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Important Role of the 3-Mercaptopropionamide Moiety in Glutathione: Promoting Effect on Decomposition of the Adduct of Glutathione with the Oxoammonium Ion of TEMPO

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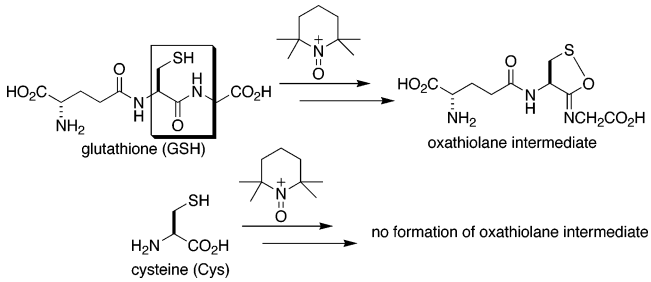

Cyclic voltammetry of TEMPO in aqueous 0.1 M NaOH in the presence of glutathione (GSH) or cysteine (Cys) indicated the following points: (i) Both of the thiols rapidly formed adducts **3** with oxoammonium ion **1** anodically generated from TEMPO. (ii) **3** generated from GSH entered a succeeding reaction that generated *N*-oxide anion $\mathbf{2}^-$ (the reduced TEMPO). (iii) **3** produced from Cys remained intact over the time scale of voltammetry. A structural feature of GSH was considered to contribute to the observed behavior of this tripeptide. Possible structural features were evaluated by screening various thiols on the basis of whether they provided GSH-like voltammetric results. The 3-mercaptopropionamide group with an amide hydrogen in GSH was determined to be responsible for the observed difference between GSH and Cys. The likely function is to transform **3** from GSH into a 5-imino-1,2-oxathiolane intermediate, thereby releasing $\mathbf{2}^-$. Product analysis for reactions of model thiols representing GSH and Cys with **1** provided support for this argument and suggested that the reaction of GSH or Cys with **1** would produce the corresponding disulfides, regardless of whether a five-membered ring intermediate was formed. The proposed function of the 3-mercaptopropionamide moiety of GSH may provide useful insight for the molecular design of exogenous thiol compounds as novel drugs for the treatment of GSH-depletion-related disorders.

Introduction

The tripeptide L-γ-glutamyl-L-cysteinylglycine (glutathione or GSH) exists in mammalian cells at the millimolar level. Although GSH is also present in its oxidized form, glutathione disulfide (GSSG), the intracellular ratio of GSH to GSSG is more than 100 in normal cells.[1] Thus, GSH acts as the most prevalent intracellular thiol, exhibiting both strong nucleophilic and strong reducing capabilities.[2] Therefore, GSH functions not only as a scavenger of reactive oxygen species (ROS) and reactive nitrogen species (RNS) but also as a redox buffer to maintain thiol−disulfide redox balances in intracellular proteins.[3] Recently, these functions have attracted much attention from the field of molecular biology. This is primarily because reactions with ROS or RNS transform GSH into its activated form, which is likely to induce S-glutathiolation between GSH and a thiol-containing protein (Scheme 1). S-glutathiolation is thought to alter the functional conformations of proteins, thereby leading to the gene expression for cellular resistance

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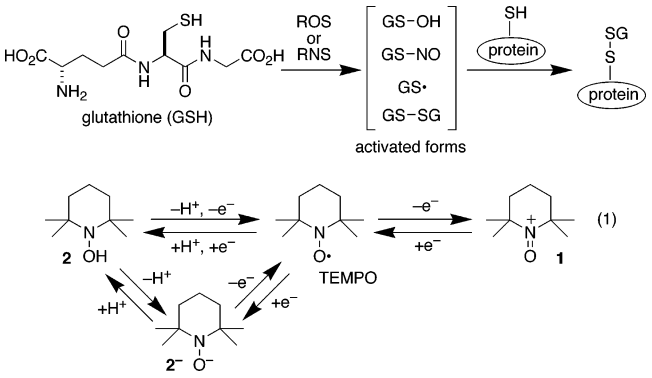
SCHEME 1. A Proposed Mechanism for S-Glutathiolation Induced by ROS and RNS



