Journal of Enzyme Inhibition and Medicinal Chemistry

http://informahealthcare.com/enz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2014; 29(4): 491–494
 DOI: 10.3109/14756366.2013.805757

informa healthcare

ORIGINAL ARTICLE

Glutathione reductase activity with an oxidized methylated glutathione analog

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Abstract

The activity of glutathione reductase with an unnatural analog of oxidized glutathione was explored. The analog, L-γ-glutamyl-2-methyl-L-cysteinyl-glycine disulfide, places an additional methyl group on the alpha position of each of the central cysteine residues, which significantly increases steric bulk near the disulfide bond. Glutathione reductase was completely unable to catalyze the sulfur–sulfur bond reduction of the analog. Additionally, enzyme kinetics experiments indicated that the analog acts as a competitive inhibitor of glutathione reductase. Computational studies confirm that the methylated analog fits within the active site of the enzyme but its disulphide bond geometry is altered, preventing reduction by the enzyme. The substitution of (*R*)-2-methylcysteine in place of natural (*R*)-cysteine in peptides constitutes a new strategy for stabilizing disulphide bonds from enzyme-catalyzed degradation.

Keywords

Computational chemistry, disulfide reduction, enzyme kinetics, GSSG, inhibition

History

Received 23 April 2013
Revised 24 April 2013
Accepted 26 April 2013
Published online 26 June 2013

Introduction

The substitution of unnatural amino acid residues within a peptide has the potential to dramatically affect its chemical behavior. In particular, the incorporation of α-methyl amino acids into peptides is known to slow their degradation by hydrolytic enzymes and increase the stability of their secondary structure[1,2]. As part of our ongoing interest in this area, we have studied the effects of substituting the unnatural α-methyl amino acid (*R*)-2-methylcysteine in place of natural (*R*)-cysteine in the peptide glutathione on its reactivity with the enzyme glutathione reductase [EC 1.8.1.7].

Glutathione reductase (GR) is the enzyme responsible for catalyzing the reduction of the disulfide bond in oxidized glutathione (GSSG) with NADPH to give glutathione in its reduced thiol state (GSH).

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H^+} \xrightarrow{\mathrm{GR}} 2\ \mathrm{GSH} + \mathrm{NADP^+}$$

This process is important for maintaining a healthy cellular ratio of glutathione in its reduced and oxidized states. In its reduced form as GSH, glutathione acts as a substrate in a number of important cellular processes. These include the detoxification of reactive oxygen species, conjugation reactions assisting with the removal of cytotoxic agents, and in the production of deoxyribonucleotides for DNA synthesis[3].

Glutathione reductase and its substrate GSSG served as a convenient model system to begin studying how enzymes that catalyze reactions of disulfides in peptides, such as disulfide reductases and transferases, respond to peptide substrates having (*R*)-2-methylcysteine substituted for natural (*R*)-cysteine. Due to the increased steric bulk of the extra methyl groups and their proximity to the disulfide bond, we postulated that these enzymes would exhibit reduced activity toward the unnatural substrates. This could then serve as a strategy for stabilizing the disulfide bond in peptides, which could have applications in the area of peptide-based therapeutics.

In a previous study, we reported an improved method for preparing differentially protected (*R*)-2-methylcysteine and elaborating it into an unnatural analog of glutathione, L-γ-glutamyl-2-methyl-L-cysteinyl-glycine disulphide[4]. This analog is shown in Figure 1 labeled as mGSSG, along with the natural glutathione substrate labeled as GSSG. The mGSSG analog differs from GSSG in that it substitutes a methyl group in place of a hydrogen atom at the *alpha*-carbon of each cysteine residue. This manuscript summarizes results of enzyme kinetics experiments and computational studies comparing the activity and binding of GR with mGSSG to GSSG.

