Akhilesh Kumar (2011). ***Studies on enzymes of the y-glutamyl cycle involved in 5-oxoproline metabolism in yeast.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Glutathione (γ-glu-cys-gly) is the most abundant low molecular weight thiol compound found in almost all eukaryotes and a few prokaryotes. The relatively low redox potential, ability to exist in both oxidized (GSSG) and reduced (GSH) forms and it's stability towards cellular peptidases, together with it's high intracellular concentration (upto 10mM) make glutathione a major redox buffer of the cell. The other functions of glutathione include a role in the assembly of iron-sulphur proteins, scavenging of free radicals and metal ions, detoxification of xenobiotics and regulation of cellular processes like signal transduction, proliferation and apoptosis. Glutathione also acts as a reservoir of sulphur and nitrogen.

Owing to it's unusual γ-glutamyl peptide bond, glutathione cannot be synthesized on ribosomes. Glutathione metabolism is carried out by a special six enzyme pathway, the γ-glutamyl cycle. γ-glutamyl-cysteine ligase and glutathione synthetase synthesize glutathione from glutamate, cysteine and glycine. γ-glutamyl transpeptidase (γ-GT), Cys-gly dipeptidase, γ-glutamyl cyclotransferase (γ-GCT) and 5-oxoprolinase degrade glutathione into it's constituent amino acids. γ-GT, a membrane bound enzyme, catalyzes the first step of glutathione degradation by removing the γ-glutamyl moiety from glutathione and transferring it to an amino acid. The γ-glutamyl-amino acids generated from γ-GT are substrates of γ-GCT and cleaved into 5-oxoproline and free amino acid. Finally, 5-oxoproline is hydrolyzed into glutamate by 5-oxoprolinase, and the Cys-gly generated from the action of γ-GT on glutathione is cleaved into free cysteine and glycine by a Cys-gly dipeptidase.

The enzymes of the γ-glutamyl cycle are well characterized in mammals. However in yeast the activities of γ-GCT and 5-oxoprolinase could not be detected. Interestingly, both the enzymes are involved in 5-oxoproline metabolism, and are responsible for the generation and hydrolysis of 5-oxoproline respectively. Thus, the γ-glutamyl cycle has been proposed to be truncated in yeast and other fungi. The discovery of a fungal-specific, alternative pathway of glutathione degradation ("DUG" pathway), further supported the truncated nature of the γ-glutamyl cycle in yeast. However, in a recent metabolome study of the yeast *Saccharomyces cerevisiae*, significant amounts of 5-oxoproline was detected. An uncharacterized homologue of mammalian 5-oxoprolinase, *YKL215c* can also be found in the *S.cerevisiae* genome. These observations suggest that the hitherto unidentified enzymes of γ-glutamyl

oxoprolinase. In comparison to Oxp1p, mouse 5-oxoprolinase appeared more sensitive for the presence of reducing agents for it's optimum activity and stability and was highly dependent on presence of reducing agents during purification and the reaction mixture.

We also initiated the first structure-function analysis of a eukaryotic 5-oxoprolinase in this thesis. In the conserved domain database, a two-domain architecture of eukaryotic 5-oxoprolinase is predicted (N-terminal, HyuA; C-terminal, HyuB). We separately cloned both the domains and discovered that when coexpressed in yeast, they could restore the function, suggesting that the domains are functionally separable. This result was interesting considering the two subunit nature of prokaryotic 5-oxoprolinase and suggesting a fusion of these subunits during evolution. The N-terminal half of eukaryotic 5-oxoprolinase was predicted to adopt an 'actin fold', even though Oxp1p did not show any significant sequence similarity with any of the actin-fold proteins. We could identify four motifs in Oxp1p, involved in the ATPase activity of actin-fold proteins. Mutations of these motifs led to loss of *in vivo* activity of Oxp1p. Moreover *in vitro* ATPase and 5-oxoprolinase activities were also lost when studied using purified mutant Oxp1p. Homology modeling confirmed the involvement of these motifs of Oxp1p in ATP binding, similar to actin fold proteins.

