Summary

Glutathione, γ-glutamylcysteinyl-glycine (GSH), is the most abundant thiol in living cells. It is present in all mitochondrial eukaryotes and several but not all prokaryotes. It is present at very high concentrations (1 mM to 10 mM) in the cell. Glutathione has a low redox potential (-240 mV) and in an equilibrium with oxidized glutathione it plays a critical role in the maintenance of the cellular redox environment. Glutathione also plays a role in the detoxification of various toxic compounds like heavy metals, xenobiotics, peroxides, and also functions as an antioxidant. Glutathione plays an essential role in iron metabolism. It is essential for the growth of eukaryotes but in prokaryotes it is essential only under stress conditions. Glutathione biosynthesis is catalyzed by two ATP-dependent enzymes γ-GCS (γ-glutamylcysteine synthetase) and GS (glutathione synthetase). The first enzyme γ-GCS is the rate-limiting enzyme in glutathione biosynthesis and plays a critical in glutathione homeostasis. This enzyme has evolved through three different lineages, Lineage I, includes enzymes from bacteria like *E. coli*, *Vibrio cholera, Pseudomonas aeruginosa etc*. Lineage II includes enzyme from non-plants eukaryote (Mammals, Fly, Yeasts) whereas lineage III includes plants and plant-associated bacteria (*Agrobacterium tumefaciens, Xanthomonas* etc). Although enzymes from all three lineages catalyze the formation of γ-glu-cys, they shares less than 10% sequence similarity among themselves. The crystal structure of representatives from each of the lineages is now available. The *E. coli* γ-GCS enzyme has been co-crystallized with the transition state analogue (2*S*)-2-amino-4-[(2*S*)-2-carboxybutyl-(*R*) sulfonimidoyl] butanoic acid. The crystal structure has clearly demonstrated the residues involve in binding with glutamate and ATP but it has only putatively predicted the possible residues interacting with cysteine. The crystal structure of the *E. coli* γ-GCS enzyme has revealed one disulphide bond between the two cysteines Cys-372 and Cys-395.

In this thesis I have focused on the two undefined aspects of *E. coli* γ-GCS enzyme. The first objective was to investigate the role of the disulphide bond of *E. coli* γ-GCS along with the role of other cysteines residues in enzyme activity. The second objective was to attempt to understand in greater detail the residues and interactions participating in formation of cysteine binding pocket.

As a cytosolic protein rarely contains disulphide bonds, the presence of a disulphide bond in this redox regulated enzyme reflected some important functions. The bacterial γ-GCS

enzyme from lineage III that includes *Agrobacterium tumefaciens* has a disulphide bond. A biochemical study with lineage III enzyme has however revealed no role for the disulphide bond. When we examined the conservation pattern of the cysteines in *E. coli* γ-GCS enzyme, we observed that among the 9 cysteine residues seen in *E. coli* γ-GCS, 3 of the residues, C106, C205 and C357 were conserved in majority of the orthologues, except bifunctional *GshF* proteins. However, surprisingly, the 2 cysteines involved in making the disulphide bond, C372 and C395 were conserved (along with C433) in only a small subset that primarily included the enterobacteria. To understand the role of the disulphide bond, we initially targeted each cysteine residue and mutated them to serine by site directed mutagenesis approach. All cysteine to serine mutants were evaluated in a *S. cerevisiae gsh1Δ* strain. The *S. cerevisiae gsh1Δ* strain is a strict glutathione auxotroph and requires glutathione from outside in order to grow. The *E. coli* γ-GCS enzyme can complement the *S. cerevisiae gsh1Δ* strain permitting growth in medium lacking glutathione. We exploited this property to functionally assess the *E. coli* enzyme. We observed that even the 7-cysteine free (7CF) mutant which had 7 of the 9 cysteines mutated to serine (barring cysteines involve in disulphide bond), was functional. Further mutating either cysteine 372and/or 395 to create 8CF or 9CF, led to a non-functional enzyme. These experiments suggested the importance of the disulphide bond. To understand in more detail the role of the disulphide bond of *E. coli* γ-GCS, we took a genetic approach by subjecting the 9CF non-functional mutants to random mutagenesis and screened for functional suppressors. During the screening for functional mutants we observed that majority of mutants had cysteine reappearing at 372 and 395, the residue originally participating in disulphide bond formation. This confirmed the importance of the disulphide bond in enzyme function. However, we were also able to isolate several mutants which did not contain any disulphide bond but were functional. These mutants had a pattern where only one of the residues, either C372 or C395 was restored as cysteine, while the other change although it also involved the corresponding 372 or 395 residues, contained an aromatic residue at the position. We were also able to obtain one mutant which lacked any cysteine but carried two changes R374Q and V375F, both of these changes were very near to cysteines involve in disulphide bond formation. We biochemically evaluated all functional disulphide bond free mutants for their redox sensitivity as well as activity. The wild type and 7CF protein have lower activity in presence of DTT and GSH as compare to protein without treatment. The comparative