Materials and methods

The activity of yeast glutathione reductase with mGSSG and GSSG as a control was measured with a commercially available GR assay kit obtained from Sigma-Aldrich Company (catalog number GRSA). This allowed the enzyme's activity to be determined by a decrease in absorbance at 340 nm due to consumption of NADPH, or by an increase in absorbance at 412 nm due to reaction between free GSH and added 5,5′-dithiobis-(2-nitrobenzoic acid) DTNB. Both the UV and colorimetric versions of the assay were used to monitor enzyme

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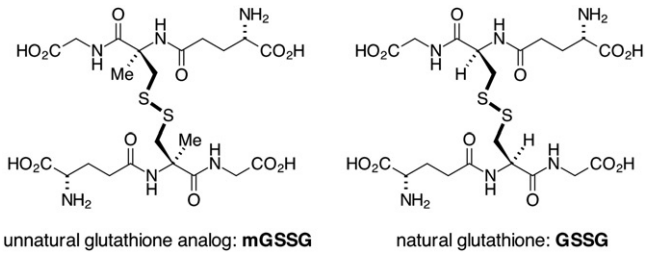

Figure 1. Structures of mGSSG and GSSG.

activity in this study. The materials used included: ''assay buffer'' pH 7.5 aqueous buffer of 100 mM in potassium phosphate and 1 mM EDTA; ''dilution buffer'' same composition as assay buffer, but with 1 mg/mL bovine serum albumin added; ''enzyme solution'' prepared by dissolving one vial of enzyme (catalog number G0665) in 1 mL of water to generate a solution of yeast GR with an activity >1 unit/mL in 100 mM potassium phosphate, 1 mM EDTA buffer solution with 38 mg/mL trehalose as a stabilizer. Solutions of GSSG, mGSSG, and NADPH were made up using assay buffer immediately before use.

Procedures for GSSG and mGSSG blank runs in UV GR assay without enzyme

A series of blank runs was carried out by mixing a solution of NADPH with either GSSG or mGSSG in the absence of enzyme and observing each solution's absorbance at 340 nm over time. Solutions were mixed in the following order: 500 µL 2 mM GSSG or mGSSG; 410 µL assay buffer; 40 µL dilution buffer and 50 µL 2 mM NADPH. After a 10 s incubation period, absorbance at 340 nm was recorded every 10 s for 170 s. The procedure was repeated in triplicate and the absorbance at each time point averaged and plotted.

Procedures for GSSG and mGSSG runs in UV GR assay with enzyme

A series of runs was carried out by mixing a solution of NADPH with either GSSG or mGSSG in the presence of enzyme and observing each solution's absorbance at 340 nm over time. Solutions were mixed in the following order: 500 µL 2 mM GSSG or mGSSG; 400 µL assay buffer; 40 µL dilution buffer; 10 µL of enzyme solution and 50 µL 2 mM NADPH. After a 10 s incubation period, absorbance at 340 nm was recorded every 10 s for 170 s. The procedure was repeated in triplicate and the absorbance at each time point averaged and plotted.

Procedures for GSSG and mGSSG runs in colorimetric GR assay with enzyme

A series of runs was carried out by mixing a solution of NADPH with either GSSG or mGSSG in the presence of enzyme and DTNB and observing each solution's absorbance at 412 nm over time. Solutions were mixed in the following order: 500 µL 2 mM GSSG or mGSSG; 190 µL assay buffer; 10 µL of enzyme solution; 250 µL 3 mM DTNB and 50 µL 2 mM NADPH. After a 60 s incubation period, absorbance at 412 nm was recorded every 10 s for 110 s. The procedure was repeated in triplicate and the absorbance at each time point averaged and plotted.

Procedures for GR inhibition assays

A stock solution of 2 mM GSSG was prepared by dissolving 1.225 mg GSSG per 1.0 mL assay buffer. This solution was used to prepare the working solutions for the assay. The working solutions ranged in concentrations from 0.02 to 1.6 mM. A stock solution of 2 mM mGSSG was prepared by dissolving 1.42 mg mGSSG per 1.0 mL assay buffer. This solution was used to prepare inhibitor solutions with concentrations of 0.02, 0.075 and 0.09 mM. Solutions were mixed in the following order: 250 µL GSSG solution; 250 µL mGSSG solution; 190 µL assay buffer; 10 µL of enzyme solution; 250 µL 3 mM DTNB and 50 µL 2 mM NADPH. After a 60 s incubation period, absorbance at 412 nm was recorded every 10 s for 110 s for a total run time of 170 s. The procedure was repeated in triplicate and the absorbance at each time point was averaged and plotted.

