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Thermodynamic Studies of Zn2+ Binding to Glutathione

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By

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Thermodynamic studies of Zn2+ binding to glutathione

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Glutathione is one of the most abundant organic compounds found in many biological systems. It is a non-protein tripeptide of Glutamic acid, Cysteine and Glycine. Due to its stability, high cellular concentrations and structural features like gamma-glutamyl linkage and sulfhydryl group, glutathione involves with many biological pathways including: cellular defense against xenobiotics and naturally occurring deleterious compounds, like free radicals, metal sequestering, and maintaining cellular sulfhydryl status. Glutathione has been broadly studied however the literature associated with Zn^{2+} coordination is not clear. This study is focused on collecting thermodynamic data of glutathione binding to Zn^{2+} metal ions using calorimetric technique. Isothermal titration calorimetry has been recommended as an excellent method to determine the association constant (K), enthalpy change (delta H), and binding stoichiometry (n) of a binding process. These parameters associated with Zn^{2+} binding glutathione deconvoluted from a series of complex equilibria provide an insight into what drives these reactions forward.

DEDICATION

I would like to dedicate this body of work to my husband (Nimanatha Dayarathna).

ACKNOWLEDGEMENTS

First and foremost I would like thank my research professor, Dr. Joe Emerson, for giving me the opportunity to join his lab. He has continuously supported me throughout my Master's career and I am grateful for the knowledge he has instilled in me. I would also like to express my gratitude to the members of my graduate committee, Dr. Nick Fitzkee and Dr. Deb Mlsna, for their advice and guidance.

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CHAPTER I

GLUTATHIONE-ZINC BINDING

1.1 Introduction to glutathione

Glutathione (GSH) is one of the most abundant biomolecules found in nature.[1] It is thought that biological systems use glutathione as a redox regulator, where both the reduced (GSH) and oxidized (GSSG) forms of glutathione are commonly found. GSSG concentrations are typically much lower inside cells, where 90 % of glutathione is found in its reduced form. Glutathione is highly concentrated in cytosol where biosynthesis and protein folding often take place, but it is also present in the nucleus and the mitochondria.[2,3] The intracellular concentration of glutathione in human cells is often as high as 1-20 mM compared to the extracellular concentrations which is about 3-4 fold lower.[4]

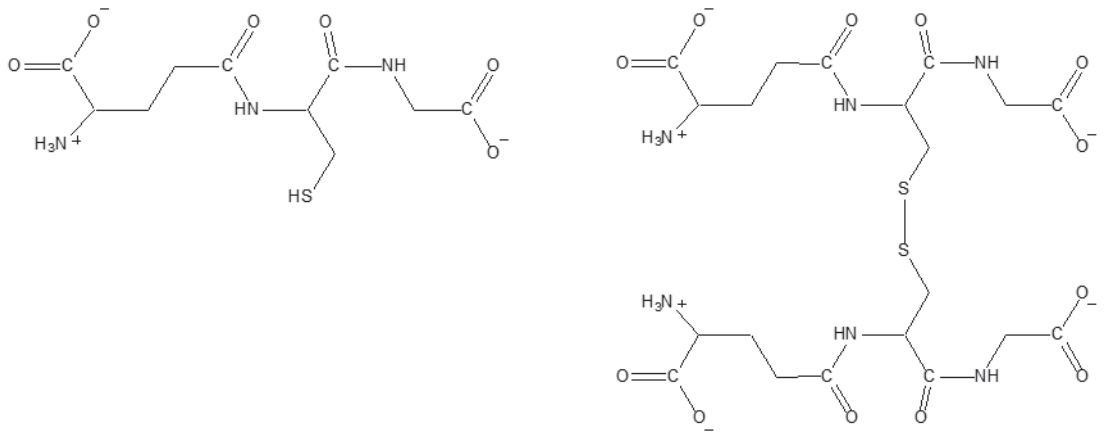

Figure 1.1 Structure of (a) Reduced glutathione (b) Oxidized glutathione

Structurally, GSH is a non-protein, tripeptide made of glutamate, cysteine, and glycine (γGlu–Cys–Gly), where the glutamic acid is connected to the structure with its γ-carboxylic side-chain bound to cysteine's N-terminal amino functional group generating an atypical amide linkage; the cysteine and glycine are linked together through a common peptide bond. In biological systems this small peptide chain of glutathione is synthesized from three amino acids, beginning with the formation of a peptide bond between γ-glutamate and cysteine by γ-glutamylcysteine synthetase (GSH1) and the later addition of glycine, catalyzed by glutathione synthetase (GSH2) using two ATP molecules for the whole process. The unique γ-glutamyl linkage is thought to protect GSH from degradation by proteases. The GSH degradation pathway is initiated enzymatically by γ-glutamyl transpeptidase.[5, 6]

1.2 Biological functions of glutathione

Glutathione is thought to play roles within sulfur biochemistry and metabolism. Glutathione is often implicated in a number of biological pathways; most of the proposed biological roles are aimed at fighting chemical challenges encountered in the life cycle.

1.2.1 Oxidative regulation of cellular sulfhydryls.

The formation of free radicals and reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) from respiration, the continuous generating of 2-oxoaldehydes from glycolysisand other fundamental metabolic pathways are considered to be significant chemical challenges encountered by living systems.[7] Glutathione is a remarkably active antioxidant where it reacts with many of these species, particularly in mitochondria, where these toxic chemicals are produced at high levels.[8] This antioxidant defense mechanism occurs twofold, glutathione can act as a free radical scavenger or it can bind to proteins to protect them against oxidation. In the cellular environment, glutathione mostly exist in its reduced form, where it can rapidly convert to the oxidized form when exposed to reactive oxidative species. GSH is commonly accepted to be the central redox agent in most aerobic organisms, and the cellular redox status depends on the relative amounts of the reduced and oxidized forms of glutathione (GSH/GSSG).[9] GSSG and GSH act as a redox coupled system that has been shown to be important for maintaining cellular redox homeostasis; this key role is evident in plants where this balance plays a role in oxidative signaling systems.[10] Experimental data indicate that GSH levels are constitutively higher in plants adapted to stress conditions, when GSH accumulates in

response to increased ROS, or to compensate for decreases in the defense capability of other antioxidants like catalase.[11,12]

An illustration of this ROS defense mechanism is the defense against hydrogen peroxide where this oxidant is reduced to water by GSH using a selenium-dependent GSH peroxidase. This process converts two glutathione molecules to one GSSG, where two liberated electrons are used to convert H_2O_2 to $2H_2O$. In the cell GSSG is rapidly reduced back to GSH by a GSSG reductase with use of NADPH, thereby forming a closed system (redox cycle) as illustrated in Figure 1.2.[13]

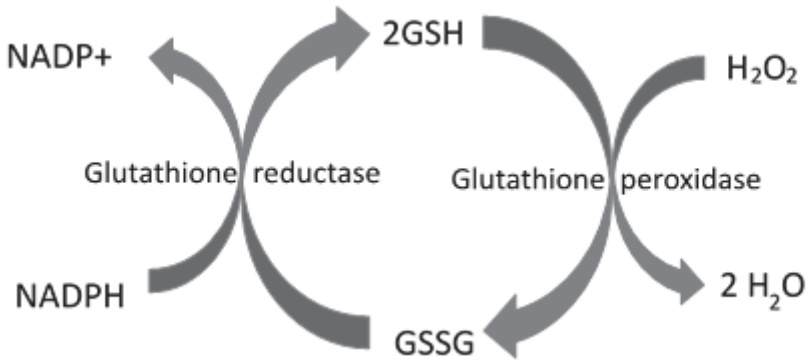

Figure 1.2 Mechanism of antioxidant activity of GSH in the presence of enzymes.

1.2.2 Thiol disulfide exchange

Cysteine residues of essential enzymes, generally need to be reduced back to their reduced, active state to fully restore their catalytic activity. This function is fulfilled by the thiol-disulfide exchange catalyzed by thiol-transferases in the presence of GSH.[14] The reaction of thiol-transferase is bidirectional and it helps to regulate certain metabolic pathways by activating or inactivating key enzymes. Many proteins are activated when a

key sulfhydryls are in the thiolate form, whereas others require them to be in the oxidized, disulfide form.[15]

1.2.3 Conjugation of electrophiles and metals.

A multitude of physiological functions, for both reduced (GSH) and oxidized (GSSG) glutathione were first identified by Hopkins, Hunter, and Eagles in the 1920s.[16] Being a versatile nucleophile glutathione also serves as the central metabolic network to remove or modify endogenous electrophilic compounds and numerous xenobiotics in most aerobic organisms.[17,18,19] Potentially dangerous xenobiotics like herbicides are bound by GSH, where GSH facilitates their sequestration away from sensitive sites in cells.[20] Additionally, almost all biologically available transition metal ions bind to thiol group of glutathione, allowing for GSH to play roles in transport, storage, and/or regulation of metal ion homeostasis in cellular systems. Interestingly, in the presence of some metal ions free cysteine can rapidly oxidize under aerobic conditions, thereby generating highly toxic hydroxyl radicals and other ROS by Fenton-like chemistry.[2] When the cysteine side chain is linked to glutamate and glycine in GSH, there is an apparent shift in the redox potential of this functional group protecting it from aerobic auto-oxidation.

1.3 Coordination Chemistry of Glutathione

Metals serve as critical micronutrients for development, growth, and persistence of almost all organisms. However higher concentrations can have adverse effects to living system, where a number of processes are required to regulate metal ion homeostasis. Moreover, as some metals likes cobalt (Co), copper (Cu), iron (Fe),

manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn) act to be essential components in cell organisms, there are a number of metals or metalloids like arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) which are toxic. Additionally, continuous up-take of metal ions increase the stress applied to the living system. Glutathione is one of the most versatile nucleophiles found in biology, and nucleophilic behavior can be used to solubilize, regulate, translocate, and store the redundant levels of metals within physiological systems. The thiol group form the cysteine residue of GSH is thought to interact with most metal ions directly or act as a cofactor for enzymes, which helps to control the cellular metal concentrations.[21]

1.3.1 Complexes of metal ions with glutathione

Metal ions bind GSH through a number of possible ligating functional groups. Oxidized glutathione has a disulfide linkage that forces two glutamic chelating systems close to each else making it a potentially strong chelator.