analysis of wild type, 7CF γ-GCS enzyme along with suppressor's mutants revealed that disulphide bond present in wild type and 7CF proteins are redox sensitive. Kinetic studies of WT and mutant γ-GCS enzymes reveal no role of the disulphide bond in determining the affinity of the enzyme towards its substrates. However we observed a lower V*max* of the mutants. Comparisons of kcat and kcat/K*m* (i.e. turnover number and catalytic efficiency) of wild type and mutant revealed that some mutants had a reduced turnover number and catalytic efficiency. We also observed that the reduced WT and 7CF (DTT treated) had lower catalytic efficiency than the untreated enzyme. More importantly, the kinetic behavior of the reduced protein was found to be very similar to the pattern shown by the mutants lacked the ability to form disulphide bond.

During our initial biochemical investigation we noticed that mutants which lack the ability to form the disulphide bond are more susceptible to elevated temperature. We made detailed investigation of this observation taking a biochemical and biophysical route. Our thermal stability assay revealed that mutants lacking the ability to form disulphide bond have lower activity after preincubation at 37°C as compared to their activity after preincubation at 4°C. Mutant lacking any cysteines had the most severely reduced activity at higher temperature. The wild type had almost similar activity at all three different preincubated temperatures (37°C, 25°C and 4°C) while the mutants lacking the ability to form a disulphide bond were more sensitive to a preincubation at 37 °C as compared to its activity when preincubated at 4°C. We were able to successfully demonstrate in wild type and 7CF protein that reduction of disulphide bond by reducing agent is major factor in reduction of stability at high temperature. Our CD experiment support above finding. Furthermore we investigated the instability of the *E. coli* γ-GCS protein under *in vivo* conditions. Pulse-chase experiments clearly demonstrated that the mutants lacking disulphide bond were more prone to degradation as compared to the wild type protein. We also demonstrate that *in vivo* that protein exists in both the oxidized form and the reduced form. The above result allowed us to conclude that *E. coli* γ-GCS enzyme has a redox sensitive disulphide bond that regulates the enzyme activity by altering the stability of the protein under different physiological conditions.

In the second part of our study, we have attempted to investigate the putative cysteine binding pocket residues in *E. coli* γ-GCS. Based on the docking studies and the crystal structure analysis published earlier, predictions were made that the cleft that accommodates the cysteine non-polar side group is lined by residues Phe-61, Tyr-131,

Arg-132, and Leu-135. The carboxyl group of cysteine was found to be within hydrogen bonding distance from Tyr-131, Gln-144, Tyr-241, Asn-297, and Tyr-300 and these were considered to contribute to substrate binding by possible hydrogen bond interactions. The amino-group of cysteine was presumed to interact with Asp-60, and the cavity was also lined with Lys-128. Similarly, the side chain Asp-60 is hydrogen bonded with WT-L-cys amino group. Apart from this, Arg-132 is poised to form polar interactions either with the sulphydryl group or carboxyl group that can play a role in higher binding affinity.