Computational procedures

Computational studies of GSSG and mGSSG docked within the GR active site used published crystallographic data for GSSG bound to human GR from the RCSB data bank as a starting point[5]. To simplify the calculations, only enzyme atoms and water within 6.0 angstroms of the GSSG ligand were included. Note this does include some atoms from the neighboring protein in the unit cell. X-ray data does not include hydrogens. These were added using the add hydrogen atoms feature of Jmol (http://www.jmol.org) and just the hydrogen positions were optimized using the MMF94F force field within the software Avogadro (http://avogadro.openmolecules.net/). The GAMESS software package[6,7] running on a computer cluster with 32 processors and 64 GB of RAM was used for the *ab initio* geometry optimization of the ligand while holding the positions of the protein atoms and water fixed. This approach has been described in previous work by Kokubo et al.[8] and Riley et al.[9] Optimizing the geometry of the natural substrate GSSG using the restricted Hartree–Fock method and a 3-21G basis set gave a good fit to the experimental crystallographic data. The computed atom positions were displaced from their X-ray positions by less than the crystallographic uncertainty. Next, the unnatural substrate mGSSG was substituted for GSSG in the calculations and its geometry optimized at the same level of theory.

Results and discussion

Blank experiments using the UV GR assay indicated that there was very little uncatalyzed background reaction that occurred between NADPH and either GSSG or mGSSG in the absence of the enzyme. Absorption data versus time in these experiments gave linear graphs that had very shallow slopes of -1.03×10^{-5} and $-8.85 \times 10^{-6}\,\Delta A/s$ for GSSG and mGSSG, respectively.

Next, the activity of the enzyme with GSSG and mGSSG was measured in the UV GR assay. This data is plotted in Figure 2 as absorbance at 340 nm versus time. In the case of GSSG and NADPH in the presence of GR enzyme, the rate of absorbance change was $-1.34 \times 10^{-3}\,\Delta A/s$. This is significantly faster than the blank experiment with GSSG confirming good enzymatic activity with the native substrate. In the case of mGSSG and NADPH in the presence of GR enzyme, the rate of absorbance change was similar to the blank experiment at $-2.21 \times 10^{-5}\,\Delta A/s$. This indicates essentially no catalytic activity of GR with the unnatural mGSSG substrate.

As a check on the results of the UV assay kinetics data, the colorimetric GR assay was also carried out with GSSG and mGSSG. The results in the colorimetric assay are shown in Figure 3. They confirmed the results obtained in the UV assay, again showing that mGSSG is not reduced by GR.

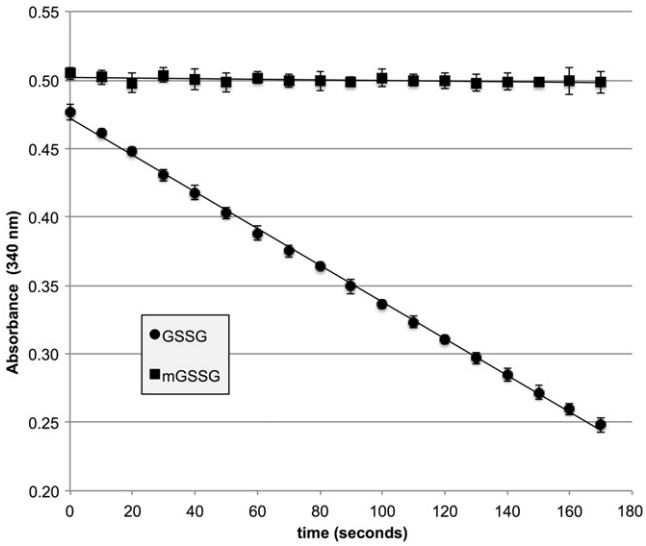

Figure 2. Enzymatic activity of GR with GSSG and mGSSG by UV absorption at 340 nm. Error bars represent standard deviations.

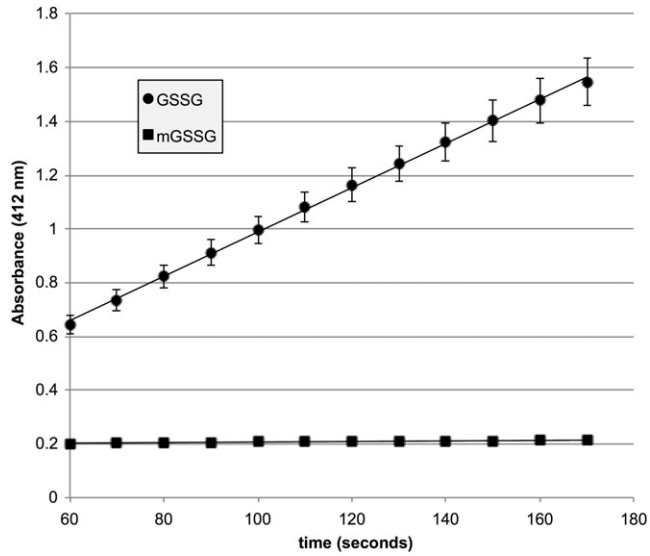

Figure 3. Enzymatic activity of GR with GSSG and mGSSG by UV-Vis absorption at 412 nm. Error bars represent standard deviations.

