Hypothesis

Glutathione Conformations and Its Implications for *in vivo* Magnetic Resonance Spectroscopy

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Abstract. Glutathione (GSH) is a major antioxidant in humans that is involved in the detoxification of reactive radicals and peroxides. The molecular structural conformations of GSH depend on the surrounding micro-environment, and it has been experimentally evaluated using NMR and Raman spectroscopic techniques as well as by molecular dynamics simulation studies. The converging report indicates that GSH exists mainly in two major conformations, i.e., "extended" and "folded". The NMR-derived information on the GSH conformers is essential to obtain optimal acquisition parameters in *in vivo* MRS experiments targeted for GSH detection. To further investigate the implications of GSH conformers in *in vivo* MRS studies and their relative proportions in healthy and pathological conditions, a multi-center clinical research study is necessary with a common protocol for GSH detection and quantification.

Keywords: Antioxidant, brain, conformation, glutathione, magnetic resonance spectroscopy, molecular dynamics, nuclear magnetic resonance

INTRODUCTION

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), synthesized in the cytosol from the precursor amino acids glutamate, cysteine, and glycine [1]. GSH is ubiquitous and one of the most abundant metabolites with millimolar (mM) intra-cellular concentration. It is the major low-molecular weight thiol compound in animal cells. Presence of thiol group in GSH makes it susceptible to oxidation [2] as well as possible source of conformational changes due to changes in its surrounding microenvironment. Published reports of molecular dynamics (MD) studies [3–6] as well as studies utilizing state-of-the-art Raman spectroscopy [7, 8] and nuclear magnetic resonance (NMR) [9, 10] techniques on GSH in various experimental conditions (solvent, pH, temperature, etc.), provide a clear insight into possible GSH conformations (Fig. 1).

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Molecular dynamics is a computer simulation method for studying movements of atoms and molecules, in which an initially created molecular structure is first energy minimized and subsequently the coordinates of structures with lower energy states as conformers are obtained by allowing the atoms and molecules to interact with each other for a fixed period of time. MD simulations over 20 ns using Gromacs all-atom force field, coupled with cluster analyses of the trajectories, have been applied to examine the distribution proportion of GSH conformations in aqueous solutions as a function of pH [3]. The results derived from MD simulations show that GSH is very flexible and does not adopt a strongly preferred conformation at any pH [3]. Calculations in another MD study with GSH [4] were carried out using a modified TINKER 4.2 molecular modeling package at T = 298 K and 1 atmospheric pressure. This study also indicated that GSH is highly flexible in an aqueous solution with transitions occurring between the extended and folded conformations [4]. In contrast to classical MD with the motion of interactive atoms, recent advancements in conformal studies are adopting quantum molecular dynamics (QMD). QMD is based on the existence of chemical bonds as a result of electron interactions and can describe the formation and breaking of chemical bonds, which cannot be accomplished using classical molecular dynamics. QMD includes elemental interactions between atoms as well as electrons. QMD simulation methods work on "first principle" based only on the laws of quantum mechanics and thus do not require any prior knowledge on inter-atomic interactions. Behavioral modeling of molecules in QMD is described in terms of density functional theory (DFT), which is presently the most successful approach to compute the electronic structure of matter [5]. The DFT approach predicts a great variety of molecular properties that include molecular structures, vibrational frequencies, atomization energies, ionization energies, electric and magnetic properties, and reaction paths. A recent report on DFT investigation of GSH has also confirmed the extended and folded conformers of GSH reported in an MD study [6].