γ-glutamyl cyclotransferase (γ-GCT) has been characterized very well in mammals. However, no report of γ-GCT study exists from bacteria or fungi. To identify a γ-GCT protein in yeast, we searched for human γ-GCT homologues in *S.cerevisiae*. However, no sequence homologues could be detected. We then searched for a protein containing a BtrG/γ-GCT fold (a unique fold, present in the proteins γ-GCT, γ-GACT and BtrG, having γ-glutamyl cyclotransferase like activity), an uncharacterized protein Yer163cp appeared as a potential candidate protein with a putative BtrG/γ-GCT fold. The homologues of this protein were present in all phyla. To investigate their possible γ-GCT activity, Yer163cp, it's prokaryotic homologue (chaC from *E.coli*), fungal homologue (from *S.pombe*) and mammalian homologues (ChaC1 and ChaC2 from mouse) were expressed in *S.cerevisiae*. Novel *in vivo* assays were set up and revealed that Yer163cp and it's homologues function as γ-GCT, as they acted on γ-glu-cys, a substrate of γ-GCT. However in contrast to γ-GCT, these proteins were inactive against γ-glu-met, another substrate of γ-GCT. More

cycle, γ-GCT and 5-oxoprolinase involved in 5-oxoproline metabolism might exist in *S.cerevisiae*.

With this background we initiated efforts to determine if the 5-oxoprolinase and γ-glutamyl cyclotransferase (γ-GCT) enzymes exist in yeast. In this study, I present genetic, molecular and biochemical evidences that the two uncharacterized ORFs *YKL215c* and *YER163c* of *S.cerevisiae* encode the missing 5-oxoprolinase and γ-glutamyl cyclotransferase enzymes respectively.

YKL215c/OXP1 was cloned into a yeast expression vector. Using 5-oxoproline and L-2-oxothiazolidine-4-carboxylic acid (OTC), a substrate analogue of 5-oxoproline and employing plate-based growth assays, 5-oxoprolinase activity of the *YKL215c/OXP1* could be demonstrated. The recombinant Oxp1p was expressed and purified from *S.cerevisiae* (as it did not express in *E.coli*). Recombinant eukaryotic 5-oxoprolinase was thus purified for the first time in this study. The yeast Oxp1p was shown to exist as a homodimer in solution composed of two 142kDa subunits. Owing to the sluggish nature of 5-oxoprolinase, an extremely sensitive, Amplex Red based fluorometric 5-oxoprolinase assay was used in this study. The sensitivity of the method depended on the regeneration and detection of glutamate, produced by 5-oxoprolinase action on 5-oxoproline. Very low levels of glutamate (as low as 1 picomole) could be detected using this assay, but had to be improvised for the 5-oxoprolinase assay. Using this assay, 5-oxoprolinase activity of Oxp1p was characterized *in vitro*. The study revealed that 5-oxoprolinase of yeast was an energy dependent metalloamidohydrolase, required ATP, monovalent (K^+/NH_4^+) and divalent (Mg^{++}/Mn^{++}) cations for it's activity. The kinetic parameters were determined for Oxp1p, and revealed a K_m = 159 μM and V_{max} = 3.5 nanomole/h/mg protein. The Oxp1p was a sulphydryl enzyme and was inactivated in presence of thiol targeting reagents. However Oxp1p was not as sensitive as mammalian 5-oxoprolinase for it's requirement of reducing agents for activity.

We also cloned and characterized the mouse 5-oxoprolinase in yeast. The recombinant mouse 5-oxoprolinase could be purified using *S.cerevisiae* as an expression host. This represented the first report of recombinant mammalian 5-oxoprolinase purification. Using the above Amplex Red based assay, kinetic parameters of mouse 5-oxoprolinase were determined (K_m = 40μM and V_{max} = 1.2 nanomole/h/mg protein) and was comparable to the reported values for rat 5-

interestingly, our *in vivo* studies also demonstrated that in contrast to γ-GCT, these proteins also act on glutathione, which is not known to be a substrate of γ-GCT.

