSH) with Light Metal Ions

(**A**) GSH biosynthetic involves a two-step reaction. (**B**) An outline of the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) assay. (**C**-**E**) The relative reaction ratio is quantified using the 0 μM of the light metal ion-treated group as a control. The data of γ-EC-treated group (left panel) and GSH-treated group (right panel) with K^+, Mg^{2+}, and Al^{3+} are illustrated in (**C**), (**D**), and (**E**), respectively. Data are presented as the mean ± standard deviation (SD) (n = 3). P-values are analyzed using one-way ANOVA following Dunnett's test.

MATERIALS AND METHODS

Reagents γ-EC and GSH were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Dojindo (Kumamoto, Japan). Potassium chloride, magnesium sulfate heptahydrate, silver (I) sulfate, and iron (III) chloride hexahydrate were purchased from FUJIFILM Wako Chemicals (Osaka, Japan). Aluminum (III) chloride hexahydrate, copper (II) sulfate pentahydrate, and zinc sulfate heptahydrate were purchased from Nacalai Tesque (Kyoto, Japan).

DTNB Assay DTNB assay was performed following the protocol. Briefly, 160 μL of the mixture containing each metal ion and γ-EC or GSH was added to each well of 96-well plates, followed by 40 μL of 250 mM Tris buffer (pH 8.0). Subsequently, 2 μL of 10 mM DTNB diluted in 1 M Tris buffer (pH 7.0) was added and mixed thoroughly. After a 15-minute incubation at room temperature, the absorbance of 5-mercapto-2-nitro benzoic acid (TNB) was quantified at 412 nm using a microplate reader (Synergy-H1; BioTek, USA).

Statistical Analysis Statistical analysis was performed using GraphPad Prism 10, with the significance set at $P < 0.05$. P-values are indicated in each figure. The p-values below 0.0001 are indicated as $P < 0.0001$.

RESULTS AND DISCUSSION

Thiol Reactivity Analysis of γ-EC with Light Metal Ions in the Complex Formation To analyze the complex formation of γ-EC with metal ions, we initially assessed the thiol reactivities of γ-EC and GSH with light metal ions (K^+, Mg^{2+}, and Al^{3+}) using the DTNB assay. In this assay, the absorbance of TNB produced by the DTNB-thiol compound reaction was measured. Therefore, the complex formation between metal ions and γ-EC or GSH reduces the absorbance of TNB (Fig. 1B). Figure 1C-E demonstrated that the reaction ratio between DTNB and γ-EC or GSH was slightly reduced by the addition of K^+, Mg^{2+}, and Al^{3+} across various concentrations. Although statistically significant reductions were observed at certain concentrations, the reaction rates did not reduce to below 50%. These results indicated that the thiol reactivities of γ-EC and GSH with light metal ions were low during the complex formation.

Because of their low density, low electronegativity, and fewer electrons, thiol complexes with light metals are generally unstable compared to those with heavy metals.[2] This study demonstrated that the reaction ratio between DTNB and γ-EC or GSH was not reduced to < 50%, even with the addition of a 20-fold higher concentration of light metals, indicating that thiol complexes between light metals and GSH or γ-EC are unstable. Additionally, the complex structures between GSH and light metals have been previously reported.[6] Therefore, γ-EC and GSH can form weak complexes with light metal ions through the thiol group.

Thiol Reactivity Analysis of γ-EC with Heavy Metal Ions in the Complex Formation Subsequently, we assessed the thiol reactivities of γ-EC and GSH with heavy metal ions (Ag^+, Cu^{2+}, Zn^{2+}, and Fe^{3+}) during the complex formation. The DTNB assays demonstrated that the reaction ratio between DTNB and γ-EC or GSH was similarly reduced in a concentration-dependent manner by adding Ag^+, Cu^{2+}, and Zn^{2+} at various concentrations (Fig. 2A-C). In contrast, the reaction ratio between DTNB and γ-EC was significantly reduced in a concentration-dependent manner by adding Fe^{3+} at various concentrations compared to that between DTNB and GSH (Fig. 2D). Because the reaction ratio was reduced to over 50% in any group, the IC_{50} value of each heavy metal ion was calculated and compared with that of the γ-EC- and GSH-treated groups. There was no significant differences in the IC_{50} values of Ag^+, Cu^{2+}, and Zn^{2+}. In contrast, the IC_{50} value of Fe^{3+} was significantly lower in the γ-EC-treated group than that in the GSH-treated group (Fig. 2E). Therefore, the thiol reactivities of γ-EC with Ag^+, Cu^{2+}, and Zn^{2+} were similar to those of GSH, whereas γ-EC exhibited a higher reactivity with Fe^{3+} than that of GSH. These data indicate that compared to GSH, γ-EC formed a stronger complex with Fe^{3+} among the tested heavy metal ions. In the future, structural and *in vivo* analyses are required to clarify the role of γ-EC during iron exposure and make it a potential drug for iron poisoning.