Interestingly the conservation pattern of these residues revealed they were highly conserved even in the distantly related bi-functional *GshF* enzyme. Phe-61 was the only residue which has been evolutionary replaced by the similar amino acid Tyr in some of the more closely related bacteria.

We took a genetic and bioinformatics approach to understand the role of each of these residues. Since we were most interested in understanding the residues involved in the interaction with the side group of cysteine, we focused on the residues lining the cleft or predicted to be involved in interaction with sulphydryl group. These residues were Phe-61, Tyr-131, Arg-132, and Leu-135. We subjected these residues to two kinds to mutagenesis. In the first place we mutated them to a conserved residue. Thus Phe-61 was mutated to Tyr-61, Tyr-131 changed to Phe-131, Arg-132 mutated to Lys-132 and Leu-135 was replaced with Val-135.

The second strategy was to randomize these residues using degenerate primers so that they may be mutated to variety of residues. We also evaluated these mutants through two approaches- a complementation growth assay using the *S. cerevisiae gsh1Δ* strain and activity assays *in vitro* using purified enzyme. The *in vivo* assay was very sensitive and detects even low enzymatic activity and it thus also indicates that protein was folded correctly *in vivo*. The *in vitro* activity assay was less sensitive but more quantitative.

When we carried out a conserved mutagenesis Phe-61 to Tyr (F61Y), we observed complementation in the plate based *in vivo* assay. However the activity assay of purified protein *in vitro* revealed that F61Y mutant (as well as F61W) had less than 10% activity as compared to wild type. However the kinetic parameters of these mutants for cysteine revealed no significant change in K*m* for cysteine (K*m* of F61W, F61Y for cysteine was 0.2 and 0.1 mM respectively which was very similar to wild type enzyme (0.1 mM)).However there was almost 20-25 fold reduced V*max* observed for these mutants. Thus a conserved change led to no significant change in K*m* but altered the catalytic

efficiency of enzyme. We also evaluated F61Y and F61W for their altered substrate specificity but we could not observe any altered specificity.

Randomization of the Phe-61 was carried out to yield lysine, aspartic acid, valine, proline, leucine, cysteine and isoleucine at this position. The *in vivo* evaluation revealed the most of the mutants showed comparable growth to wild type except F61P and F61K mutants which had poor growth. F61I failed to complement. We also evaluated these mutants (*in vitro*) for activity toward cysteine as well as other substrate like serine, valine, glycine, DL-allylglycine, ethylamine and α-amino butyric acid but did not observe any altered activity as compared to wild type. All these mutants displayed compromised activity for cysteine and other substrates.

Tyr-131 was also mutated first to the conserved residue Phe-131. The Y131F mutation showed *in vivo* complementation. The *in vitro* activity revealed only 3.8% activity. The K*m* had increased nearly 60-fold and V*max* decreased by 25 fold for cysteine. Y131F mutant showed no altered substrate specificity Randomizing Y131 residue yielded arginine, threonine, serine, phenylalanine, isoleucine, lysine, glutamine, and asparagine. All these mutants complemented well *in vivo* except Y131R which had poor growth phenotype, while Y131Q failed to grow. The *in vitro* activity of these mutants revealed that these mutants marginally retain the activity as compared to the wild type enzyme.

R132 and L135 residues were also mutagenized to 132K, and 135V respectively. R132K and L135V were both able to complement *in vivo*. The R132K mutant under *in vitro* condition revealed that unlike other conserved mutants was able to retain nearly 90% activity but surprisingly had very low substrate affinity for cysteine with K*m* 8.5 mM (which was nearly 85 fold higher than wild type) whereas there was only 8-fold change in V*max*. We also subjected R132 to randomization and changed it to glycine, serine, alanine, leucine, proline, glutamine and tryptophan. Only R132W mutant was unable to show *in vivo* activity. We observed no change in substrate specificity for different amino acids. *In vitro* analysis of L135V mutant protein showed only 8.7 % activity with not much change in K*m* for cysteine. Interestingly we observed approx. 25-fold reductions in the rate of reaction. The other mutants of L135 residue, the L135S and L135P mutants were found to complement poorly. The *in vitro* analysis of L135S mutant indicated that it had very high K*m* for cysteine (K*m*=12.0 mM).