toward oxidative stress and the modulation of signal-transduction pathways such as tyrosine phosphorylation, regulation of transcription, proteolytic processes, ubiquitination, and degradation of proteins.[3] When the intracellular concentration of GSH falls below a critical level, the S-glutathiolation-dependent and other functions of GSH are partially lost. Further, GSH deficiency has been demonstrated to have a correlation with pathological conditions such as cancer, neurodegenerative disorders, HIV, and aging.[4] These observations have prompted research to elucidate a molecular link of GSH with GSH-dependent biological and pathological processes. However, no studies have been done to address why the thiol functionality of GSH has been naturally selected to perform crucial in vivo functions. GSH is thought to contain structural features that preferentially allow this thiol to maintain cellular homeostasis. The only available information on the structural features of GSH is that it contains an exceptional γ-peptide linkage between the glutamyl and cysteinyl moieties that aids GSH in resisting intracellular aminopeptidases.[3d] Understanding the structural chemistry of GSH in connection with its chemical reactivity will help provide a criterion for the molecular design of novel exogenous thiol compounds with GSH-like biological acitivity. This information can then be used for the treatment of GSH-depletion-related disorders.

A previous investigation to develop an electrochemical detection−HPLC system with the recourse of TEMPO as an electrochemical mediator for anodically inactive compounds (such as alcohols and sugars)[5] initiated the application of this system toward the analysis of biological thiols. To date, alkanethiols have been converted into their corresponding disulfides by anodic oxidation with TEMPO as an electrochemical mediator.[6] However, no voltammetric behavior of TEMPO in the presence of thiols, such as GSH and Cys, in aqueous media has been examined. In an effort to determine whether these thiols can be detected using an electrochemical response due to the catalytic oxidation of the thiols by a redox coupling

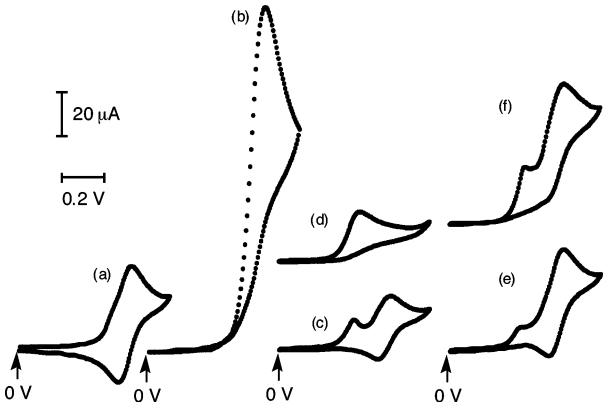
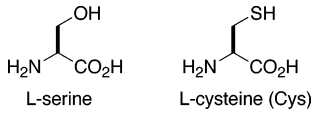


FIGURE 1. Cyclic voltammograms of (a) 1.0 mM TEMPO, and 1.0 mM TEMPO in the presence of (b) 1.0 mM serine, (c, d) 0.3 and 1.0 mM Cys, and (e, f) 0.3 and 1.0 mM GSH, respectively, in 0.1 M aqueous NaOH.

CHART 1



of TEMPO and its oxoammonium ion **1** (eq 1), cyclic voltammetry for aqueous solutions of TEMPO in either the absence or the presence of GSH or Cys was performed. When aqueous 0.1 M NaOH was used as the medium, GSH and Cys were determined to exhibit uniquely different effects with respect to the voltammetric response of TEMPO. The results implied that the adduct of GSH and **1** smoothly decomposed, thereby generating *N*-oxide anion $\mathbf{2}^-$ (eq 1) (the reduced TEMPO). In contrast, the reaction of Cys with **1** formed a similar adduct, which remained intact during the time scale of voltammetry. This interesting observation was investigated more thoroughly because the difference in the chemical structures between GSH and Cys was thought to be responsible for the voltammetric results. In this paper we describe an important function of the 3-mercaptopropionamide group, located on the cysteinylglycine moiety of GSH, in promoting the adduct of GSH and **1** to decompose almost certainly into $\mathbf{2}^-$ and GSSG. The proposed mechanism provides a reasonable explanation for the recent report that *N*-acetylcysteinamide scavenges peroxides more effectively than *N*-acetylcysteine itself.[7]

