Summary :

Cysteine, as one of the 20 amino acids, is essential for protein synthesis and the growth of the cell. Cysteine is also a constituent of the cellular redox buffer, glutathione (γ-glu-cys-gly), present in almost all eukaryotes and some prokaryotes. The requirements of cysteine can be met by assimilation of the abundant sulphate in the environment and its subsequent reduction to organic sulphur compounds such as cysteine, homocysteine and methionine. This assimilatory pathway is seen in bacteria, yeast, fungi and plants, but is absent in mammals. An alternate source of cysteine is by the direct uptake of organic sulphur compounds such as cysteine, methionine, homocysteine, glutathione. Compounds other than cysteine can then be converted to cysteine by either the trans-sulphuration or reverse trans-sulphuration pathway or by degradation (in case of glutathione). Specific transporters exist for each of these compounds. This method of assimilation is found in all organisms, including humans. In case of bacteria, yeast fungi and plants, specific, as well non-specific transporters of cysteine have been identified. In some oxidizing environment such as blood plasma, high levels of cystine are found and pathogenic microbes have evolved highly specific cystine transporter for its utilization. Thus for example, the yeast *S. cerevisiae* lacks a cystine transporter, but the pathogenic yeast *Candida glabrata* and *Candida albicans* contain cystine transporters in addition to cysteine transporters. In mammals also, cysteine is mostly taken up as cystine to be then reduced to cysteine within the cell. Several cystine transporters have been identified in mammals. These include transporters at the plasma membrane, as well as cystine transporters that function to transport cystine between locations in the cell (such as from lysosome to cytosol).

In addition to their role in protein synthesis, cysteine as mentioned earlier is also a precursor for the cellular redox buffer, glutathione and also participates in some systems such as, cysteine /cystine redox system which is independent of glutathione. Despite the importance of cysteine within the cell, and its involvement in these processes, the levels of cysteine are regulated very tightly within the cell. In fact, for glutathione biosynthesis, cysteine is the rate limiting substrate.

We have been interested in understanding different aspects of cysteine /cystine metabolism within the cell. Although cysteine transporters have been studied extensively, very little is known about cystine transporters. We have thus investigated two cystine transporters, *CgCYN1* and *CTNS* in this study. *CgCYN1* is the plasma membrane cystine

transporter from *Candida glabrata* that was recently described in our lab, and the mammalian cystine transporter, *CTNS* that is found in mammalian lysosomes, and for which we have tried to develop yeast based screen for assaying the protein and for setting up genetic screens. Finally, we have tried to investigate how the yeast *S. cerevisiae* tackles an overload of cysteine in the cell since it lacks the conventional pathways of cysteine degradation.

Thus this thesis has essentially three parts to it. The first is the study of the plasma membrane cystine transporter *CgCYN1* of *C. glabrata* which we have investigated in S. *cerevisiae*. The second is the human lysosomal cystine transporter, *CTNS* that we have also investigated in *S. cerevisiae* and have also set up screens and assays to investigate this protein. Finally utilizing specific cysteine and cystine transporters to pump elevated levels of cysteine and cystine in to the cell, we have attempted to investigate how the yeast cell responds to cysteine overload.

Results and discussion:

1. **Identification of residues involved in cystine binding in the *C. glabrata* cystine transporter *CgCYN1***

In pathogenic fungi such as *C. glabrata*, *C. albicans* and *H. capsulatum*, cystine has been shown to be transported into the cells by a specific transporter (*CYN1*). Cystine transporters are a very important class of transporters but, there is a very little information about the mechanism of transport for the known cystine transporters. We decided to carry out a detailed structure-function characterization of *C. glabrata CgCYN1* to obtain the insights into the substrate binding site of the transporter.