Metals like Pt, Pd, Hg, Pb, Cd, and Tl exhibit a similar binding mode in glutathione, which is typically govern thought hard/soft acid/base interactions. Late transition metals are generally strongly attracted to the thiolate functional group, which is considered a soft base. These metals then chelate with other Lewis bases functional groups. This processes is thought to start by anchoring to the thiolate group to the metal ion. Among the known adducts, the strongest association is seen with mercury, where the coordination is limited to thiol donation from two GSHs to make linear structures (GS-Hg-SG, CH3-GS-Hg).[22,23]

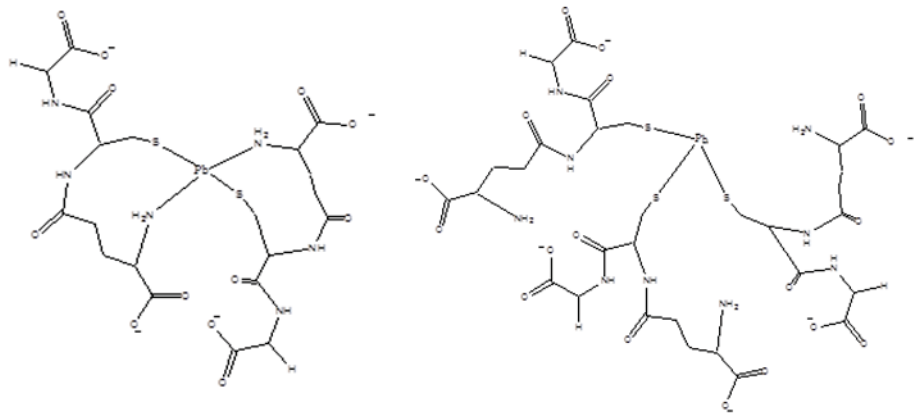

Figure 1.3 Proposed Structures for Lead(II) Glutathione Complexes $Pb(GSH)_2$ (Left) and $Pb(GSH)_3$ at pH 8.5, (reproduced from literature)

Lead is another heavy metal found in cellular systems, at high concentrations of Pb^{2+} ions a decrease in approximately 50 % of the activities of glutathione-based enzymes in blood was observed.[24] Different $Pd(GSH)_x$ species have been observed at various pH values, with thiol and amino coordination modes being the primary chelates to the metal ion. The Lewis acidity of the metal ion changes the pK_a values for many ionizable groups in GSH.[25] Additionally concentration of GSH impacts the species formed between Pb^{2+}-glutathione complexes, this phenomena become very apparent at pH 8.5. At high ligand concentrations the stoichiometry of coordinating species increases from two to three ligands, so that $Pb(GSH)_2$ and $Pb(GSH)_3$ complexes are in equilibrium with each other at pH 8.5 (Figure 1.3).[26]

At low pH thiol coordination is favorable for soft metal ions. Cadmium metal binds to sulfhydryl of glutathione at pH 3.5 with subsequent chelation with the carboxylates of glycine and glutamate, respectively. Seven possible stoichiometric forms of glutathione and cadmium ions have been reported at various pH values.[27] More

recently a study on the cadmium-glutathione adduct in solution suggest that $Cd(GSH)_2$ is the most stable and predominant form of this adduct in solution at physiological pH. Despite the fact that there are two non-chelating carboxylate residues from the two ends of the peptide chain, only water molecules have been proposed to occupy the empty or labile coordination sites of the tetrahedral metal center.[28]

Earlier studies done using NMR and EPR of Ni^{2+}-Glutathione complexes the Ni-GSH complex favors octahedral geometry with only carboxylic acid donor groups up to pH 5. Between pH 5-8 there exist an equilibrium between a diamagnetic, square-planar complex formed from interactions between Ni and glycine, and an octahedral complex when the glutamate is involved in coordination to Ni. At pH higher than 11, the predominant geometry is the square planar complex, where Ni^{2+} is bound to only thiol and Glu-Cys amide groups.[29] Similar geometries and coordination modes have been observed when a study was reported on the redox activity of Ni^{2+}-glutathione complexes.[30] This report further concludes that Ni^{2+} accelerate the air oxidation of glutathione at alkaline conditions, and discusses the most susceptible molecular form of GSH is the HL^{2-} form, where the ionic interactions of its protonated amine and deprotonated thiol dominate the interactions with the metal ion.[31] More interestingly, Ni^{2+} showed different coordination modes with different molar ratios of ligands. Ni^{2+} chelation by oxidized glutathione (GSSG) has been also studied, where this species was found to be a low spin complexes.[32]

Spectrophotometric and EPR studies have shown that copper binds to both reduced (GSH) and oxidized (GSSG) glutathione.[33] It is suggested that GSH makes tetragonal complexes having CuHL and $Cu(HL)_2$ prospective stoichiometry at pH 6.5 and

lower, whereas the glutamate moiety and at higher pH solutions helps to facilitate the Cu(II) reaction to generate Cu(I) by oxidizing GSH to GSSG.[34,35] An X-ray absorption study revealed that the Cu(I) at higher GSH concentrations binds to 2 or 3 sulfur donors with possible structures of Cu(I)-glutathione polymeric features similar to Cu(I)-metallothioneins binding.[36] Formation of Cu(II) complexes with oxidized glutathione is also observed at low pH systems having Cu(I)-$[GSH]_2$ stoichiometry. Copper is the only metal ion found to bind with the disulfide bond of GSSG. At lower pH Cu only binds to the glutamic moiety and the thiol coordination only occurs at neutral or alkaline pH where the coordination geometry is completely changed.[37,38] Additionally Fe(III) is another metal ion that undergoes a reduction reaction coupled to GSH binding and oxidation. Mössbauer spectroscopic studies of a reaction started with Fe(III) and glutathione was found to be unstable and Fe(III) rapidly reduced to Fe(II). Ultimately complex with both reduced and oxidized glutathione coordinated via carboxylate groups in pH around 3-7. Much weaker complex formation has been observed at alkaline conditions, pH ~ 8, resulting in a $Fe(OH)_2$ precipitate.[39,40]

Cobalt forms two kinds of complexes, an octahedral amide like complexes similar to the Ni tetrahedral type coordination mode, where the Cys-Gly peptide bond was found to be a donor. X-ray absorption spectroscopy data show structure of Co-GSH in solution is primarily in the Co(III) state in highly alkaline conditions. However the most stable structure formed involves the carboxylate of Gly, thiol, and Glu-Cys amide stabilizing an octahedral coordination geometry surrounding the cobalt ion. No dependence on concentrations have shown in complexing and the stoichiometry was always 1:1 at both pH systems.[41]

Zinc complexation of glutathione has been studied in a number of ways, and reasonable models of Zn-GSH of solution structures have been proposed. Based on pH titration studies Zn^{2+} ions in acidic pH coordinates with the N-terminal amine and carboxylate for the glutamate residue allowing the thiol to remain free and active.[42] More recently it was determined that even though Zn^{2+} is first row transition metal, its malleable coordination geometries often look similar to Cadmium(II). Zn^{2+} is a borderline soft metal ions, while Cd^{2+} is a soft metal. An NMR study revealed the involvement of a Zn—S coordination mode in the entire pH range studied. The coordination mode is highly dependent on both the molar ratio and pH of the sample, where a number of structures have been proposed (Figure 1.4.) At more alkaline conditions, a binuclear structure was common and Glu-Cys peptide nitrogen is deprotonated and bind Zn^{2+} ions.[43,44]

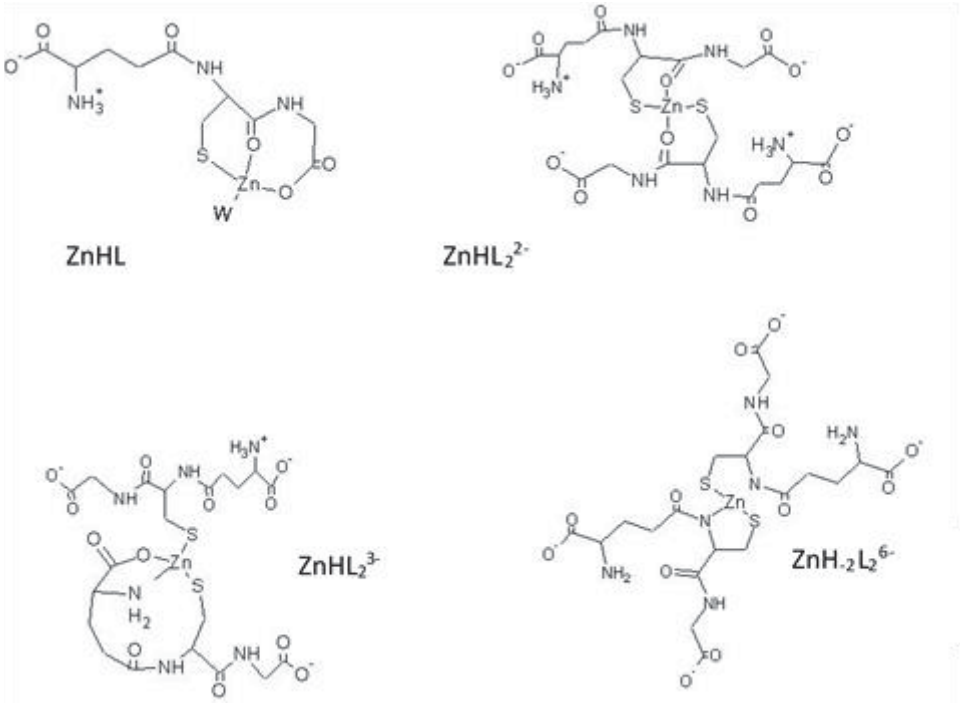

Figure 1.4 Proposed structures of Zn-GSH complexes using NMR, (reproduced from literature)

CHAPTER II

MATERIAL AND METHODS

Understanding the thermodynamics of metal ion binding to GSH and GSSG may provide some insight regarding the structure and functions of this biological molecule. Here we report our efforts to characterizing the thermodynamics of Zinc(II) coordination to GSH and GSSG by isothermal titration calorimetry. This highly sensitive technique is as an excellent method to determine the association constant (K_{ITC}), enthalpy change (ΔH_{ITC}), and binding stoichiometry (n) of an equilibria, especially between macromolecules and its substrates. Here the thermodynamic parameters of zinc(II) binding to glutathione have been studied using ITC techniques along with use of Raman spectroscopy.