Results and Discussion

Figure 1 compares cyclic voltammograms of TEMPO (1.0 mM) in aqueous 0.1 M NaOH either in the absence or in the presence of L-serine, Cys (Chart 1), or GSH. TEMPO itself exhibited a redox wave with anodic and cathodic peaks at 530 and 460 mV vs SCE, respectively (Figure 1a). The addition of serine brought about an increase of the anodic peak and the disappearance of the cathodic peak on the redox wave of TEMPO (Figure 1b). These voltammetric results clearly indicate that serine undergoes catalytic oxidation by the redox cycle of TEMPO. This finding is in good agreement with known

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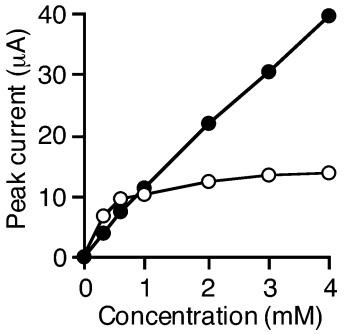


FIGURE 2. Changes in the peak current for the wave at 350 mV in the voltammograms of 1.0 mM TEMPO plotted against the concentration of (closed circles) GSH or (open circles) Cys.

results for primary and secondary alcohols.[8] The thiol counterpart of serine, Cys, produced a different effect on the voltammetric response of TEMPO. This conflicted with the expectation that Cys could be oxidized catalytically by the redox cycle of TEMPO. Cys itself exhibited no voltammetric waves between 0 and 0.7 V. When 1.0 mM TEMPO in the presence of 0.3 mM Cys was conducted, a new anodic peak emerged at 350 mV, with a contracted redox wave (Figure 1c). The new peak became larger and the redox wave for TEMPO disappeared when the concentration of Cys was increased to 1.0 mM (Figure 1d). These results suggested that Cys entered a stoichiometric reaction with TEMPO or its oxidized form **1**. As with Cys, GSH yielded no voltammetric waves in the examined potential window and did not interfere with observing the 350 mV anodic peak. However, the anodic wave of TEMPO always remained fairly intact, regardless of the concentration of GSH added to the TEMPO solution (Figure 1e,f). In contrast to Cys, GSH likely entered a catalytic process with a redox cycle of TEMPO. To distinguish the electrode processes of TEMPO in the presence of these thiols, a relationship between the concentration of added Cys or GSH and the peak current of the new anodic wave at 350 mV in the voltammetry of TEMPO was established (Figure 2). When the concentration of Cys was greater than or equal to TEMPO, the peak height became almost constant. This observation was in contrast to that for GSH. The anodic peak at 350 mV became larger as the concentration of GSH increased, and continued to increase even when GSH was added at higher concentrations than TEMPO.

The following two pathways could be envisioned for the resulting peak at 350 mV, which was observed in the presence of the thiols GSH and Cys (Scheme 2). (Route 1) A chemical reaction followed by an electrode reaction: Both Cys and GSH are likely to exist as the corresponding thiolate anion in aqueous 0.1 M NaOH. These thiolate anions would be oxidized by TEMPO, yielding the corresponding thiyl radical and the reduced TEMPO $\mathbf{2}^-$ (eq 1), and anodic oxidation of either product might be responsible for the peak at 350 mV. (Route 2) An electrode reaction followed by a fast chemical reaction: The oxoammonium **1** (eq 1), generated through anodic oxidation of TEMPO, would be subjected to a markedly fast reaction with the thiolate anion of Cys or GSH,