In the absence of prior information we first delineated the putative trans-membrane domains and its topology. We examined the potential cystine binding residues by both modeling and multiple sequence alignment. 18 residues conserved only in cystine transporters from trans-membrane domains (TMD) TMD1, TMD3, TMD6, TMD8 and TMD10 were identified and subjected to alanine mutagenesis. Using *S. cerevisiae met15Δ* strain, which is an organic sulphur auxotroph. Functional analysis of these mutants was initially carried out by plate-based growth assays on cystine containing media. These studies revealed that N134A, D146A, L150A (all of TMD 3), F247A, G255A (all of TMD6), T339A, S340A, H347A (all of TMD8), and Q409A, G424A (all of TMD10)

showed defects in functionality. Protein expression analysis of these mutants revealed negligible changes in the protein levels in these mutants. The mutants were subsequently evaluated by radioactive uptake assays, using radiolabeled ^{14}C-cystine.

In case of N134A, D146A, F247A, Q409A measurement of the kinetic parameters showed only marginal increase in K*m* (<2 fold) and with no significant change in the V_{max} and these were categorized as moderately defective mutants, and did not appear to play a role in cystine binding.

In case of the defective mutants S340A and H347A there was a very minimal uptake of radiolabeled cystine. Uptake levels were too low for determination of kinetic parameters. Among the other mutants, L150A (K*m* 78.5 μM and V_{max} 6.4 μM), G255A (K*m* 95.2±16 μM and V_{max} 4.9±1.7), T339A (K*m* 178.7±43.3μM and V_{max} 10.6±6.7), G424 (K*m* 54.45±9.5μM and V_{max} 2.7±0.2) an increase in K*m* was observed suggesting that they may be involved in cystine binding.

Kinetic analysis of severely defective mutants such as L150, G255A, T339A, G424 revealed that compared to WT *CgCYN1*, they had increased K*m* but the V_{max} was not altered. Thus L150, G255, T339, and G424 appear to play a key role in cystine binding. Alanine mutants of S340 and H347 also show a severe defect and are likely to be involved in substrate binding as well, but this could not be ascertained owing to the inability to conduct the kinetic studies.

In conclusion, the results of these investigations have provided the first insight into the substrate binding residues of the *C. glabrata* cystine transporter, *CgCYN1*.

2. **Development of a yeast based assay for the investigating the human lysosomal cystine transporter *CTNS* and mutational studies using this assay.**

In humans, apart from plasma membrane based cystine transporters $b^{0,+}$ and Xc^-, cystinosin (*CTNS*) a lysosome based cystine transporter is involved in efflux of cystine from lysosome to the cytosol. Very little information about the mechanism of lysosomal cystine transporter *CTNS* is available. The C-terminal tail of *CTNS* contains a lysosomal targeting motif GYDQL deletion of which leads to the partial mis-localization to the cell surface. The *CTNS* protein which is a proton/cystine symporter also has a duplicated motif termed "PQ Loop" which is a key functional element involved in substrate

translocation and the residue D305 from PQ loop 2 has been found to be crucial for proton translocation

Human *CTNS*, if mislocalized to the plasma membrane, allows cystine uptake. Using this principle, we expressed mutated *CTNS* in yeast and found that mutations in the GYDQL motif led to partial mislocalization of protein to plasma membrane in yeast and thus growth on cystine. Growth could be observed in these transformants at high concentrations of cystine (300μM). We have exploited the inability of a *S. cerevisiae met15Δ* strain (an organic sulphur auxotroph) to grow on cystine owing to the lack of cystine transporter in *S. cerevisiae* and used this strategy as the basis for the growth based screen.

Development of such a functional assay for *CTNS* in yeast also served as a genetic screen for the isolation of gain-of-function mutants which would help us get new insights into the functioning of the cystinosin transporter. By using random mutagenesis, gain-of-function mutants for the cystinosin with an ability to grow on lower concentrations of cystine were isolated. These mutants are G131S, G131D, G197R, S270T, L274F, G309S, and G309C. By means of plate based growth assays and radiolabeled cystine uptake assays, it was established that they are indeed showing gain-of-function phenotype. For the WT *CTNS*-ΔGYDQL, the uptake was still too low. Using a vacuolar protein mutant *vps1Δ* we were able to get a better growth and this background was used for the radioactive uptake studies as well. We proceeded to determine the kinetic parameters in this background for the gain-of-function mutants. Gain-of-function mutant G197R and G309C which showed the better uptake, were characterized and the K*m* for G197R was determined to be 41.6 μM and that for G309C was found to be 59.6 μM. The reported K*m* for the *CTNS*-ΔGYDQL is 278±49μM. The full validation of the yeast screen will be possible once we compare the kinetic parameters of the mutants with the mammalian system as well.