Next, a series of enzyme kinetics experiments were carried out to test whether mGSSG might act as an inhibitor of GR. Given the close structural similarity of GSSG and mGSSG, competitive inhibition was a possibility. However, mGSSG also adds a significant amount of steric bulk compared to the natural substrate and it wasn't immediately clear that mGSSG would fit into the active site of GR. The colorimetric assay of enzyme activity was carried out at varying concentration of GSSG in the presence of 90, 75, 20 and 0 μM mGSSG inhibitor. Values for initial velocity (V_0) versus GSSG concentration are plotted in Figure 4 at the different inhibitor concentrations. The data obtained in the assays was plotted in a Lineweaver–Burk plot (Figure 5), which was consistent with a competitive inhibition model. The data reported in Figures 4 and 5 were imported into GraphPad Prism® software. Using the built in data analysis programs, the data was fit to the model for competitive inhibition and gave a global fit value of $R^2 = 0.9228$, a K_i value of 14.64 ± 1.768 (μM), and $V_{max} = 0.007962 \pm 0.0002527$.

An *ab initio* computational study of GSSG and mGSSG docked within the GR active site was undertaken to better understand the behavior of the mGSSG substrate. The results indicate that despite the increased steric bulk of additional methyl substitution, mGSSG is still able to fit within the active site of the enzyme. Figure 6 shows the optimized geometries for GSSG and mGSSG bound within the active site and overlaid with the atoms of GSSG represented using standard colorings while the atoms of mGSSG are shown in purple. The two structures overlap very closely with the exception of the disulfide region, which is twisted in mGSSG with respect to GSSG. The calculated C-S-S-C dihedral angles for GSSG and mGSSG in the active site were 84.5° and 62.2°, respectively (Figure 7). This distortion in dihedral angle likely accounts for the enzyme's inability to catalyze the reduction of mGSSG.

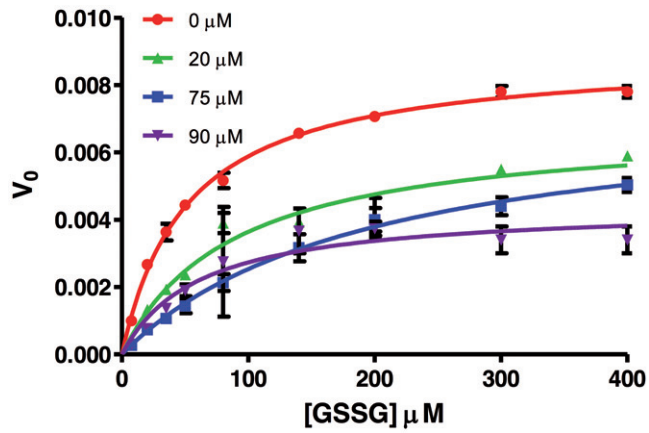

Figure 4. Enzymatic activity of GR at various inhibitor concentrations. Error bars represent standard deviations.

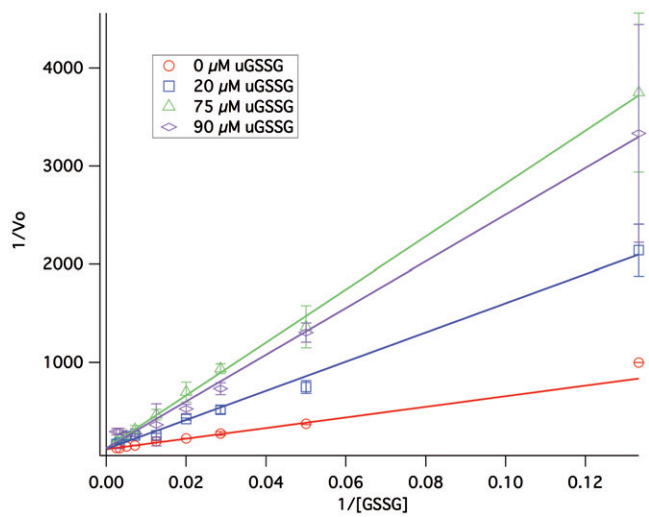

Figure 5. Lineweaver–Burk plot. Error bars represent standard deviations.