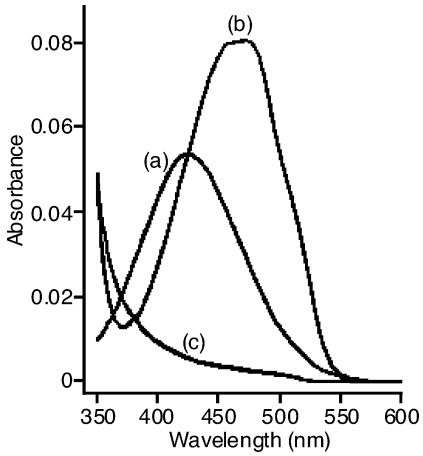
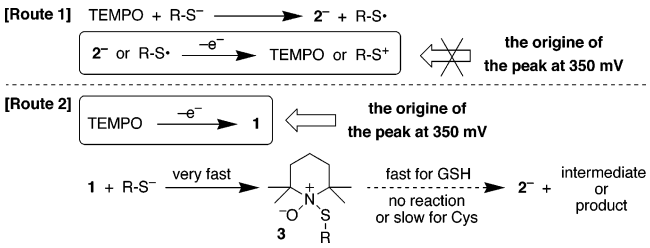


FIGURE 3. Absorption spectra observed for CH_3CN-H_2O (5:95) solutions of (a) 4 mM TEMPO, and 4 mM **1** (b) before and (c) immediately after addition of 8 mM GSH in aqueous 24 mM NaOH.

SCHEME 2. Qualitative Description for the Observed Voltammetric Results of TEMPO in the Presence of Cys or GSH



leading to a shift of the anodic peak of TEMPO to the negative direction.[9] In an effort to examine which route causes the thiol-induced voltammetric peak at 350 mV, ESR spectra of TEMPO and absorption spectra of **1** as well as TEMPO were measured either in the absence or in the presence of Cys or GSH. No changes in the ESR signal observed for 10 μM TEMPO in aqueous 0.1 M NaOH were induced upon addition of 40 μM Cys or GSH (data not shown). TEMPO itself exhibited a well-defined peak at 424 nm (Figure 3a). Essentially the same spectrum was observed for a mixture of TEMPO and the thiolate anion of Cys or GSH (data not shown). These results negated the possibility of a direct chemical reaction between TEMPO and the thiolate anions of Cys and GSH, that is, route 1. In the spectra of **1** electrochemically generated from TEMPO, a poorly defined peak around 464 nm was observed (Figure 3b). This peak due to **1** disappeared totally and immediately after addition of Cys or GSH in aqueous NaOH (Figure 3c). Similar phenomena were recognized even upon reaction of **1** with Cys or GSH in the absence of NaOH (data not shown). It is worth commenting that no peak due to TEMPO was observed after reaction of **1** with Cys or GSH. These observations suggested that Cys or GSH rapidly enters reaction with the oxoammonium **1**, which accompanies no regeneration of TEMPO. Thus, route 2 is thought to be responsible for the 350 mV peak; the anodic oxidation

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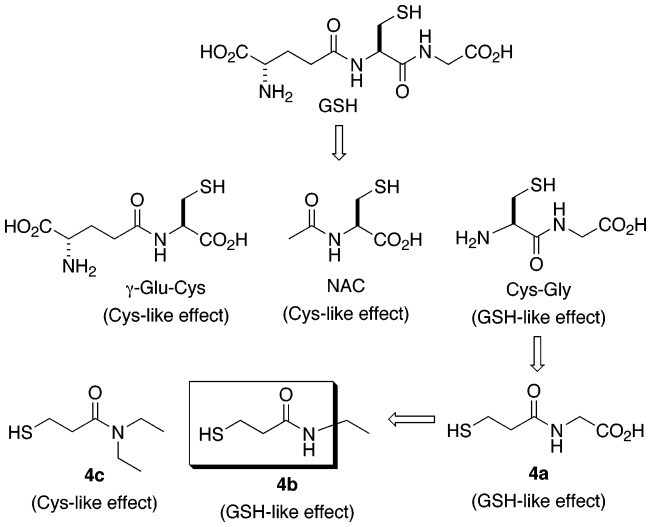
CHART 2



