Neha Kasturia (2009). ***Genetic studies on the glutamylcysteine synthetase enzyme involved in glutathione biosynthesis in the yeast S. cerevisae.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Glutathione is the most abundant low molecular weight cellular thiol compound found in almost all eukaryotes and some prokaryotes. Glutathione plays a key role in the maintenance of intracellular reducing environment in response to oxidative, metal and xenobiotic stress. It is also an important storage molecule for sulphur and nitrogen. Glutathione metabolism essentially proceeds through the γ-glutamyl cycle. The *de novo* biosynthesis of glutathione proceeds via a two-step ATP dependent process. The first step is catalyzed by γ-glutamylcysteine synthetase enzyme which catalyses the ligation of L-glutamic acid and L-cysteine to form γ-glutamylcysteine. The second step is catalyzed by glutathione synthetase enzyme, which ligates glycine to γ-glutamylcysteine to form glutathione again in an ATP dependent manner. The enzyme of the first step, γ-glutamylcysteine synthetase, is feedback inhibited by reduced glutathione (GSH) but not by oxidized glutathione (GSSG) to prevent over-accumulation of glutathione. This enzyme, which catalyzes the rate-limiting step of glutathione biosynthesis, is under transcriptional and post-translational control and also the focus of much investigation and also has been the focus of this study.

The γ-glutamylcysteine synthetase enzyme appears to have evolved in different organisms in to three distinct lineages on the basis of pair-wise sequence identities at the amino acid level. The Lineage I includes enzymes from gram negative bacteria like *E.coli, S.typhimurium* etc and the crystal structure of *E.coli* γ-GCS has been recently solved by Hibi *et al.*, 2004. The Lineage II includes enzymes from non-plant eukaryotes like mammals, flies, protozoans and yeasts. The crystal structure of members belonging to this lineage has not been elucidated so far. The Lineage III mainly consists of γ-GCS enzymes present in plants like *B.juncea* and plant associated bacteria. The crystal structure of the plant γ-GCS has been recently elucidated by Hothorn *et al.,* 2006. The crystal structure of *E.coli* γ-GCS enzyme has shown the presence of an intramolecular disulphide bond, however biochemical studies elaborating on its role in response to the redox conditions of the cell have not been reported so far. In the absence of structural

information on lineage II γ-GCS, biochemical studies in the enzymes from higher eukaryotes have reported that the enzyme is a heterodimer and the subunits are covalently linked by an intermolecular disulphide bond. These disulphide bonds are redox sensitive and modulate the activity of enzyme *in vivo* in response to the redox condition of cell. The *B.juncea* γ-GCS crystal structure has been reported to have two intramolecular disulphide bonds which have been shown to be responsive to the redox conditions of the cell.

In the present study, in the absence of structural information on the γ-GCS enzyme of Lineage II (non-plant eukaryotes), we have sought to gain insights into the structure/functional aspects of the enzyme in the yeast *S.cerevisiae* via a genetic approach. Unlike the γ-GCS enzyme present in higher eukaryotes of Lineage II, which are heterodimeric in nature, the γ-GCS enzyme present in yeasts has been suggested to be a monomer. Initial efforts in our study were directed towards developing a sensitive plate based screen for γ-GCS functionality. Subsequently, we have initiated an investigation into the putative cysteine-binding pocket of the enzyme. A previous study (Abbott *et al.*, 2001) had already predicted residues present in the putative Mg^{2+}, MgATP and L-glutamate binding sites in the substrate-binding pocket of the enzyme on the basis of PSIBLAST analysis. However, no clues were available on the cysteine-binding pocket of the enzyme. To identify the putative cysteine-binding pocket in *S.cerevisiae* γ-GCS we have adopted a site directed mutagenesis approach followed by *in vivo* functional evaluation of the mutants. Residues to be mutated were picked up on the basis of *E.coli* crystal structure and the putative alignments reported by Hibi *et al.*, 2004. We have also undertaken the selection of residues for mutagenesis by independently examining the PSIBLAST alignment published by Abbott *et al.*, 2001. The residues that were identified from both these alignments were selected and mutated to alanine. These were T201, G319, W445, Y362, R205, R193, E189, N215, V190. The mutant enzymes were functionally evaluated using our plate based assay. The study revealed W445A, T201A, R205A, R193A, N215A and G319A as being defective to differing extents. Among these, the completely non-functional W445A mutant was subjected to an intragenic suppressor

analysis. However, no second site suppressor mutants were obtained for this primary mutation, suggesting that W445 might be critical for activity. A structural model of the *S.cerevisiae* γ-GCS was created using the PHYRE server. This model has placed all these residues in the substrate-binding pocket of the enzyme near the glycine rich P loop, which binds to the phosphate group of ATP during catalysis.

A second line of investigation that we chose to initiate and which we thought might be interesting as it could give insights into *H.sapiens* γ-GCS SNP's, was to reproduce the *H.sapiens* SNP's in *S.cerevisiae* γ-GCS enzyme. The three *H.sapiens* γ-GCS SNP's which we chose to study in the *S.cerevisiae* γ-GCS were valine 55 to serine, praline 462 to serine (SNP's of no known phenotype as reported in the NCBI database) and arginine 127 to cysteine responsible for hemolytic anemia (Hamilton *et al.,* 2003). The corresponding residues mutated in *S.cerevisiae* γ-GCS were V55S, H509S and R125C. Among these H509S showed complete loss in activity of the enzyme and R125C significantly decreased functionality of the enzyme. An intragenic suppressor analysis of H509S revealed a compensatory G319A mutation. Interestingly, G319 has been observed to be present in the putative cysteine-binding pocket of the enzyme in the present study and its interaction with the C-terminal region have indicated that this region might be crucial for the maintenance of the overall structure/functional aspects of the enzyme, either in terms of contributing residues as part of the cysteine-binding pocket of the enzyme and/or in terms of maintenance of overall structural stability of the enzyme.