2.1 General procedures for the thermodynamic observation of Zn binding to Glutathione

All solutions and media were made using 18 MΩ water filtered using a Millipore Ultra purification system. All media, buffers, and salts purchased were of the highest grade available and used as received. GSH (Reduced glutathione) and GSSG (Oxidized glutathione) were purchased through Sigma Aldrich at 99 % purity and used as received.

2.1.1 Isothermal titration calorimetry (ITC)

Reduced glutathione solutions were prepared by dissolving the glutathione sample in buffer at a concentration of 0.4 mM. Samples were buffered at pH 7.4 with 100 mM MOPS. GSH was titrated with 4.0 mM $Zn(NO_3)_2$ under anaerobic conditions. All solutions were septum sealed and made anaerobically by purging with argon for 15 minutes. The MicroCal VP-ITC instrumentation was sealed in a Plas-Labs anaerobic chamber with a constant dinitrogen flow during the course of the experiment.

ITC experiments were carried out at 25 ˚C unless otherwise indicated, using 10 μL injections of $Zn(NO_3)_2$, with 300 s spacing between injections to allow full return of power to baseline. Data directly taken from calorimeter was corrected for dilution and integrated. The corrected isotherms were fitted with the Origin 8.0 software package provided by MicroCal, and an ITC calorimetry software developed by Edwin Lewis and co-workers at Mississippi State University (CHASM), which uses nonlinear least squares algorithms.[45] Binding was performed in four different buffers, MOPS, HEPES, Tris, and PIPES. Experiments were replicated at a minimum of twice, however most experiments were repeated three to four times.

2.1.2 ITC data analysis

Isothermal titration calorimetry is a well-known technique that uses to directly characterize thermodynamics of binding interactions of biomolecules (protein, DNA) to ligands (metal ions or substrates). ITC is highly sensitive and fairly rapid technique.[46] The general workings of the ITC instrument can be simplified to a syringe that contains the titrant, typically small molecule or the ligand which is added into the macromolecule solution. The instrument the adds or removed power to maintain the reaction temperature

with respect to a reference cell. From this power measurement over time, three parameters can be determined the heat of binding for the reaction (ΔH), molar ratio (n), and binding constant (K) can be directly extracted from the fitted model curve, which allows for calculation of Gibbs free energy (ΔG) and entropy (ΔS) for the system using the following thermodynamic equations:

$$\Delta G = - RT \ln K \qquad 2.1$$

$$\Delta G = \Delta H - T\Delta S \qquad 2.2$$

It is important to note that all calorimeters, measure the sum of the heat associated with all processes occurring within the cell which is not necessarily limited to dilution, hydrolysis, and sometimes redox reactions, occurring upon addition of aliquots of the titrant. If M, P and B are metal, protein, and buffer, respectively, and ΔH is change in enthalpy associated with the reactions, all individual equilibria that contribute to heat extracted from ITC can be summarized as;

$M + P \rightleftarrows MP$	1	ΔH_{MP}
$MB \rightleftarrows M + B$	X	$-\Delta H_{MB}$
$HP^+ \rightleftarrows H^+ + P$	Y	$-\Delta H_{HP}$
$B + H^+ \rightleftarrows HB^+$	Y	ΔH_{HB}

An equation for the overall enthalpy can be written as follows, by adding all the enthalpies according to the Hess's law;

$$\Delta H_{ITC} = \Delta H_{MP} - x\Delta H_{MB} - y\Delta H_{HP} + y\Delta H_{HB} \qquad 2.3$$

The condition independent enthalpy (ΔH) and the number of proton release of binding can be determined following this equation.

CHAPTER III

RESULT AND DISCUSSION

Coordination chemistry of metal binding to small molecules like glutathione can be studied using isothermal titration calorimetry to measure heat of interaction for a binding process. Isothermal titration calorimetric studies of the formation of Zn-GSH and Zn-GSSG were carried out in four buffers at pH 7.4 and at 25 ˚C to interrogate thermodynamic parameters ΔG, ΔH, and ΔS for each equilibrium. The reactions were carried out under anaerobic conditions as glutathione autoxidizes in the presence of O_2. The raw heats and integrated isotherms for the binding reactions of Zn^{2+} to reduced glutathione in MOPS, HEPES, Tris, and PIPES buffers are shown in Figure 3.1 to 3.4.

The thermodynamic parameters extracted from these curves cannot be taken as intrinsic values for the particular binding event. These data also include heats contributing from other chemical phenomena in addition to desired reaction. Metal ions can undergo a number of interactions in solution such as redox reactions, dilutions, hydrolysis, and precipitation. The heat of any or some of these undesired interactions are often associated with total heat measured in an ITC experiment and can add to the complexity of their system. Metal dilution heats need to be measured independently and then subtracted before the isotherms are fitted to model curves. Subtraction of Zn-buffer titration heats from the integrated raw data gives baseline-corrected isotherms. Figure 3.1 to 3.4 showed in here are baseline subtracted and integrated. Metal dilution heats of

MOPS, PIPES, and HEPES were endothermic and quite similar in size. In Tris however, the dilution heat was very exothermic.

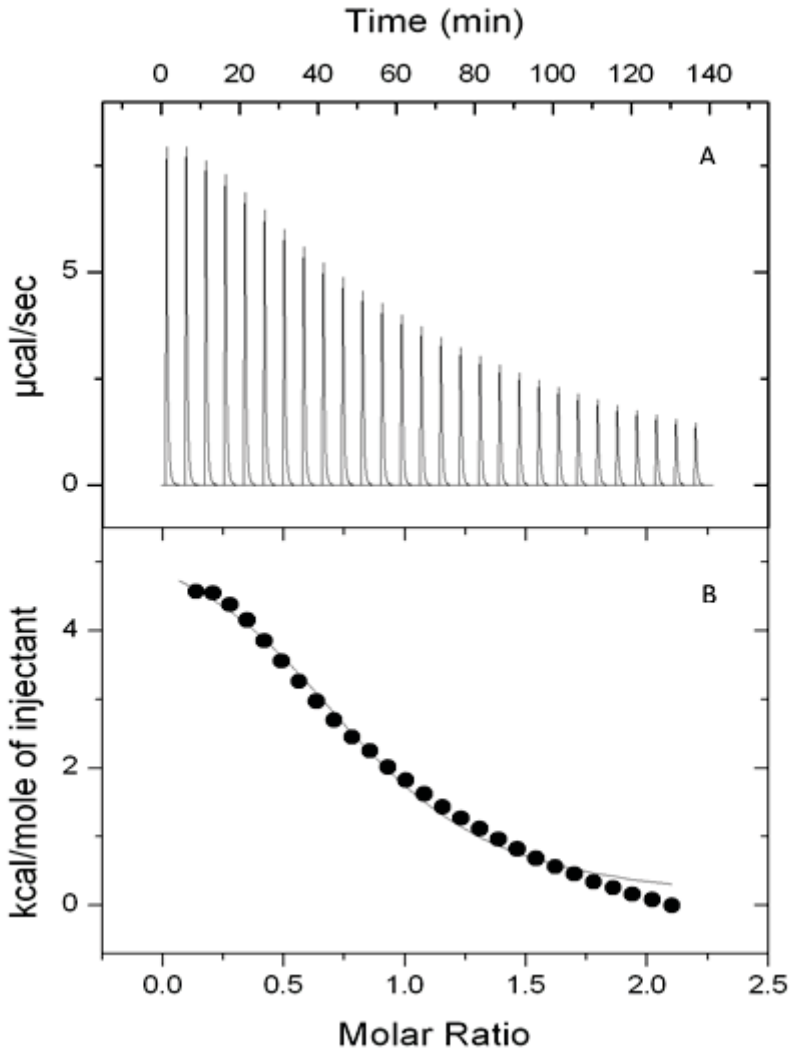

Figure 3.1 ITC data zinc binding to GSH in 100 mM PIPES buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$ 28 × 10 µL with 0.4 mM GSH
(Bottom) Integrated isotherm and the best associated fit for one site binding model