of TEMPO is observed as a prewave in the voltammograms, due to the significantly higher reactivity of the sulfhydryl groups of Cys and GSH toward **1** compared with the hydroxyl group in serine. It is well-known that the oxidation of alcohols with **1** is initiated by the attack of a hydroxyl oxygen atom on the nitrogen atom of **1**.[8,10] A similar initiation step would be included in the present reaction of thiols with **1**. The common effects of Cys and GSH on the voltammetry of TEMPO and spectrophotometry of **1** are consistent with this adduct formation between **1** and Cys or GSH. Therefore, the way in which Cys and GSH react with **1** is likely linked to the decomposition rates of their adducts **3** (Scheme 2). The adduct of GSH and **1** will smoothly decompose into $\mathbf{2}^-$ and an intermediate or product, whereas **3** from Cys and **1** will be stable and not susceptible to further transformations on the voltammetric time scale. Anion $\mathbf{2}^-$ will be oxidized to **1** at a potential similar to that of TEMPO, allowing the turnover of the catalytic cycle. This is the reason the stoichiometry of the reaction of Cys with **1** was 1:1, whereas **1** reacted catalytically with GSH.

The susceptibility of **3** from GSH to a succeeding reaction was expected to correlate with the structural features of GSH. In an attempt to determine a structural moiety in GSH, as a factor in promoting the catalytic reaction with **1**, cyclic voltammetry of TEMPO was conducted in the presence of various thiols with partial structures of GSH (Chart 2). The voltammetric evaluation was based on whether they provided GSH-like voltammetric results (GSH-like effect). The first screening of a key structural feature of GSH was performed with L-γ-glutamyl-L-cysteine (Glu-Cys), L-cysteinylglycine (Cys-Gly), and *N*-acetyl-L-cysteine (NAC). As shown in Figure 4, Glu-Cys exerted a Cys-like effect on the voltammetric response of TEMPO, whereas the addition of Cys-Gly produced results essentially identical to those of GSH. In addition, NAC induced the same changes as Cys as far as the voltammetric behavior of TEMPO. These results suggested that the key partial structure for the observed reactivity inherent in GSH is included

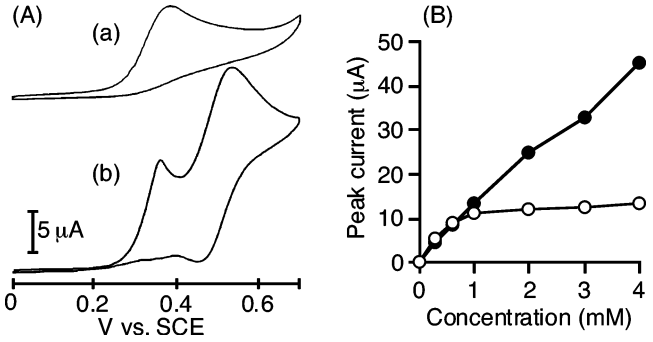
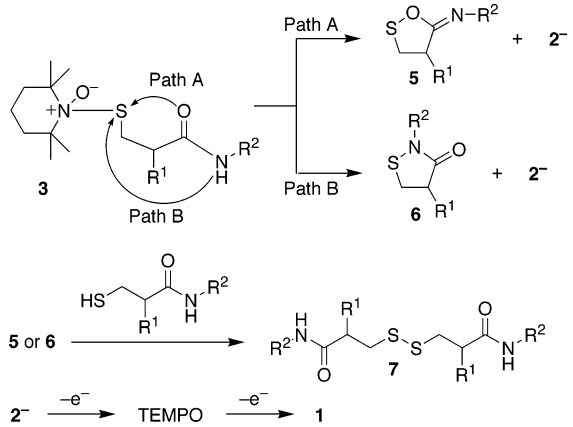


FIGURE 4. (A) Cyclic voltammograms of 1.0 mM TEMPO in the presence of (a) 1.0 mM Glu-Cys or (b) 1.0 mM Cys-Gly in 0.1 M aqueous NaOH and (B) changes in the peak current of the wave at 350 mV in the voltammograms of 1.0 mM TEMPO plotted against the concentration of (open circles) Glu-Cys or (closed circles) Cys-Gly.