Conformations of GSH in aqueous solutions have been investigated using Raman spectroscopic methods that rely on vibrational and/or rotational frequency differences. Raman spectroscopy provides detailed information on vibrational frequencies specific to chemical bonds as well as on molecular symmetry, thereby giving a unique fingerprint of the molecule of interest. Since Raman spectroscopy is a non-destructive technique, it is useful for the analysis of chemical structures and their molecular conformations [7]. The amide III band components, between 1308 cm^{-1} and 1288 cm^{-1}, show three distinct distribution of conformations of GSH (P_{II}, α_R and β at 0.5 M GSH in H_2O, pH 7 [8], and these conforma-
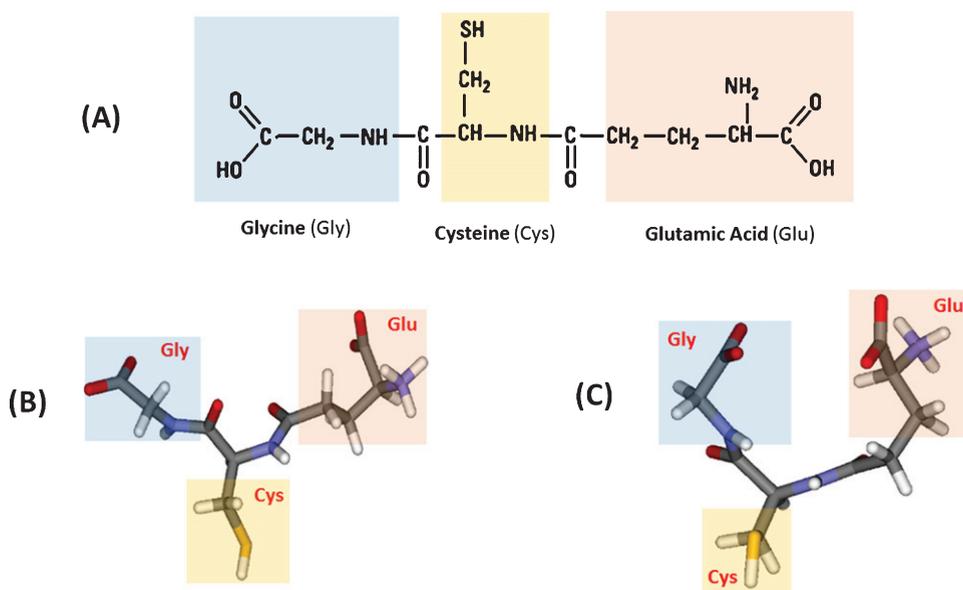
Fig. 1. Glutathione and its structural conformations. (A) Molecular structure of glutathione, (B) the extended, and (C) the folded conformations (modified from [4]; copyright permission obtained from the publisher).

tions have been used by numerous experimental and theoretical studies. The Raman spectroscopic bands at $1305\,cm^{-1}$, $1298\,cm^{-1}$ and $1288\,cm^{-1}$ correspond to the P_{II}, α_R, and β conformations, respectively, of GSH in aqueous medium [8]. The relative population of β, P_{II}, and α_R conformations of GSH is reported as 60%, 25%, and 15%, respectively, based on calculations of the amide III regions in Raman spectra of GSH [8].

NMR spectroscopy provides information on the conformational changes of peptides and proteins through measurement of chemical shifts of molecular groups and coupling constants of the chemical bonds in them. Conformational analysis of a compound at a given experimental condition (e.g., pH, temperature and solvent) is accomplished using NMR spectroscopy to determine the relative populations of conformers in solution. Proton NMR chemical shift values of the molecular groups of GSH were determined without ambiguity using one dimensional and/or two-dimensional NMR studies performed at high magnetic fields (400–700 MHz) [9–12] in various physiological conditions (e.g., pH, temperature, and solvent).

The (two) published NMR reports of GSH indicate that there are major differences in the way the samples were prepared [11, 12]. One report [11] used a degassed aqueous GSH solution in order to reduce the amount of dissolved oxygen in the solution, thereby minimizing the oxidation of GSH, while another report [12] used a non-degassed GSH aqueous solution in the NMR studies. GSH is susceptible to oxidation due to the presence of a thiol group, and thus it is highly sensitive to external environment. Therefore, the sample preparation method plays a key role in deciding the conformational state of GSH. The two NMR studies [11, 12] that have used a degassed or a non-degassed aqueous GSH solution report distinct conformational states of GSH. NMR studies of a non-degassed aqueous GSH solution show the chemical shift of GSH cysteine H_α and H_β at 4.56 and 2.95 ppm [12–14]. In contrast, the studies that have used degassed samples report GSH cysteine H_α and H_β at 4.40 and 2.80 ppm [11, 15]. One of the studies reported the chemical shifts of GSH cysteine H_α and H_β at 4.37 and 2.77 ppm in aqueous solution using DMSO as an internal reference [10]. The chemical shift values [10] of the cysteine H_α/H_β are close to the values reported in studies that used degassed GSH aqueous solutions [11, 15]. A combined MD and NMR study reveals that GSH in DMSO solution

remains in the stable folded conformation, whereas in aqueous solution it is highly flexible with transitions between extended and folded conformations [4]. The stability of the folded GSH form has been confirmed by a recent advanced DFT calculations report [6].