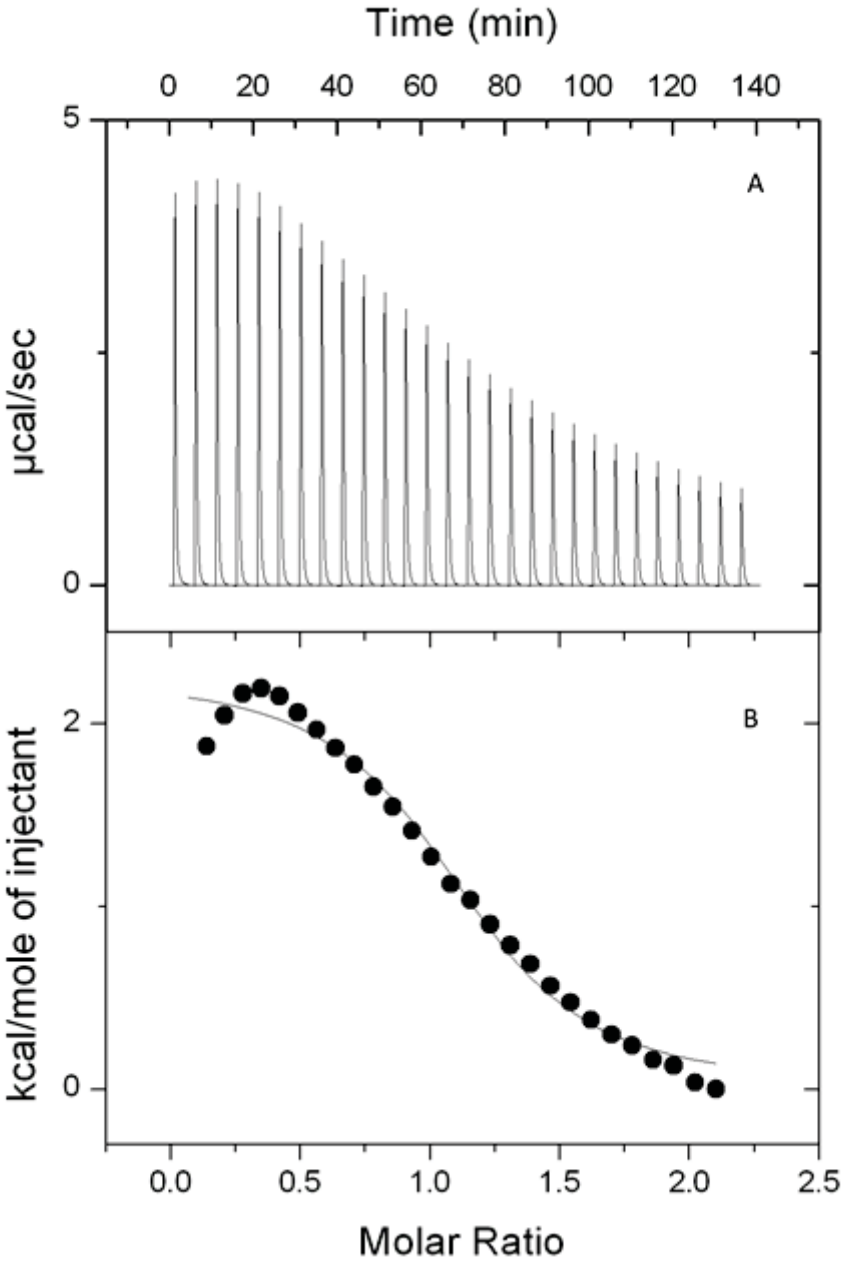

Figure 3.2 ITC data zinc binding to GSH in 100 mM MOPS buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$ 28 × 10 µL with 0.4 mM GSH
(Bottom) Integrated isotherm and the best associated fit for one site binding model

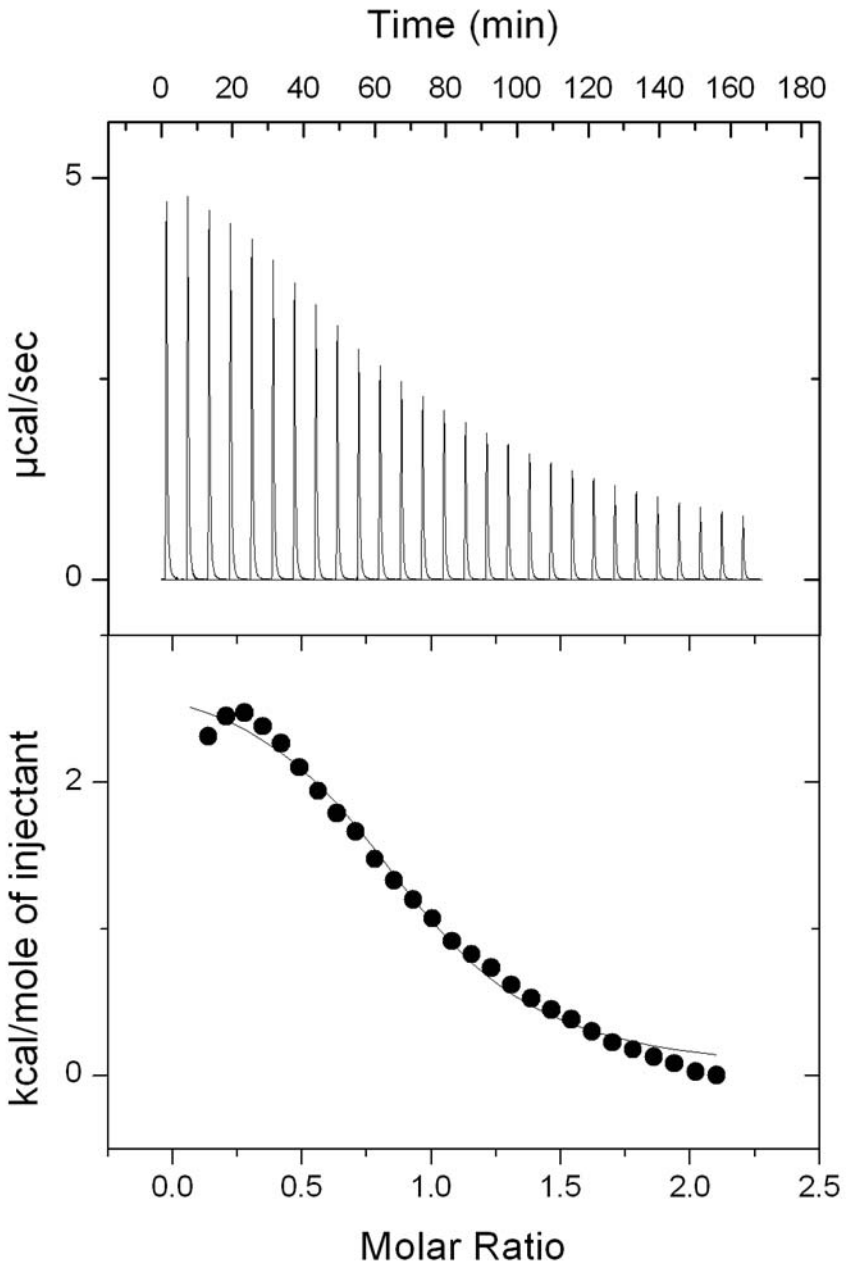

Figure 3.3 ITC data zinc binding to GSH in 100 mM HEPES buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$ 28 × 10 µL with 0.4 mM GSH
(Bottom) Integrated isotherm and the best associated fit for one site binding model

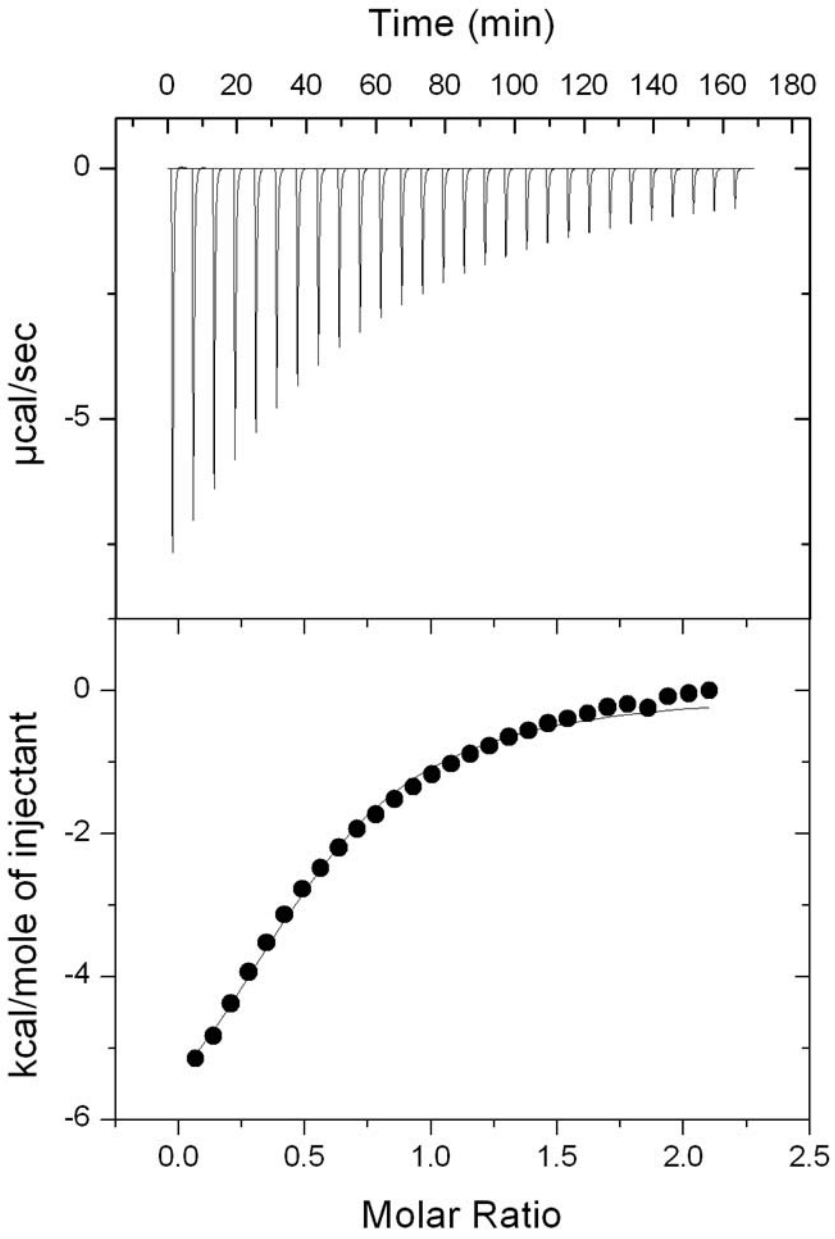

Figure 3.4 ITC data zinc binding to GSH in 100 mM Tris buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$ 28 × 10 µL with 0.4 mM GSH
(Bottom) Integrated isotherm and the best associated fit for one site binding model

The experimental thermodynamic values for the binding reaction in each buffer are listed in Table 3.1. The K_{ITC} and ΔH_{ITC} was directly extracted from the fitted curve

and the change in free energy, ΔG_{ITC} and change in entropy, ΔS_{ITC} of glutathione Zn^{2+} binding equilibrium are calculated using the following equations 2.1 and 2.2.