Conclusion

In conclusion, we have described the behavior of a methylated analog of glutathione (mGSSG) with glutathione reductase. The enzyme is completely unable to reduce this analog. Kinetics experiments indicated that mGSSG also acts as a mild competitive inhibitor of the enzyme. Finally, computations indicated that mGSSG fits within the active site of the enzyme, but with distorted geometry about the disulfide bond, which presumably is the reason that the enzyme does not reduce it. This is the first example of a new strategy to protect disulfide bonds from enzyme catalyzed degradation, and could be useful for stabilizing other disulfide containing peptides. Future work will involve studying

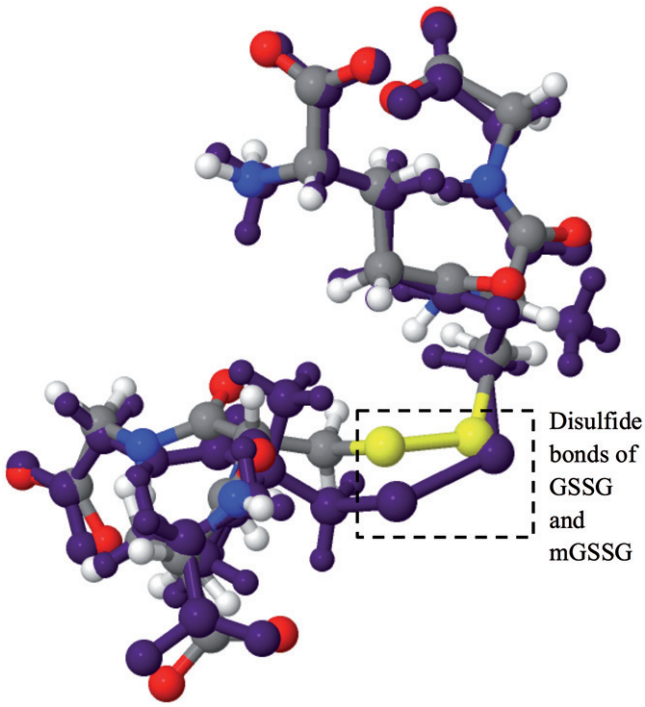

Figure 6. Overlaid computational structures of GSSG and mGSSG in the active site of glutathione reductase. The atoms of GSSG are represented using standard colorings while the atoms of mGSSG are shown in purple.

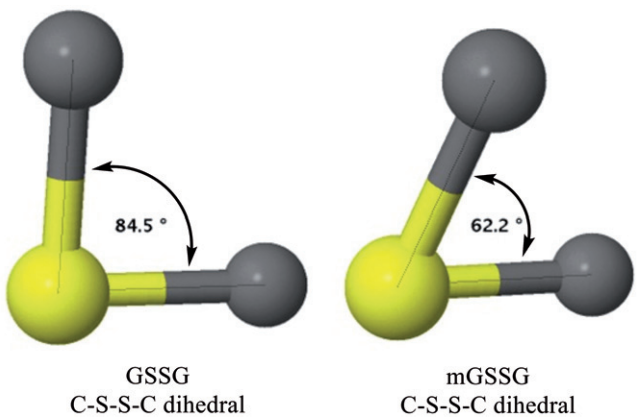

Figure 7. Newman projections of GSSG (left) and mGSSG (right) within the active site looking down the disulfide bond in each.

the effects of substituting 2-methylcysteine in other significant disulfide containing peptides. Additionally, the usefulness of mGSSG and related analogs as potential antimalarial agents will be explored. The plasmodium parasite is known to be more sensitive to oxidative stress than human erythrocytes. Therefore, the inhibition of glutathione reductase is a common strategy for therapeutically targeting it[3,10,11].

Acknowledgements

The authors thank the University of Wisconsin Oshkosh Department of Chemistry and the University of Southern Mississippi Department of Chemistry and Biochemistry.

Declaration of interest

We thank the National Science Foundation for support of this work (NSF-CAREER, MCB-0844478). Maureen Smith thanks the NSF for providing her with a GK-12 Fellowship (Award 0947944). Chondrea Jordan thanks ACS Project SEED for summer financial support. We thank NSF S-STEM grant # 0806501 for support of Gorman Stock and partial support of this project.

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