The hard and soft acids and bases (HSAB) principle is defined by Lewis acids and bases, which accept and donate electrons, respectively.[7] According to the HSAB principle, soft, borderline, and hard acids bind with soft, borderline, and hard bases, respectively. Ag^+, Cu^{2+}/Zn^{2+}, and Fe^{3+} are classified as soft, borderline, and hard acids, respectively. Additionally, the thiol and amino groups derived from the cysteine residues of γ-EC and GSH are classified as soft and borderline bases (Fig. 3A). Therefore, the Ag^+-GSH complex model, in which Ag^+ as soft acid bind with GSH containing thiol group as soft base, are reasonable. Moreover, the Cu^{2+}/Zn^{2+}-GSH complex model, in which Cu^{2+}/Zn^{2+} as borderline acid bind with GSH containing thiol group as soft base and amino group as borderline base, are reasonable, as previously reported [2,3,8] (Fig. 3B). Furthermore, in Ag^+- and Cu^{2+}/Zn^{2+}-treated groups, there were no significant differences between the thiol reactivities of γ-EC and GSH. Therefore, the Ag^+-γ-EC and Cu^{2+}/Zn^{2+}-γ-EC complex models were predicted to be similar to the Ag^+-GSH and Cu^{2+}/Zn^{2+}-GSH complex models, respectively (Fig. 3B).

In contrast to Ag^+, Cu^{2+}, and Zn^{2+}, Fe^{3+}-γ-EC and Fe^{3+}-GSH complex models have not yet been proposed. According to the HSAB principle, a hard base is required to bind with Fe^{3+} as a hard acid. Therefore, we focused on the carboxyl group as a hard base and proposed Fe^{3+}-γ-EC and Fe^{3+}-GSH complex models (Fig. 3B). In the Fe^{3+}-γ-EC complex model, Fe^{3+} binds to the thiol and carboxyl groups derived from the cysteine residue of γ-EC, forming a six-membered ring (Fig. 3C, left panel). In the Fe^{3+}-GSH complex model, Fe^{3+} binds to the thiol and carboxyl groups derived from the cysteine and glycine residues of GSH, respectively, thereby forming a nine-membered ring (Fig. 3C, right panel). The formation of a six-membered ring is generally more stable than that of a nine-membered ring.[9] Therefore, the thiol reactivity of γ-EC with Fe^{3+} may be higher than that of GSH.

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Conflict of interest The authors declare no conflict of interest.