To characterize these proteins *in vitro*, recombinant Yer163cp and it's mammalian homologues ChaC1 and ChaC2 were purified from *E.coli*. Oligomeric studies revealed that Yer163cp and ChaC2 existed as monomer in solution. However ChaC1 appeared in different sizes of 10 to 20-mer, suggested a tendency to form aggregates. The products of Yer163cp and it's mammalian homologues ChaC1 and ChaC2 acting on different γ-glutamyl compounds were investigated using HPLC. The analysis revealed that glutathione was the only substrate efficiently cleaved by these proteins. γ-glu-ala was the only γ-glutamyl amino acid cleaved to some extent by Yer163cp while other γ-glutamyl compounds were totally inactive for these proteins.

Two highly sensitive assays for γ-GCT activity were also devised in this study. The first assay was 5-oxoprolinase coupled and depended on the detection of glutamate using Amplex Red kit. The second assay was coupled to Cys-gly peptidase (Dug1p) and depended on the detection of cysteine by acidic ninhydrin. Using the 5-oxoprolinase coupled assay, it was found that glutathione was the preferred substrate of these proteins and γ-glu-ala was cleaved to a very limited extent. All other γ-glutamyl amino acids tested were inactive. As glutathione was the preferred substrate, the kinetic parameters of these proteins against glutathione were determined using the Cys-gly peptidase coupled assay (For Yer163cp, K_m = 1.5 mM; V_{max} = 110 μmole/h/mg protein, for ChaC1, K_m = 3 mM; V_{max} = 980 μmole/h/mg protein and for ChaC2, K_m = 3 mM; V_{max} = 22 μmole/h/mg protein). The data revealed that the K_m of all these proteins for glutathione were similar and were in the physiological range of glutathione. However the V_{max} of ChaC1 was nearly 50 fold higher than the ChaC2 and nearly 10-fold higher than the Yer163cp. Based on it's activity on glutathione we have renamed the *S.cerevisiae* ORF *YER163c* as GCG1 (**G**amma-glutamyl **C**yclotransferase specific for **G**lutathione).

As Yer163cp and it's homologues, ChaC1 and ChaC2 were predicted to contain the BtrG/γ-GCT fold, we modeled these protein using Phyre sever. The homology models contained BtrG/γ-GCT fold and showed very high similarity to human γ-GCT, when superimposed. Moreover the orientation of the active site and catalytic residues of these proteins were similar to γ-GCT. We also demonstrated that mutation of putative catalytic glutamate residue of Yer163cp and it's homologue

ChaC1 led to loss of the activity of these proteins. This data suggested that Yer163cp and it's homologues not only adopted the fold similar to γ-GCT, they also showed similarity in their reaction mechanisms. The phylogenetic analysis of BtrG/γ-GCT fold proteins revealed that these proteins can be classified into four different families namely γ-GCT, γ-GACT, BtrG and ChaC, on the basis of their amino acid sequences. These proteins adopt similar fold, contain similar active sites, and show similar activity, despite extensive divergence in their sequences. These proteins also differ in their substrate preferences. In contrast to the other BtrG/γ-GCT families, the ChaC family is found in all phyla.

To investigate the physiological function of Yer163cp and it's homologues, these proteins along with γ-GCT were overexpressed in *S.cerevisiae*. The overexpression led to significant depletion in glutathione levels. The glutathione depletion was highest when ChaC1 was overexpressed. Interestingly ChaC1 has also been reported as a proapoptotic factor during ER stress, although the molecular function of ChaC1 has not been established. Our results explain how ChaC1 performs it's proapoptotic function by depleting glutathione. Glutathione depletion is a well established factor of apoptosis initiation and execution.