Cystinosis in developing countries has been poorly studied. Till now very few patients from India are diagnosed with cystinosis. Due to its rare occurrence the disease goes almost unnoticed and thus the actual numbers are expected to be much higher.

We have analyzed the molecular basis of cystinosis in 5 patients from South India. Molecular analysis of *CTNS* in the patients led to identification of S141F mutation in 3

patients, but in two of the patients the mutations could not be identified. Out of these three patients, two were brothers. S141F mutation which has been previously identified in few South African patients is known to cause severe defect in functionality of the transporter and thus falls under a category of infantile cystinosis. Using the yeast based assay, we were also able to demonstrate that the mutation leads to loss of function.

3. Yeast cellular response to high levels of cysteine and cystine

The intracellular cysteine levels of cells are highly regulated. Cysteine levels in the cell should be adequate to meet the needs of the cell, but should also be below a threshold level to prevent cytotoxicity. In case of humans excess levels of cysteine has been shown to cause toxicity. In addition to mammals, cysteine toxicity has also been shown to occur in bacteria, yeast and fungi. In pathogenic fungi and humans, in order to maintain homeostasis excess cysteine is subjected to degradation by cysteine dioxygenase (*CDG1*). In many non pathogenic fungi, cysteine dioxygnase is absent so, the fate of excess cysteine and cystine in the system is unknown. In order to understand the fate of excess cysteine and cystine in the model eukaryote *S. cerevisiae* lacking *CDG1* this investigation was initiated.

In the experimental setup, we exploited the specific cysteine (*YCT1*)/cystine (*CgCYN1*) transporters. Constitutive expression under strong promoters was used to generate high cysteine/ cystine levels in the cell. Microarray analysis was carried out on cells exposed to cysteine/cystine to identify genes involved in maintenance of cysteine/cystine homeostasis. It was followed by sulphur containing metabolites of the cells exposed to cysteine/cystine at different time intervals to determine the changes in concentration of different metabolites since it will help us to identify how cells can handle cysteine or cystine overload .

The initial growth experiments on in cells transformed with either *YCT1* or *CgCYN1* revealed that, toxicity to cysteine or cystine begins to be seen at 0.5mM concentrations in the medium. Microarray experiments were also carried out on the cells exposed to 0.5mM cysteine or cystine for 5 hours. Microarray results revealed that both in cysteine treated cells and cystine treated cells when high levels of cysteine and cystine accumulate a few pathways are induced. Almost the entire arginine biosynthesis pathway was derepressed. The genes which were found to be upregulated were Ornithine carbamoyltransferae

(*ARG3*), Acetyl ornithine transferase (*ARG8*), Vacuolar cationic amino acid transporter (*RTC2*), mitochondrial ornithine transporter (*ORT1*), Acetyl glutamate kinase and N-acetyl-gamma-glutamyl-phosphate reductase (*ARG5,6*), Argininosuccinate lyase (*ARG4*),small subunit of carbomoyl phosphate synthetase (*CPA1*) . It appears that the entire pathway of arginine biosynthesis was de-repressed. These results suggest that the cells were facing a condition of arginine limitation. Since cystine is similar to arginine in structure, one possible explanation may be that cystine inhibits or blocks one of the biosynthetic enzymes leading to arginine limitation. A second more possible explanation is that the polyamines which are known sink for the thiols are synthesized at higher levels and since ornithine (Intermediate of arginine biosynthetic pathway) acts as a precursor for polyamine biosynthesis, upregulation of this pathway is been observed.

A subset of iron homeostasis genes related to iron acquisition was also found to be up-regulated. Genes such as *TIS11*, *FMP23*, *FIT2*, *SIT1*, and *ARN1* were found to be significantly up regulated. Most of these are related to siderophore mediated iron uptake, but the exact reason for their upregulation is still not clear at this stage.