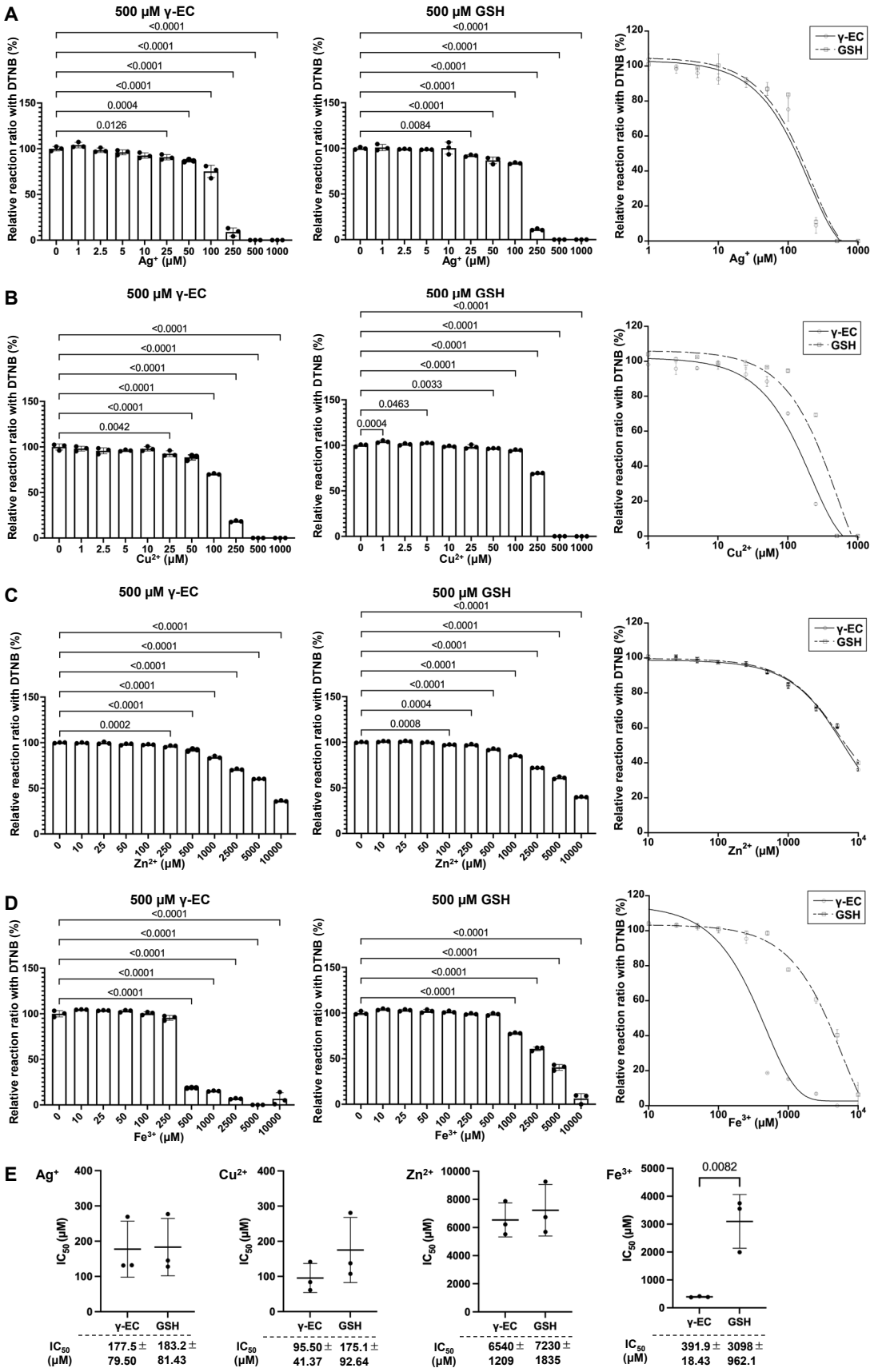

Fig. 2. The Thiol Reactivities of γ-Glutamylcysteine (γ-EC) and Glutathione (GSH) with Heavy Metal Ions

(**A**-**D**) The relative reaction ratio is quantified using the 0 μM of heavy metal ion-treated group as a control. The data of the γ-EC-treated group (left panel) and GSH-treated group (right panel) with Ag^{+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} are illustrated in (**A**), (**B**), (**C**), and (**D**), respectively. Curve-fitting graphs are drawn using the KaleidaGraph 5. Data are presented as the mean ± standard deviation (SD) (n = 3). P-values are analyzed using one-way ANOVA following Dunnett's test. (**E**) The IC_{50} values of Ag^{+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} in the γ-EC- and GSH-treated groups are calculated using the KaleidaGraph 5. Data are presented as the mean ± SD (n = 3, experiments). P-values are analyzed using Student's t-test.

A

	Soft	Borderline	Hard
Acid	Ag^+	Cu^{2+}/Zn^{2+}	Fe^{3+}
Base	-SH (Cys in γ-EC and GSH)	$-NH_2$ (Cys in γ-EC and GSH)	-COOH (Cys in γ-EC and Gly in GSH)
Another bond	-	-SH (Cys in γ-EC and GSH)	-SH (Cys in γ-EC and GSH)

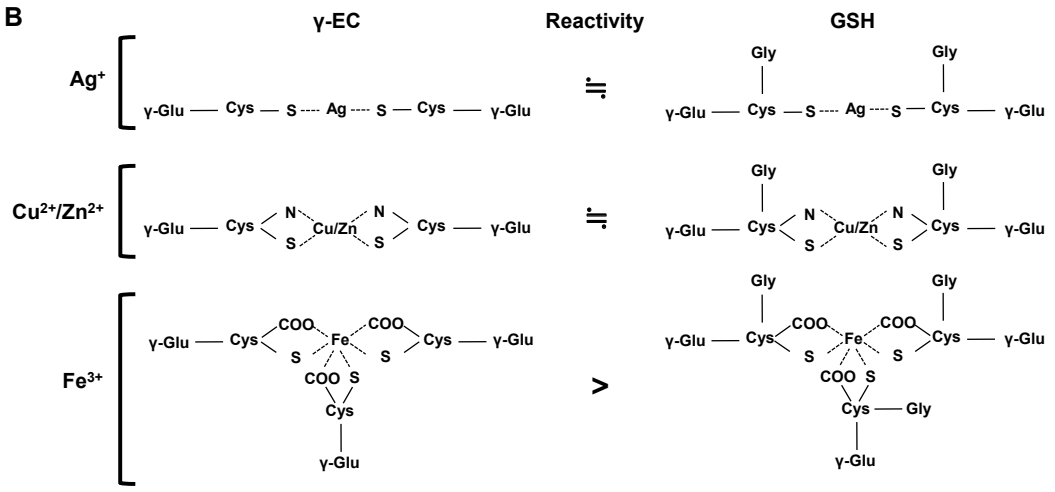

Fig. 3. Proposed Complex Models of γ-Glutamylcysteine (γ-EC) and Glutathione (GSH) with Heavy Metal Ions

(A) The interaction with the metal ion in each complex model is summarized based on the hard and soft acids and bases (HSAB) principle. **(B)** An outline of the proposed complex models between γ-EC or GSH and Ag^+, Cu^{2+}/Zn^{2+}, or Fe^{3+}. **(C)** In the Fe^{3+}-γ-EC/GSH complex model, the interaction around Fe^{3+} is illustrated in atomic detail.

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