Table 3.1 Best fit values for Zn^{2+} binding to GSH from ITC experiments

Buffer	n_{ITC}	$K_{ITC} \times 10^4$	ΔH_{ITC} (kcal/mol)	ΔG_{ITC} (kcal/mol)	$-T\Delta S_{ITC}$ (kcal/mol)
MOPS	0.9	4.2 ± 0.2	2.2 ± 0.2	-6.3 ± 0.1	-8.5 ± 0.1
HEPES	1.0	3.4 ± 0.4	2.3 ± 0.1	-6.2 ± 0.3	-8.5 ± 0.2
Tris	0.9	1.2 ± 0.1	-7.4 ± 0.1	-5.5 ± 0.1	1.8 ± 0.1
PIPES	1.0	1.0 ± 0.2	5.6 ± 0.3	-5.5 ± 0.1	-11.1 ± 0.2

Corrected data in all buffers were best fitted to a one-site binding model. As described in the methods section, during a chemical binding experiment, protons are either released or consumed by the system and these protons are regulated by the buffer to maintain the pH. Heat of buffer ionization and metal-buffer interactions produced during this event is well quantified and recorded at specific pH values for different buffers. Using these previous reported energies, we calculated the number of protons released by the following mathematical relationship.[47]

$$\Delta H_{ITC} + \Delta H_{MB} = n\,(\Delta H_{HB}) + \Delta H_{(HP + MP)} \qquad 3.1$$

The observed heat for the binding reactions, ΔH_{ITC} and metal buffer interaction energy ($\Delta H_{ITC} + \Delta H_{MB}$) were plotted against the change in enthalpy of ionization of each buffer (ΔH_{HB}) obtaining a linear relationship which can be fit to Equation 3.1 to get the number of proton release (n) from the slope of the line. The heat for the reaction, independent of the ionization of the buffer ($\Delta H_{(HP + MP)}$), is 11 kcal/mol, as indicated by

the y-intercept, and approximately 1.8 proton release could be seen upon binding Zn^{2+} to GSH.

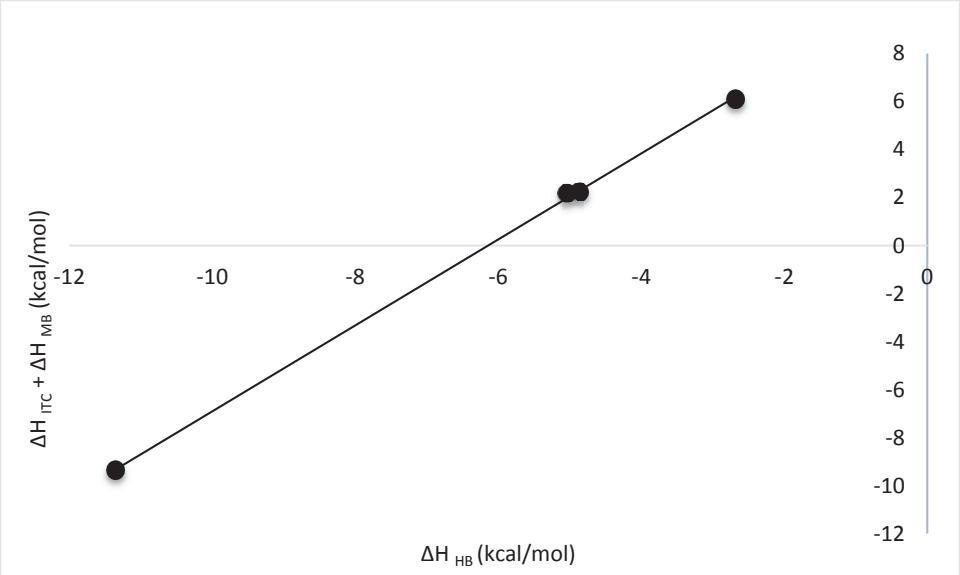

Figure 3.5 Proton release from binding reaction of Zn^{2+} to GSH

Plot of observed enthalpies from ITC and metal-buffer interactions, $\Delta H_{ITC} + \Delta H_{MB}$ versus buffer ionization enthalpies, ΔH_{HB}, for various buffers at pH 7.4. The resulting linear relationship can be fit to the equation $y = 1.79x - 10.99$ with an R^2 value of 0.99.

The values for ΔH_{ITC}, ΔH_{HB}, and ΔH_{MB} in various buffers are listed in Table 3.1 and Table 3.2. Once the number of protons released to system was clarified the binding energies were further elucidated using a thermodynamic cycle similar to those developed by Wilcox and co-workers.[48] The overall energy extracted from ITC can be donated as summation of dissociation of Zn^{2+}-Buffer complex and association of Zn^{2+}-GSH complex.

Table 3.2 Summarized binding constants and formation enthalpies for Zn-Buffer and H-Buffer.

Buffer	$\log K_{H\text{-}Buffer}$[a]	$\Delta H_{H\text{-}Buffer}$[a] (kcal/mol)	$\log K_{Zn\text{-}Buffer}$[b]	$\Delta H_{Zn\text{-}Buffer}$[b] (kcal/mol)
MOPS	7.18	-5.04	3.22	-0.09
HEPES	7.17	-4.86	3.23	-0.08
Tris	8.1	-11.35	2.27	-2.02
PIPES	6.93	-2.68	3.07	0.46

a Value from reported literature,[49] b value from reported literature.[50,51]

All the possible individual equilibria were considered, to examine each of the energies associated to explicate the absolute enthalpy and binding constant of Zn^{2+} ion binding to GSH molecule. That consist of four different equilibria as follows: (1) Dissociation of Zn^{2+}-Buffer complex (2) Deprotonation of GSH and/or surrounding molecules (3) Binding of Zn^{2+} ions to GSH molecule (4) Interaction of buffer with protons released while Zn-GSH complexation. Equilibria 1 and 4 are documented in literature for various buffers and only Zn-HEPES association heat was experimentally determined. These values could be easily referred, as all our experiments were carries out in 100 mM buffer at pH 7.4. The higher concentrations of buffer allows for only slight reduction in the buffering capacity from Zinc-buffer complex formation while maintaining pH.

Table 3.3 Thermodynamic cycle for Zn^{2+} binding to GSH in 100 mM MOPS at pH 7.4

	Coef	ΔH (kcal/mol)	ΔG (kcal/mol)
Zn^{2+}MOPS + $H^+{}_x$(GSH) ⇄ xH^+(MOPS) + GSH-Zn + (1-x) MOPS		2.2[a]	-6.3
H^+ + MOPS ⇄ H^+(MOPS)	1.8	-5.04[b]	-9.78
Zn^{2+}(MOPS) ⇄ MOPS + Zn^{2+}	1	0.09[c]	-4.40
H^+-GSH ⇄ H^+ + GSH	1.8		
-SH ⇄ H^+ + S^-	0.8	6.2[d]	12.6
$-NH_3^+$ ⇄ NH_2 + H^+	1	10.0[d]	12.8
Zn + GSH ⇄ Zn-GSH	1	-3.8	-7.1

a value from direct ITC measurement (Table 3.1), b and c value from Table 3.2, d value from literature[52]

Table 3.4 Thermodynamic cycle for Zn^{2+} binding to GSH in 100 mM PIPES at pH 7.4

	Coeff	ΔH (kcal/mol)	ΔG (kcal/mol)
Zn^{2+}PIPES + $H^+{}_x$(GSH) ⇄ xH^+(PIPES) + GSH-Zn + (1-x) PIPES		5.6 [a]	-5.46
H^+ + PIPES ⇄ H^+(PIPES)	1.8	-2.68 [b]	-9.44
Zn^{2+}(PIPES) ⇄ PIPES + Zn^{2+}	1	-0.46[c]	-4.20
H^+-GSH ⇄ H^+ + GSH	1.8		
-SH ⇄ H^+ + S^-	0.8	6.2[d]	12.6
$-NH_3^+$ ⇄ NH_2 + H^+	1	10.0[d]	12.8
Zn + GSH ⇄ Zn-GSH	1	-4.0	-7.1

a value from direct ITC measurement (Table 3.1), b and c value from Table 3.2, d value from literature[51].

Table 3.5 Thermodynamic cycle for Zn^{2+} binding to GSH in 100 mM HEPES at pH 7.4

	Coeff	ΔH (kcal/mol)	ΔG (kcal/mol)
Zn^{2+}HEPES + H^+_x(GSH) ⇄ xH^+(HEPES) + GSH-Zn + (1-x) HEPES		2.3[a]	-6.1
H^+ + HEPES ⇄ H^+(HEPES)	1.8	-4.86[b]	-9.78
Zn^{2+}(HEPES) ⇄ HEPES + Zn^{2+}	1	0.08[c]	-4.40
H^+-GSH ⇄ H^+ + GSH	1.8		
-SH ⇄ H^+ + S^-	0.8	6.2[d]	12.6[d]
$-NH_3^+$ ⇄ NH_2 + H^+	1	10.0[d]	12.8[d]
Zn + GSH ⇄ Zn-GSH	1	-3.9	-7.0

a value from direct ITC measurement (Table 3.1), b and c value from Table 3.2, d value from literature[51].

The estimated thermodynamic parameters associated with Zn^{2+} binding to GSH were $\Delta H_{Zn\text{-}GSH}$ = -3.8 kcal/mol and $\Delta G_{Zn\text{-}GSH}$ = -7.1 kcal/mol in MOPS buffer system. Upon this reaction of Zn^{2+} ion binds to the glutathione molecule approximately 1.8 protons are released from the system. These protons can be attributed to several different groups surrounded in the reaction sphere. These can either be coming from the glutathione molecule itself or other solvents that deprotonate upon coordination Zn^{2+} ion. Among the protential binding groups of glutathione, two caboxylates are already deprotonated at working pH and the thiol and the protonated amine group can be considered as next candidate ligands. Zinc bound waters or buffers can also participate in H^+ release, but pK_a of water is fairly higher than that of glutathione donar groups and all of the buffers expect Tris might have already deprotonated as of their pK_a values. In deciding the best possible complex structure of this one site binding event we see in ITC, previously proposed complex models, mainly based on NMR studies and the results from Raman spectroscopic studies we collected were taken into consideration. Though the

binding event of Zn-GSH is fitted to one site binding model, the residual heat that is not well fit from the curve was observed in the isotherm. This should be a small contribution of some other Zn binding equilibrium. One possiblity might be Zn binding to two different spicies of gluthione present in the ITC cell with different ionized state or perhaps a small amount of GSSG is protect in the samples. The observation of multiple states of glutathione was reported by the Bal group at physiological pH.[4] However these points in the ITC data do not warrant multiple independent binding models, because that only appeared in vey small percentage which is neglegible when considering the whole process. Some of the reported complex structures of Zn-GSH which were shown in introduction (Figure 1.3), especially by Bal and the group, were taken into the consideration as those agree with the experimetal conditions and results of ours.[4] Both amine and thiol groups are considered to be feasible where they undergo deprotonation at the working pH. The pK_a of thiol is found to be 8.93 and amine is 9.28 and it has been established binding of metals induces a pK_a shift due to their Lewis acidity.[53,54] The Raman data provides many vibrational modes of the metal. But it is clear the reduction of thiol peak in at 2585 cm^{-1} where the S-H stretch is, and Zn-S is generated when Zn^{2+} binds GSH.