SCHEME 3. Proposed Mechanism for the Succeeding Reaction of Adduct 3



in Cys-Gly. The deamino derivative of Cys-Gly, **4a**[11] (Chart 2), exhibited a GSH-like effect on the voltammograms of TEMPO. Its decarboxy derivative **4b** also induced essentially the same voltammetric results as GSH. However, Cys-like voltammetric results were observed in the presence of **4c** bearing no amide hydrogen. These results suggested that a 3-mercaptopropionamide group with an amide hydrogen is the key chemical structure for the observed voltammetric behavior of TEMPO in the presence of GSH.

The observed importance of the amide hydrogen in GSH, **4a**, and **4b** suggested that deprotonation of the amide hydrogen would be included in the decomposition process for the adducts generated from **1** and these thiols. On the basis of this speculation, a plausible two-pathway succeeding reaction of **3** from GSH, **4a**, or **4b** was proposed in Scheme 3. One pathway yields the 5-imino-1,2-oxathiolane intermediate **5** via attack of the amide oxygen atom on the sulfur atom (path A). The other proceeds via bond formation between the sulfur and the amide nitrogen atom, providing the 1,2-thiazolidin-3-one intermediate **6** (path B). Both intermediates are expected to react with another molecule's thiol group, producing the corresponding disulfide **7**. Formation of five-mem-

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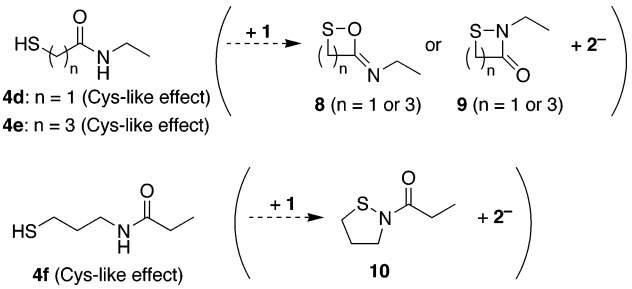
SCHEME 4. Model Thiols for Elucidating Which Is a Plausible Intermediate, 5 or 6

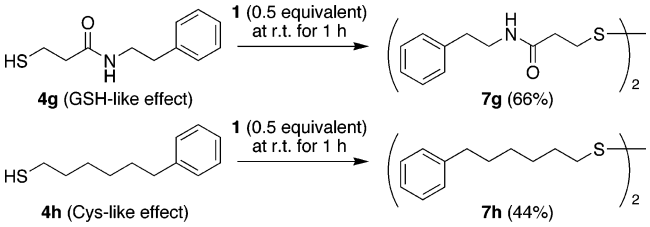


bered ring intermediates is accompanied by the release of $\mathbf{2}^-$ (the reduced TEMPO), and this anion is oxidized to **1** via TEMPO, allowing the turnover of the TEMPO redox cycle.