To understand the above altered *in vitro* properties of mutants, we attempted to map the conformational changes occurring in these mutants due to mutation through molecular

modeling and docking. We evaluated F61, Y131, R132 and L135 residues separately and analyzed their individual contribution in formation of cysteine binding pocket. Phe-61 contributes partially to overall hydrophobicity of cysteine binding pocket since it is surrounded by hydrophobic residues (Ala-62, Leu-65, Leu-116, Ala-117, Leu-135 and Ala-141) or hydrophobic part of side chain (Arg-132 and Lys-136). Y131 forms hydrogen bond with carboxyl group of cysteine and it is located almost in the middle of the α-helix. Arg-132 and L135 are also in the middle of the α-helix that interacts with side chain of residues lying on its helix face. R132 residue interacts with Lys-128, Leu-135 and it is oriented in a way that facilitates its interaction with Tyr-131. In addition, the Arg-132 side chain also forms a hydrogen bond with the main chain carbonyl oxygen of Asp-60, which might help in its spatial orientation to interact with substrate. Any attempt to replace these residues even with similar amino acid could have affected local conformation of the helix as well as their interaction with the neighbor residues or alteration in the active site cavity volume. Any such changes in and around the active site could affect the accommodation and binding of the substrate. The *in vitro* studies clearly demonstrate that the observed properties were possibly due to disruption of active site architecture.

Based on the docking studies the residues predicted to interact with carboxyl group of cysteine included Tyr-131, Gln-144, Tyr-241 and Tyr-300 and were suggested to contribute significantly towards overall binding energy. Moreover, these residues were also proposed to help in the orientation of L-cys for nucleophilic attack toward the carbonyl of the γ-glutamyl phosphate intermediate. The residues Q144, Y241, Y131 and Y300 were mutated to similar amino acid as well as alanine. *In vitro* characterization of these mutants demonstrate that unlike mutants involved in interaction with the sulfhydryl group of cysteine (F61, Y131, R132, and L135), mutations in Q144, Y241, and Y300 only minimally affected the activity of the enzymes. These mutants were able to retain more than 50% activity except Y300A mutant which lost nearly 75% activity as compared to the wild type. It appeared that apart from this specific role, these residues impart robustness to cysteine binding site against single residue mutations and such a feature would be advantageous for Ecγ-GCS to ensure biosynthesis of glutathione. Additionally, this network might help in substrate specificity as the cysteine binding site is rather shallow cleft, which is mostly occupied by cysteine side chain.

In *S. cerevisiae* an arginine residue has been shown to be catalytically important. The catalytically important Arg-472 residues of *S. cerevisiae* superimposed well with Arg-330 of *E. coli*. A conserved R330K mutation led to reduced rate of catalysis whereas change to alanine completely abolished the enzymatic activity. These finding suggested that the R330 residue may be a catalytic important residue in *E. coli* γ-GCS enzyme.

A remarkable feature that emerged from this study is that cysteine can bind with high affinity (K*m* = 0.1mM) in the cys-cavity even though, the cysteine binding pocket is shallow with similar sized pockets lying close to it. This is most likely achieved by a network of side chain interactions present in the binding pocket that helps in binding and proper orientation of cysteine for optimal activity. This is also evident with experimental studies wherein mutation of binding site residues (even to similar amino acids), usually, results in lower enzyme activity.

In conclusion, the work described in this thesis has thrown light on two important aspects of the *E. coli* γ-GCS enzyme. Firstly, a role for the disulphide bond of γ-GCS enzyme was proposed where it was shown to confer 'redox dependent stability' and thereby confer a novel type of redox regulation. Secondly the residues important for the binding of cysteine substrate have been defined by docking and mutagenesis. Unique feature of the cysteine binding pocket have been revealed, chief among them being the involvement of a network of side chain interaction that assists in accommodation of cysteine for optimum activity.