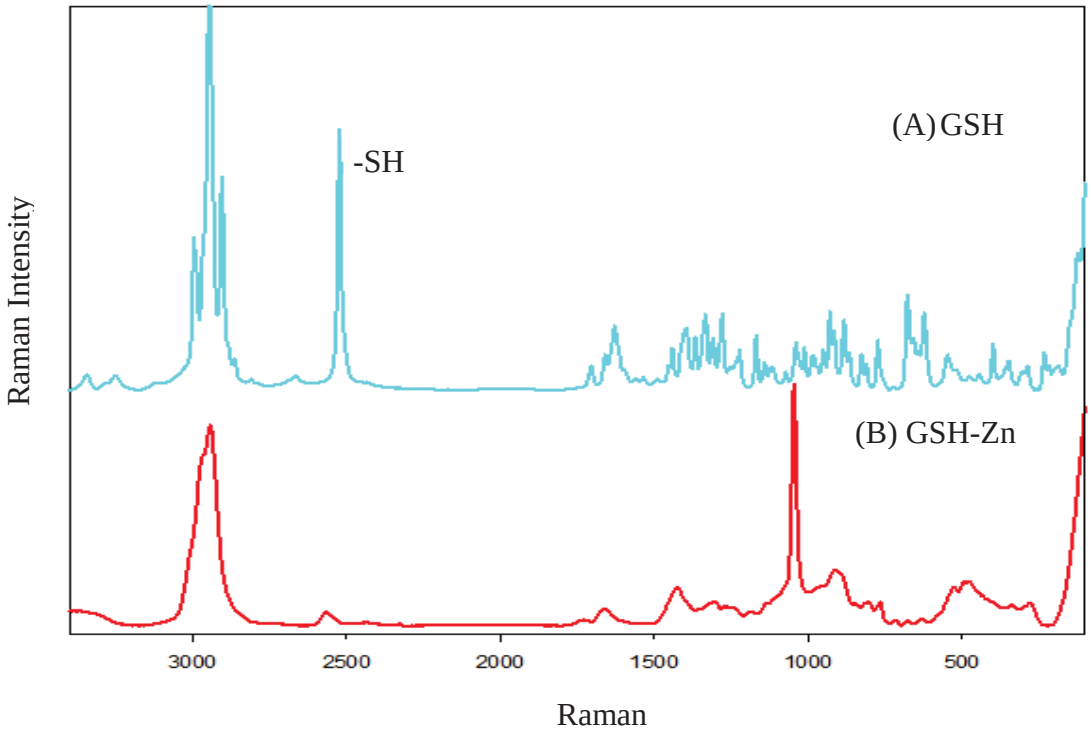

Figure 3.6 Raman spectra of (A) GSH, (B) Zn-GSH (excitation λ= 532 nm)

Solid samples of C= 0.1625M, Zn-GSH reacted 1:1 molar ratio, lyophilized for 48 hrs.

Table 3.6 Summary of pH and buffer independent thermodynamic values for Zn^{2+} binding to GSH

	$K \times 10^5$	ΔH (kcal/mol)	ΔG (kcal/mol)	-TΔS (kcal/mol)
MOPS	1.8	-3.8	-7.1	-3.4
HEPES	1.5	-3.9	-7.0	-3.0
PIPES	1.7	-4.0	-7.1	-3.1
Average	1.7 ± 0.2	-3.9 ± 0.1	-7.1 ± 0.1	-3.1 ± 0.2

Thermodynamic parameters calculated for the binding reaction of Zn^{2+} to glutathione in various buffers are consistent with one another as showed in Table 3.6. The average pH and buffer independent association constant (K_a) is 1.7×10^5. The Gibbs free

energy for the binding reaction is favorable at -7.1 kcal/mol. The energy is distributed into both entropic and enthalpic factors indicating that Zn^{2+} binding is both an enthalpically and entropically driven process, as indicated by the negative values of -3.9 kcal/mol and -3.1 kcal/mol for ΔH and –TΔS, respectively. This result provide insight into how glutathione interact with Zn^{2+} metal ions. Even though this is a spontaneous process, the experimentally measured binding constant is not as high as those reported for some heavy metals. As an essential metal ion present in biological systems Zn^{2+} binding of glutathione might require this weaker interaction to allow for efficient regulation and transportation of Zn^{2+} ions rather than very strong interactions that would lead to excrete out from the system. A moderate binding constant will facilitate both association and dissociation of zinc ions where ever it is needed.

Figure 3.7 The Thermodynamic Profile for Zn^{2+} binding to reduced glutathione (GSH)

The entropy term is reported herein as –TΔS to provide a direct comparison to the other thermodynamic terms in kcal/mol.

Similarly, oxidized glutathione (GSSG) was subjected to the same set of experimental conditions as those used in Zn-GSH binding to determine the thermodynamic parameters of Zn^{2+} binding to GSSG. ITC titrations of binding event was carried out in same four buffer systems, MOPS, PIPES, HEPES, and Tris. Any heat of dilution from the control experiments was subtracted from the relevant isotherms.

Zn-GSSG binding isotherms in each buffer were fit to a one site binding curve and the binding constants and enthalpies were directly determined from the model curves while the rest of the thermodynamic parameters are calculated using thermodynamic equations and are tabulated in Table 3.7.

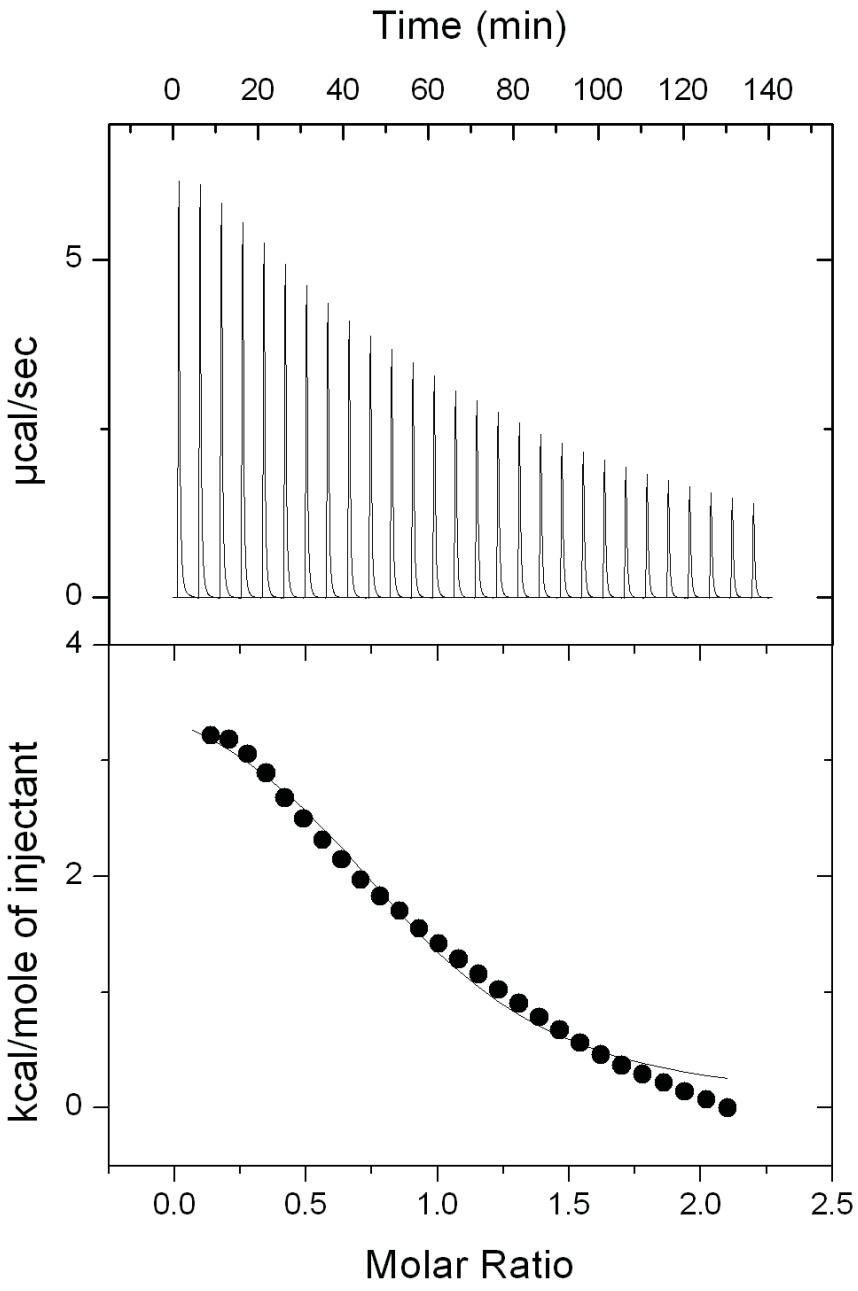

Figure 3.8 ITC data zinc binding to GSSG in 100 mM PIPES buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$ 28 × 10 µL with 0.4 mM GSH
(Bottom) Integrated isotherm and the best associated fit for one site binding model.