Based on these combined studies [4, 6], it can be inferred that chemical shifts of cysteine H_α and H_β of GSH at 4.40 and 2.80 ppm, respectively, in degassed aqueous solution is similar to the one obtained in DMSO solution, are indicative of the stable folded GSH conformation. We hypothesize that reduced glutathione in aqueous solution has two conformations as indicated by MD studies: *the extended conformation* is characterized by the chemical shifts of cysteine H_α and H_β at 4.56 and 2.95 ppm, respectively, and *the folded conformation* is characterized by the chemical shifts of H_α and H_β at 4.40 and 2.80 ppm, respectively. However, there is need to perform a thorough and well-controlled experimental study to understand the reasons behind the changes observed in the chemical shifts of cysteine hydrogens of GSH and to ascertain if this is to do with the change in its conformations or other factors.

Glutathione is a free radical scavenger, which plays a key role in maintaining the oxidative and redox balance in human cells and exists in both the reduced (GSH) and oxidized (GSSG) forms. GSH can be converted to GSSG by the enzyme glutathione peroxidase, and this can be reconverted to GSH by glutathione reductase [16]. Measurements of GSH, GSSG and their enzyme catalyzed reactions are thus important for evaluating the redox and antioxidant status [16]. GSH serves as a marker of oxidative stress that is an important factor in Alzheimer's disease (AD) [17]. GSH in the brain is reported to be $1.18 \pm 0.09\,mM$ from the parietal cortex of autopsy brain [18], which decreases with age and age-associated disorders [19]. A clinical study indicated the link between oxidative damage, mild cognitive impairment (MCI) and AD using the plasma levels of GSH and GSH/GSSG ratio of 34 subjects with MCI, 45 subjects with AD, and 28 age-matched control subjects [20]. The results showed a significant decrease in GSH levels and GSH/GSSG ratios in AD and MCI patients compared to age matched control subjects [20]. The depletion of GSH was specific to AD patients compared to patients with other neurodegenerative disorders such as Parkinson's disease and dementia with Lewy body disease [21]. This clinical study reported that the mean GSH levels in the cingulate cortex brain region of AD patients were

decreased (49%) compared to age matched control subjects [21]. These disease specific studies [20, 21] stress the importance of quantitation of GSH in the brain regions patients with different pathological conditions (e.g., AD and MCI), which may serve as an early surrogate marker of diseases.

DISCUSSION

It has been established that the tripeptide structure of GSH is quite sensitive to its surrounding environment (e.g., pH, temperature, and solvent). Molecular dynamics studies have shown that the conformation of GSH is very flexible in aqueous solution by converting it from the extended form to the folded form [4]. Furthermore, NMR and Raman spectroscopic studies of GSH clearly support the existence of various conformers of it. The results of these studies allude to the presence or absence of anaerobic or aerobic environment indeed influence the antioxidant potential of GSH by modulating the conformational changes of GSH. At present, it is not known which of the two GSH conformations (i.e., extended or folded) is present in the human brain. It is pertinent to know the proportion of the two GSH conformations in healthy brain and in pathological conditions. Answers to these questions will have major implications in the way GSH levels are detected and quantitated using *in vivo* MRS methods.

It is prudent to report that the accuracy of the NMR spectral parameters (chemical shifts and coupling constants) of glutathione can be limited for its molecular groups with complex and overlapping spectral patterns. Furthermore, the values for these parameters may be altered in *in vivo* conditions as a result of local physiological and cellular environment [22]. Detection of GSH in human brain *in vivo* is commonly accomplished by ^{1}H magnetic resonance spectroscopy (MRS) using spectrally-selective editing schemes that employ J-couplings of cysteinal hydrogens. We hypothesize that the proton NMR spectral peak positions of cysteine H_α and H_β are highly associated with the conformational states of GSH. The QMD study of GSH [6] has not got sufficient attention, and further studies with *in vivo* physiological conditions are necessary to understand possible conformations of GSH in the brain. A clear understanding for the conformations of GSH in the brain will help us to come up with optimal experimental data acquisition parameters for MRS studies performed *in vivo* for selectively detecting GSH.

An important first step is to do thorough *in vitro* NMR experiments of GSH in different physiological conditions that will clarify some of the unanswered questions. Towards this, we plan to acquire proton MRS data at high magnetic fields (e.g., >11 T) using phantoms containing GSH and/or GSSG at physiological and non-physiological (or pathological) conditions as well as *ex vivo* intact animal or human brain tissue samples. The high magnetic field MRS data will allow us to measure the chemical shifts and coupling constants of the hydrogens in the extended and folded GSH conformations at much higher accuracy than at 3T clinical MRI scanner.

Previous task force reported eight neurochemical (not GSH) with their specific chemical shifts and *in vivo* concentration range. Subsequently, a multicenter *in vivo* research project with a common data acquisition protocol will help us to unravel the associations between the conformations of GSH *in vivo* and the observed spectral patterns in healthy controls and pathological conditions.

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