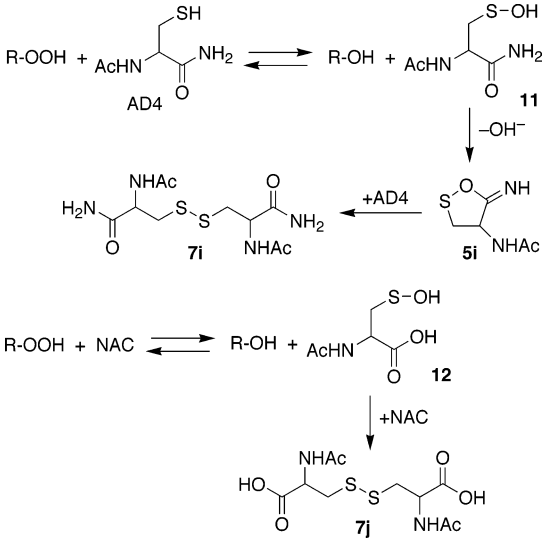
The sulfhydryl and amide groups at positions that permit the closure of a five-membered ring such as **5** or **6** is important. Compounds **4d**[12] and **4e**[13] (Scheme 4) are able to form four- or six-membered ring analogues such as **8** and **9** if the corresponding adducts **3** from these thiols behave similarly to GSH. However, **4d** and **4e** exerted Cys-like effects. The formation of four- and six-membered ring intermediates from adduct **3** appeared to be much less favored than the production of **5** or **6**, as is generally observed in ring-closure reactions. The voltammetric results on **4f** (Scheme 4) were informative to regard **5** rather than **6** as a plausible intermediate. The corresponding adduct of **1** and **4f** might undergo ring formation by attack of the amide nitrogen atom on the sulfur atom, yielding the five-membered ring intermediate **10**. However, Cys-like results were obtained in cyclic voltammetry of TEMPO in the presence of **4f**, suggesting that a five-membered intermediate such as **6** would not be formed through the attack of an amide nitrogen atom in **3**. Thus, it was concluded that the voltammetric behavior of TEMPO, in the presence of GSH, originates from the formation of **5**, as an activated form of GSH toward attack of another molecule of GSH, via path A accompanied by release of $\mathbf{2}^-$.

The formation of a disulfide compound as the final product in the reaction of **1** with a thiol was confirmed by the following experiments. The oxoammonium **1** was prepared as a 50% aqueous CH_3CN solution by constant-current electrolysis of TEMPO in a divided cell at room temperature, in which 1 F/mol of electricity was passed. The obtained solution was added to a solution of **4g** or **4h**[14] (Scheme 5) in aqueous NaOH. The resulting mixture was stirred at room temperature for 1 h, and product analysis was performed on the reaction mixture. Compounds **4g** and **4h** were used as model thiols because it was easy to isolate the products. Cyclic voltammetry experiments revealed that **4g** and **4h** represented GSH and Cys, respectively. The reaction of **1** with both of these thiols provided the corresponding disulfides **7g** and **7h**. Thus, it was demonstrated that thiols react with **1** to provide the corresponding disulfides, regardless of their

SCHEME 5. Product Analysis for Reaction of 1 with 4g or 4h as a Model Compound of GSH or Cys, Respectively



SCHEME 6. Proposed Mechanism for the Reported Difference in Scavenging Ability of Peroxides between AD4 and NAC



classification (GSH- or Cys-like) with respect to their effects on the voltammetric response of TEMPO. Although it was reported that various types of 1,2-thiazolidin-3-one compounds **6** could be prepared and isolated,[15] no formation of the corresponding **6** from **4g** was noted. Further, this supports path A via **5** as the route for adduct **3** from GSH. It is important to note that the isolated yield of **7g** was higher than that of **7h**. These results implied that disulfide formation from a thiol by a reaction with **1** proceeds more effectively when a thiol has an amide group that permits formation of **5** from the corresponding **3**. Adduct **3** from a thiol with Cys-like reactivity was thought to react directly with another thiol molecule on the preparative time scale, producing the corresponding disulfide.

The proposed mechanism for the reaction of GSH and GSH-like thiols with **1** generated from TEMPO suggests that **5** is the activated form of GSH in biological reactions induced by ROS or RNS. Moreover, 3-mercaptopropionamides with amide hydrogens are potent candidates as exogenous thiol compounds which can be used for the treatment of GSH-depletion-related disorders. Recently, it was reported that *N*-acetylcysteinamide (AD4 in Scheme 6) reduced H_2O_2 and *t*-BuOOH much more effectively than NAC and was stronger as a peroxide scavenger by

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(15) Takahaki, S.; Inaba, T.; Matsunaga, A.; Sakurasawa, M.; Ichikawa, S. Japanese Patent Appl. JP 2002-173079, 2002; *Chem. Abstr.* **2003**, *139*, 44173.