The γ-GCS enzyme appears to be under redox control (involving cysteine residues) as seen from studies of the plant and mammalian enzymes. We thus investigated the role of cysteine residues in *S.cerevisiae* γ-GCS enzyme as well. In *S.cerevisiae* γ-GCS there are nine native cysteine residues of which only cysteine 266 is conserved amongst Lineage II enzymes. However, mutation of cysteie 266 to serine did not affect the *in vivo* functionality of the enzyme, suggesting that a cysteine-free molecule should also be functional. A 9-Cys free γ-GCS enzyme was created by mutagenizing all 9 cysteines sequentially to serine. However, the 9-Cys free γ-GCS was completely non-functional. To identify if a functional cysteine-free molecule might be possible, we considered a

genetic suppressor strategy. The 9-Cys free intragenic suppressor analysis was carried out and several functional suppressors were isolated, but none were without a cysteine residue. Amongst the suppressors that were isolated, interestingly no single pattern of reverting cysteines was observed. Multiple combination of cysteines allowed for restoration of functionally active form of the enzyme. Four independent combinations of different cysteines were observed to be minimally important for the functionality of the enzyme: (i) C213 (ii) C70 C86 (iii) C571 C643 and (iv) C264 C266 C571. The intragenic suppressor results of *S.cerevisiae* γ-GCS allow us to propose a working model for the *S.cerevisiae* γ-GCS enzyme. We propose the enzyme to be essentially having three distinct domains: the N-terminal domain (1-200 aa residues), the substrate binding domain (201-533 aa residues) and the C-terminal domain (534-678 aa residues). The cysteines present in these domains i.e C70 C86 (present in the putative N-terminal domain), C213 C264 C266 (present in the putative substrate binding domain) and C571 C643 (present in the putative C-terminal domain) by virtue of direct/indirect interactions with each other and/or other residues are largely responsible for bringing these domains together in a functionally optimal conformation *in vivo*. Our genetic studies indicate that the cysteines in the substrate binding glycine rich P-loop (C264 and/or C266) and the cysteines in the C-terminal domain (C571) are involved in direct interaction with each other. The suppressor analysis study suggests that the substrate-binding domain is in close proximity to the C-terminal domain of the enzyme by virtue of these interacting residues. This proposition is further strengthened by the suppressor analysis of H509S mutant, which picked up a G319A (present in the substrate binding domain). The *S.cerevisiae* γ-GCS PHYRE server structural model also predicts the substrate-binding domain to be occupying the central core region of the enzyme and to be anchoring the C-terminal domain.

To examine if the *S.cerevisiae* γ-GCS results (where multiple combinations of cysteines yield a functional enzyme) was a general phenomenon in this class of enzymes or specific to *S.cerevisiae* γ-GCS, we carried out a similar intragenic suppressor analysis study on the 9 native cysteines of *E.coli* γ-GCS whose crystal structure is known. However, the

results were surprisingly different from those obtained for the analogous *S.cerevisiae* γ-GCS. The 9-Cys free *E.coli* γ-GCS was non-functional like the 9-Cys free *S.cerevisiae* γ-GCS. However, despite the apparent essentiality of the single C372-C395 disulphide bond, a mutant was obtained with no cysteine residue reverting back. The functional revertant had an R374Q mutation, which was capable of suppressing the loss of function phenotype of 9-Cys free enzyme. Examination of the crystal structure of *E.coli* γ-GCS reveals that the disulphide bond between C372 (present on an α-helix) and C395 (present on a loop) tether these two secondary structural elements together. Mutagenesis of these two cysteines to serine and subsequent removal of the disulphide bond predicts that a readjustment of the helix takes place such that the arginine 374 gets close to the tyrosine 241 and 300 (present in the cysteine binding pocket). Since on arginine to glutamine mutation at position 374 allows for restoration of wild type functionality of the enzyme, it can be speculated that the shortening of the side chain of glutamine can circumvent the affect of helix readjustment and allow for optimal interactions with the tyrosine residues in the substrate binding pocket of the enzyme, thus restoring functionality.

Efforts were made to purify *S.cerevisiae* γ-GCS enzyme from *S.cerevisiae* and *E.coli* as the host system to further investigate and kinetically characterize the different mutants. However, we were not successful in obtaining a functional purified enzyme. Despite this lacuna, several interesting insights have emerged from this genetic study. Firstly, the study identifies key residues that possibly line the putative cysteine-binding pocket of the enzyme present in Lineage II (non-plant eukaryotes) γ-GCS. Secondly, it reveals a surprising finding that the native cysteine residues in different combinations essentially involving the three putative domains of the enzyme can allow for functionally active conformation of the enzyme *in vivo*. Since cysteine residues are playing a role in restoration of functionally active form of the enzyme, a redox dependent regulation of function of *S.cerevisiae* γ-GCS becomes a distinct possibility. Thirdly, from the studies on the role of cysteine residues in *E.coli* γ-GCS, it has been demonstrated that it is possible to obtain a functional γ-GCS enzyme devoid of any cysteine residues. This

indicates that the cysteine residue in γ-GCS enzyme are not mandatory for structure maintenance of γ-GCS enzyme in *E.coli*.