In both the cysteine and cystine cells we were able to observe the induction of the sulphite efflux pump, *SSU1*. In the case of cystine treated cells we observed *STR3* gene getting up-regulated. STR3 encodes for cystathionine beta lyase. While in the case of cysteine treated cells we observed, in addition to *SSU1*, the *STR2* gene being up-regulated. *STR2* encodes for the gene cystathionine gamma synthase. *SSU1* is critical for detoxification of cysteine in *Candida albicans* which contains a cysteine dioxygenase. *CDG1* converts cysteine to cysteine sulphonate that is eventually converted to sulphite that is effluxed. *S. cerevisiae* lacks CDG1. However recent studies with knockout *CDO* (cysteine dioxygenase) mice along with earlier biochemical studies have revealed that cysteine can be converted to sulphite by cystathionine gamma lyases and cystathionine beta synthatase. This explains the induction of these enzymes and along with *SSU1* it would enable some efflux of cysteine as sulphite to the extracellular medium.

Cysteine is also known to be a chelator of zinc and explains the upregulation of the plasma membrane zinc transporter, *ZRT1* in both the cysteine and cystine overload experiments. *ZRT1* which is regulated by *ZAP1* is known to be up-regulated during zinc limitation.

The upregulation of a number of genes of unknown function was also observed.

Analysis of genes down-regulated by cysteine or cystine overload revealed that the sulphur pathway was strongly down-regulated by cysteine and mildly down-regulated by cystine.

The metabolite experiments were carried out to examine the levels of the different metabolite involved in sulphur metabolism in a time course manner, and to determine how the excess metabolites were being handled or channeled. In both cysteine and cystine we could see a dramatic increase in intracellular levels of these metabolites suggesting that indeed the system of over expressing *CgCYN1* or *YCT1* was a valid method to study the effect of excess of these metabolites in the cell.

The metabolite experiments with *YCT1* cells with added cysteine led to high levels of cysteine and also interestingly, to high levels of cystine and suggested that the oxidation to cystine was a means by which the cysteine was being detoxified, and the free sulphur was trapped and stored in the cells till it is needed further for use in the cell. Unlike cysteine which has a free thiol group that is reactive and that can interfere with cellular processes, cystine is comparatively inert. Thus is appears that the cell immediately is able to convert excess cystine to cysteine as a mechanism to detoxify, and possibly store excess cysteine, an otherwise valuable nutrient to the cells. We refer to this method of detoxification by the cells as 'thiol trapping'.

Excess cysteine was trapped not only as cystine but, part of it was also converted to other cysteine metabolites that were intermediates, and lacked a free thiol group. Thus cystathionine and S-adenosyl homocysteine accumulated at significantly higher levels and stayed at these elevated levels even after 5 hours. These are cysteine intermediates in which the free thiol is blocked. Thus the method of thiol trapping is not limited to cystine, but to other intermediates that can be tolerated at higher levels and yet lack a free thiol group. It is also interesting that SAM was accumulated higher in cells treated with cysteine as compared to cystine, and explains the strong down regulation of the sulphur regulation genes, since SAM is considered a key regulatory metabolite in the process. In contrast, methionine, which also lacked a free thiol group, did not change much. Since SAM is directly linked to the polyamines biosynthetic pathway, it explains the need for higher levels of polyamines and arginine.

Since cystine was used as a sulphur source its metabolism required reduction to cysteine and regarded to be a reducing metabolite in the cell. We attempted an analysis of cystine reduction using deletion constructs of the different thioredoxins and glutaredoxins. These studies revealed that no single enzyme was entirely responsible for the reduction. Although a slight defect was found in *grx3*Δ disruptant the defect was only minimal. Further determination of *in vitro* cystine reduction activity by using purified thioredoxins or glutaredoxins will enable us to determine if a few of them are more specifically involved in cystine reduction.

In conclusion, cysteine and cystine overload experiments revealed several new leads about how the cells responded to excess cysteine and cystine. It also provided several new insights into how the toxicity of these compounds is handled by the cells.