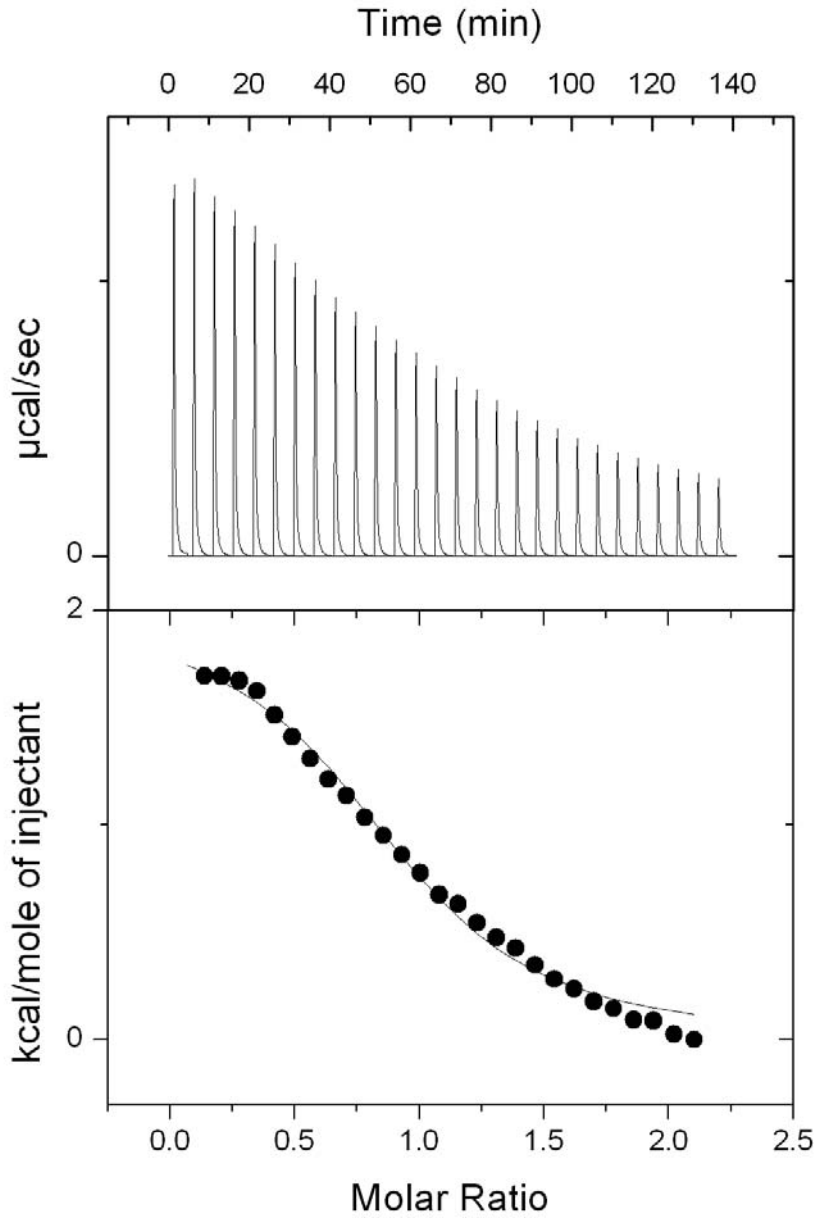

Figure 3.9 ITC data zinc binding to GSSG in 100 mM MOPS buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$, 28 × 10µL with 0.4 mM GSSG
(Bottom) Integrated isotherm and the best associated fit for one site binding model

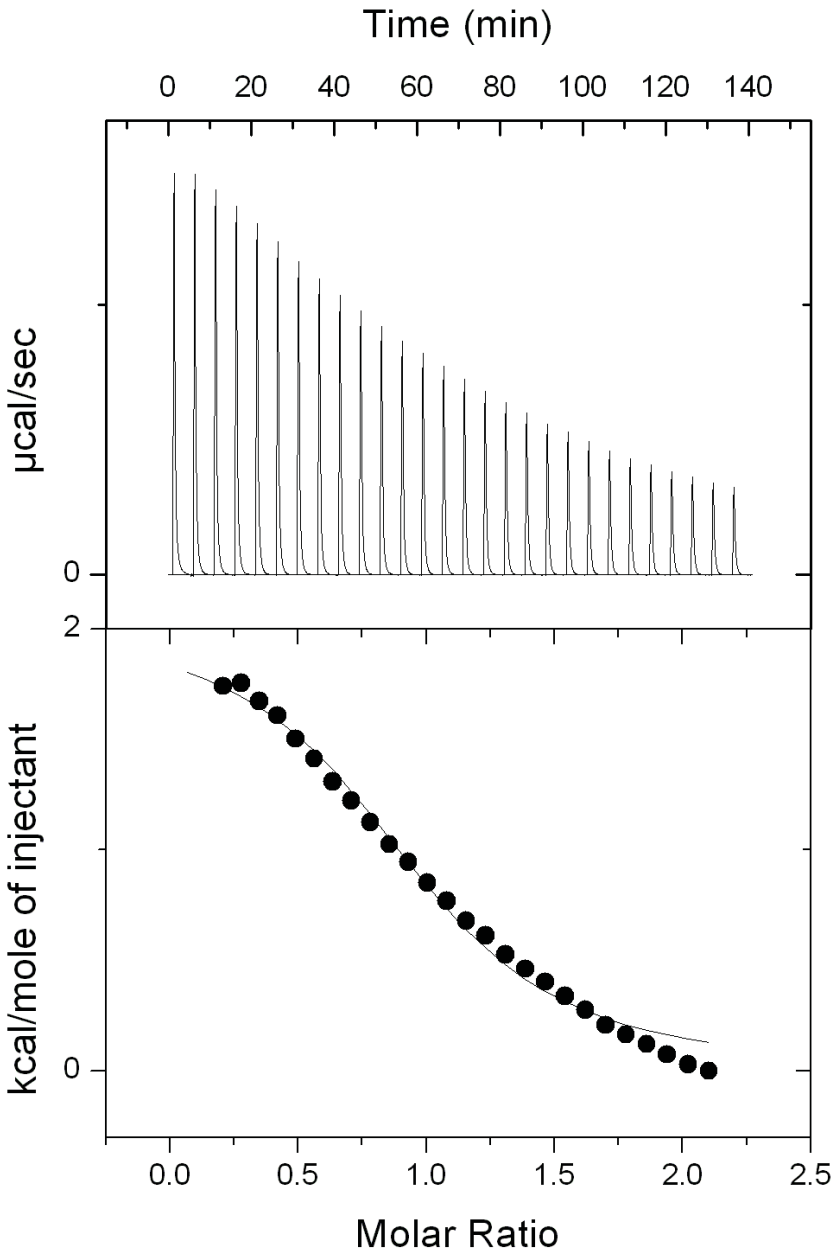

Figure 3.10 ITC data zinc binding to GSSG in 100 mM HEPES buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$, 28 × 10µL with 0.4 mM GSSG
(Bottom) Integrated isotherm and the best associated fit for one site binding model.

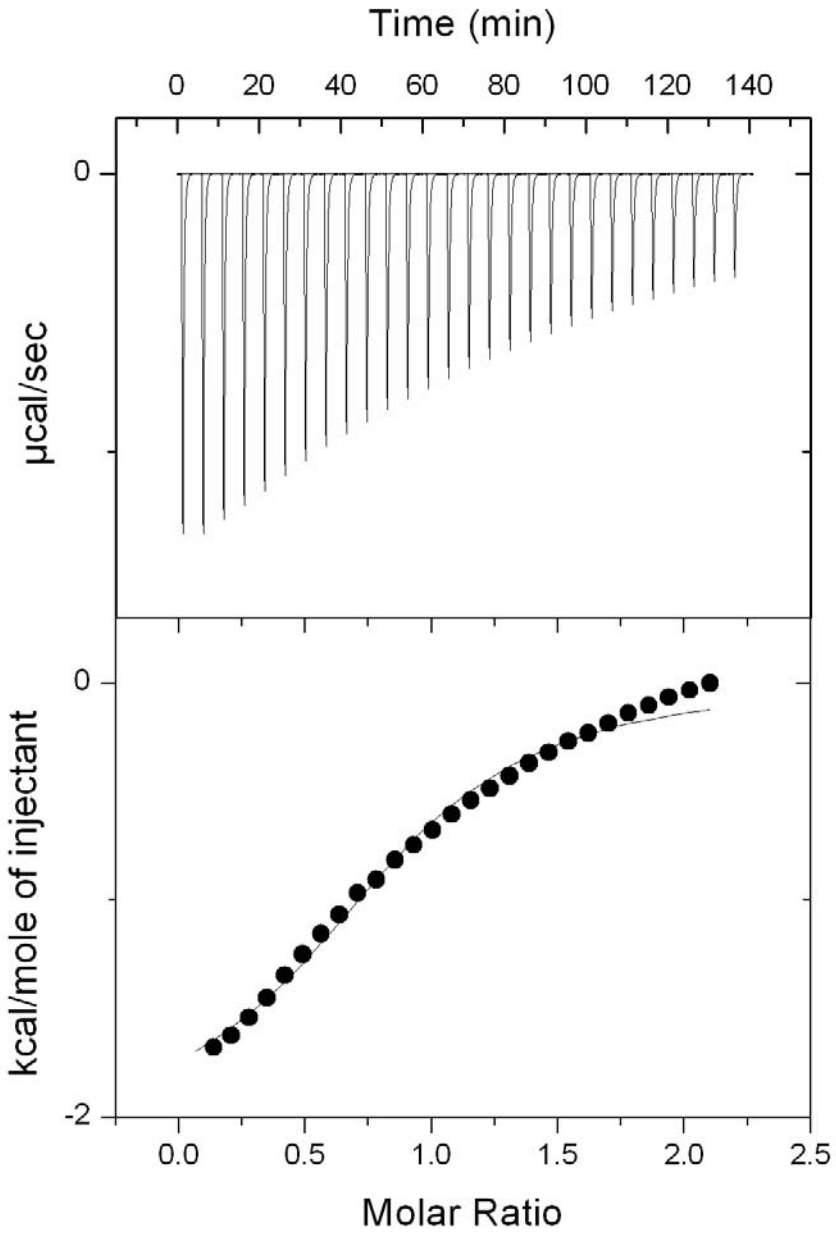

Figure 3.11 ITC data zinc binding to GSSG in 100 mM Tris buffer, pH 7.4

(Top) Raw data from titration of 4 mM $Zn(NO_3)_2$, 28 × 10µL with 0.4 mM GSSG
(Bottom) Integrated isotherm and the best associated fit for one site binding model

The number of protons released in GSSG can be estimated based on the linear dependence of the addition of observed heat from ITC (ΔH_{ITC}) and metal-buffer

interaction enthalpy (ΔH_{MB}) versus the change in ionization enthalpy each buffer (ΔH_{HB}) as shown in Figure 3.12.