a factor of about 2 than GSH.[7] These observations can be explained by the effective formation of an oxathiolane intermediate such as **5i** from AD4. Provided that the reaction of a thiol with a peroxide is reversible, the reducing ability of the thiol will depend on the rate of transformation of a sulfenic acid, such as **11** or **12**, to the corresponding disulfide. The amide group of AD4 is thought to promote disulfide formation via **5i**, while NAC lacks a promoting functional group. NAC exerted the same influence on the voltammograms of TEMPO as Cys; therefore, AD4 is a better reducing agent of peroxides than NAC. The observed increase in reactivity of AD4 with respect to GSH toward peroxides is related to the presence of two amide hydrogen atoms, which increases the likelihood of forming a cyclic intermediate (**5**).

Conclusions

The biological thiols GSH and Cys produce different effects on the voltammetric behavior of TEMPO in aqueous NaOH. The characteristic effect of GSH is that it allows the adduct **3** of GSH and **1** (the oxidized TEMPO) to form an initial intermediate which readily decomposes, thereby generating $\mathbf{2}^-$ (the reduced TEMPO) and allowing the turnover of the TEMPO redox cycles via anodic oxidation of this anion. Examination of various thiol compounds based on whether a GSH-like voltammetric result was provided, revealed that the 3-mercaptopropionamide group with an amide hydrogen is essential for GSH to exhibit its inherent reactivity toward **1**. The proposed function of this structure is to form the oxathiolane **5** from **3** of GSH and **1**, resulting in an activated form of GSH. This, in turn, reacts with another molecule of GSH, producing GSSG. This mechanism provides a reasonable explanation for the observation that AD4 works as a stronger scavenger for peroxides than NAC and GSH. Thus, the present investigation provides acceptable criteria for the molecular design of exogenous thiol compounds as novel drugs for the treatment of GSH-depletion-related disorders.

Experimental Section

Materials. Milli-Q water and HPLC-grade CH_3CN and MeOH were used. All other chemicals were purchased from commercial suppliers and used without further purification.

General Procedure for Cyclic Voltammetry. A three-electrode configuration was employed: a glassy carbon (∅ = 3 mm) electrode as a working electrode, a saturated calomel electrode (SCE) as a reference electrode, and a platinum wire electrode as a counter electrode. The glassy carbon electrode was polished with polishing paper (no. 1500) and alumina powder (0.05 μM) on a polishing cloth, sonicated in water, MeOH, and water, and dried with a stream of N_2 gas prior to use. Test solutions were deoxygenated by bubbling N_2 gas for several minutes and subjected to voltammetric measurements at room temperature and 100 mV/s over a potential range between 0.0 and 0.7 V vs SCE under a N_2 atmosphere.

General Procedure for Spectrophotometry of 1. TEMPO (156 mg, 1.0 mmol) was subjected to constant-current electrolysis (1.5 mA, 1 F/mol) in 25 mL of CH_3CN-H_2O (1:1) containing 0.1 M $NaClO_4$ at room temperature under a N_2 atmosphere in a divided cell equipped with a glassy carbon anode (15 × 30 mm) and a platinum foil cathode (12 × 20 mm). After the electrolysis, the analyte was used as a stock solution (40 mM) of **1**. Absorption spectra of **1** itself were obtained after the stock solution (1 mL) was diluted by a factor of 10 with H_2O. Effects of Cys or GSH on the absorption spectra of **1** were evaluated as follows. A thiol solution (40 mM, 2 mL) in H_2O, NaOH (1 M, 240 μL) in H_2O, and the stock solution (1 mL) of **1** were added in this order to the appropriate volume of H_2O. The total volume of the mixture was adjusted to 10 mL by addition of H_2O, and the thus obtained solution was immediately subjected to spectrophotometry.

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Supporting Information Available: Synthesis and characterization of **4a**−**h** and experimental procedures for product analysis on reaction of **1** with **4g** or **4h**. This material is available free of charge via the Internet at http://pubs.acs.org.

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