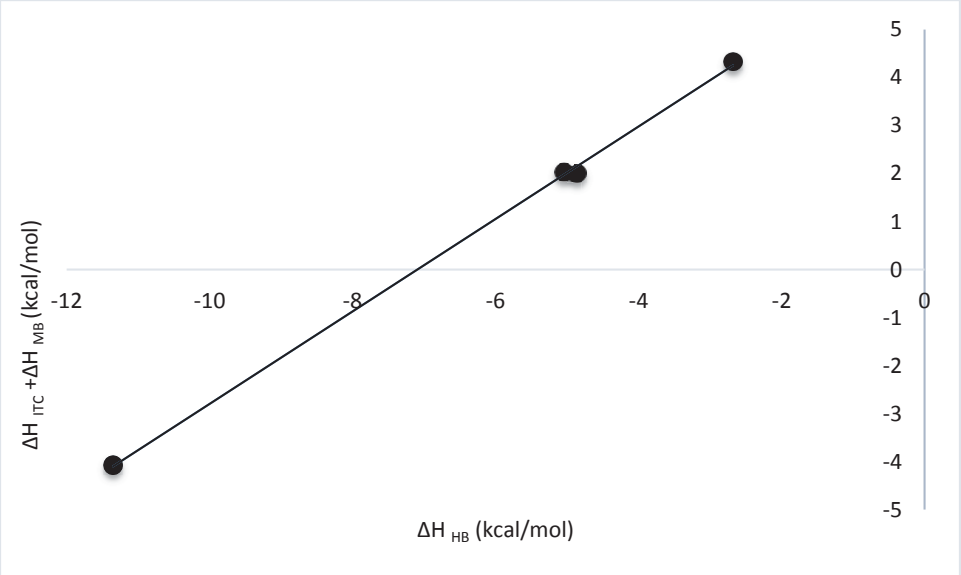

Figure 3.12 Proton release from binding reaction of Zn^{2+} to GSSG

Plot of observed enthalpies from ITC and metal-buffer interactions, $\Delta H_{ITC} + \Delta H_{MB}$ versus buffer ionization enthalpies, $\Delta H_{HB,}$ for various buffers at pH 7.4. The resulting linear relationship can be fit to the equation $y = 0.96x – 6.83$ with an R^2 value of 0.99.

The linear relationship is fit to Equation 1.2, the slope of the curve suggests approximately one proton is displaced by binding reaction. The pH and buffer independent thermodynamic parameters of Zn^{2+} binding to oxidized glutathione can be evaluated using the thermodynamic cycle defined previously with more structural information.

Table 3.7 Best fit values for Zn^{2+} binding to GSSG from ITC experiments

Buffer	n_{ITC}	$K_{ITC} \times 10^4$	ΔH_{ITC} (kcal/mol)	ΔG_{ITC} (kcal/mol)	$-T\Delta S_{ITC}$ (kcal/mol)
MOPS	0.9	2.1 ± 0.1	2.1 ± 0.1	-5.9 ± 0.2	-8.0 ± 0.1
HEPES	1.0	1.7 ± 0.4	2.1 ± 0.3	-5.8 ± 0.3	-7.9 ± 0.2
TRIS	0.9	1.1 ± 0.1	-2.0 ± 0.1	-5.4 ± 0.1	-3.4 ± 0.1
PIPES	1.0	1.2 ± 0.2	3.9 ± 0.3	-5.5 ± 0.2	-9.4 ± 0.2

Our best model suggest this one proton is donated to one of the amine (NH_3^+- R) groups from the N-terminal glutamate residues. The resulting thermodynamic parameters are consistent across all buffer systems except for Tris. All these calculation models are illustrated in Table 3.8 to 3.10, the latter being a summary of all buffers and average values.

Table 3.8 Thermodynamic cycle for Zn^{2+} binding to GSSG in 100 mM MOPS at pH 7.4

	Coef	ΔH (kcal/mol)	ΔG (kcal/mol)
$Zn^{2+}MOPS + H^+_x(GSSG) \rightleftarrows xH^+(MOPS) + GSSG\text{-}Zn + (1\text{-}x)\ MOPS$	1	2.10[a]	-5.9
$H^+ + MOPS \rightleftarrows H^+(MOPS)$	1	-5.04[b]	-9.78
$Zn^{2+}(MOPS) \rightleftarrows MOPS + Zn^{2+}$	1	0.09[c]	-4.40
$H^+\text{-}GSSG \rightleftarrows H^+ + GSSG$	1		
$-NH_3^+ \rightleftarrows NH_2 + H^+$	1	8.1[d]	13.4
$Zn + GSH \rightleftarrows Zn\text{-}GSH$	1	-1.0	-5.1

a value from direct ITC measurement (Table 3.1), b and c value from Table 3.2, d value from literature[48]

Table 3.9 Thermodynamic cycle for Zn^{2+} binding to GSSG in 100 mM MOPS at pH 7.4.

	Coeff	ΔH (kcal/mol)	ΔG (kcal/mol)
Zn^{2+}PIPES + $H^+{}_x$(GSSG) ⇄ xH^+(PIPES) + GSSG-Zn + (1-x) PIPES	1	3.87[a]	-5.48
H^+ + PIPES ⇄ H^+(PIPES)	1	-2.68[b]	-9.44
Zn^{2+}(PIPES) ⇄ PIPES + Zn^{2+}	1	-0.46[c]	-4.20
H+-GSSG ⇄ H+ + GSSG	1		
$-NH_3^+$ ⇄ NH_2 + H^+	1	8.1[d]	13.4
Zn + GSSG ⇄ Zn-GSSG	1	-1.1	-5.24

a value from direct ITC measurement (Table 3.1), b and c value from Table 3.2, d value from literature[48]

Table 3.10 Thermodynamic cycle for Zn^{2+} binding to GSSG in 100 mM HEPES at pH 7.4

	Coeff	ΔH (kcal/mol)	ΔG (kcal/mol)
Zn^{2+}HEPES + $H^+{}_x$(GSSG) ⇄ xH^+(HEPES) + GSSG-Zn + (1-x) HEPES	1	2.08[a]	-5.8
H^+ + HEPES ⇄ H^+(HEPES)	1	-4.86[b]	-9.78
Zn^{2+}(HEPES) ⇄ HEPES + Zn^{2+}	1	0.08[c]	-4.40
H^+-GSSG ⇄ H^+ + GSSG	1		
$-NH_3^+$ ⇄ NH_2 + H^+	1	8.1[d]	13.4
Zn + GSSG ⇄ Zn-GSSG	1	-1.2	-5.0

a value from direct ITC measurement (Table 3.1), b and c value from Table 3.2, d value from literature[48]

Table 3.11 Summary of pH and buffer independent thermodynamic values for Zn^{2+} binding to GSSG

	$K \times 10^4$	ΔH (kcal/mol)	ΔG (kcal/mol)	-TΔS (kcal/mol)
MOPS	5.7	-1.0	-5.1	-4.1
HEPES	4.9	-1.2	-5.0	-3.8
PIPES	6.9	-1.1	-5.2	-4.1
Average	5.8 ± 1.0	-1.1 ± 0.1	-5.1 ± 0.1	-4.0 ± 0.2

Binding event is favorable in -5.1 kcal/mol Gibbs free energy change. The binding constant for Zn^{2+} binding to oxidized glutathione was found to be $K_a = 5.8 \times 10^4$, which indicates comparatively lower affinity of oxidized glutathione to Zn^{2+} when compared to reduced glutathione at this pH. This change in driving force can be ascribed to the coordination of the thiol group in Zn-GSH complexation. This argument further supported by thermodynamic reaction of Zn-GSSG being a less enthalpy driven, but it is highly entropically driven process as nicely shown in thermodynamic profile in Figure 3.13.

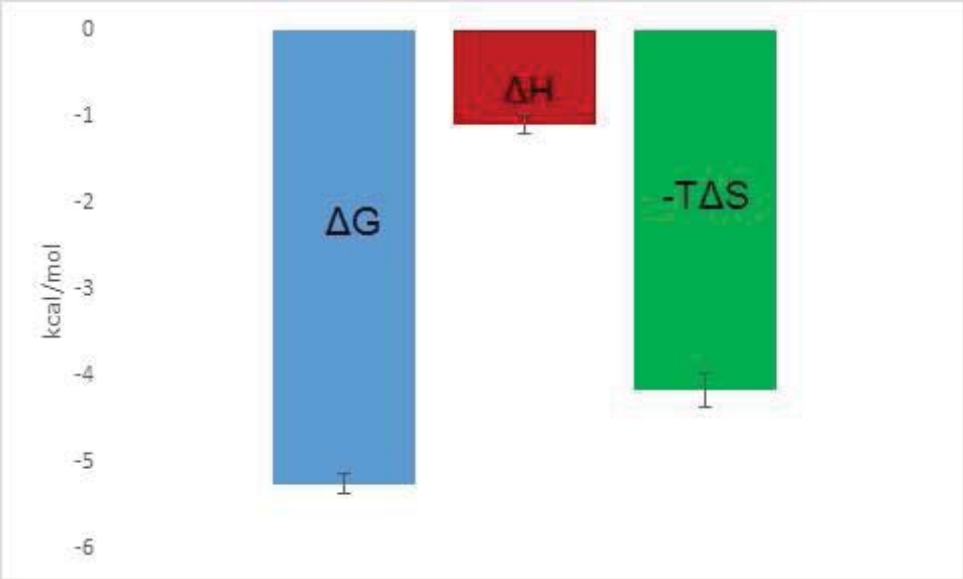

Figure 3.13 The Thermodynamic Profile for Zn^{2+} binding to Oxidized glutathione (GSH)

The favorable entropic factor could rise from increasing mobility of dissociated water and buffer molecules from GSSG and the Zn metal ion when Zinc is coordinated. Alternatively this profile could also be from breaking the electrostatic interactions

between protonated amine groups and deprotonated carboxylates in oxidized glutathione molecule, which are needed to overcome to unfold the structure to increase the entropy.

3.2 Conclusion

Glutathione is a well-studied biomolecule due to its important roles in biological systems. We have collected new data that helps to refine the solution structure of glutathione along with the thermodynamic data of Zn^{2+} binding to both reduced and oxidized glutathione at physiological pH. The values refined from a thermodynamic model showed that both reduced and oxidized glutathione binds Zn^{2+} ions with moderate association constants and reduced glutathione has comparatively higher affinity than oxidized one. The driving forces behave the reduced glutathione-zinc binding reaction is both enthalpically and entropically driven, whereas the similar binding event with oxidized glutathione binding is driven mainly through entropic interactions. In both cases, additional structural data would benefit our thermodynamic analysis.

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