6.1. Summary and Conclusions

Cysteine biosynthesis in *M.tuberculosis* has been a subject of attention in recent times. This is because the identification of new drug targets in *Mtb* is now a global pursuit and sulphur metabolism in *Mtb* is one of the key arenas. Although the disease may be controlled by the current drug regimen, they have several shortcomings. Besides the lengthy regime of the treatment, adverse side effects, drug resistance and inability of the drugs to target persistent *Mtb* population are the major drawbacks.

In view of targeting the persistent *Mtb* population, which is one of the prime reasons of the emergence of the disease even long after the treatment has ceased, research is undergoing in some key areas like fatty acid catabolism, metabolic enzymes, stress related products and respiratory enzymes. The genes involved in these pathways are observed to be either required for or are over expressed during latency. Therefore, we have chosen to focus on one of the crucial pathways ie: the cysteine metabolism in *Mtb*. The genes of the sulphur metabolic pathway are consistently upregulated during the mycobacterial life in the granuloma, in conditions of oxidative stress, nutrient starvation or adaptation to dormancy.

The metabolism of cysteine in *Mtb*, although interesting, remains largely undiscovered. Unlike other intracellular pathogens in humans, *Mtb* posseses many routes for synthesis of this essential amino acid. Moreover, *Mtb* has three cysteine synthase enzymes, CysM (OASS-B) (Rv1336), CysK1 (Rv2334) and CysK2 or CysM2 (Rv0848), where the role of CysK2 has been recently identified as a S-sulfocysteine synthase besides providing an additional route of cysteine biosynthesis (Steiner et al., 2014). We have chosen to study the sulfide dependant pathway of cysteine metabolism, primarily focussing on the role of the last two enzymes in this pathway, ie: CysK1 or OASS-A and CysE or SAT. Although the sulfide dependant pathway is well characterized in other plants and bacteria, it is not well studied in *Mtb*. We started with the purification of the components of the cysteine synthase complex (OASS-SAT). The expression and purification of the enzyme *Mt*SAT was met with a lot of challenges, owing to the nature of the protein which is insoluble, has a tendency to oligomerize, subject to proteolysis, nuclease contamination, low expression levels besides many other problems. Finally, we were able to purify *Mt*SAT to homogeneity and checked for the enzyme activity.

*Mt*SAT had a turnover number (k_{cat}) of 76.66 sec^{-1}, while *Mt*OASS turnover (k_{cat}) value is 254 sec^{-1}. In the extensive enzyme kinetics which followed, we undertook a detailed investigation of the sulfide dependant pathway in *Mtb*; where we have explored unique features regarding this route of cysteine biosynthesis.

In the biochemical characterization of the components, we observe that *Mt*OASS follows a simple Michaelis-Menten kinetics with respect to its substrate OAS. But significant differences are observed for the substrates of *Mt*SAT, as while binding isotherm for serine is monophasic, the binding isotherm of acetyl CoA is biphasic. Therefore, while the K_d value for serine is 0.05 ± 0.01, the K_d values for acetyl CoA are 0.06 ± 0.014 mM for the first binding site and 5.15 ± 3.96 for the second binding site, respectively. Hence, acetyl CoA binds to two sites, and we propose that a secondary site other than active site is the inhibitory site and binding of acetyl CoA to this site at higher concentration inhibits the SAT activity. From our steady state kinetic experiments, we further conclude that acetyl CoA acts as a inhibitor beyond a concentration of 0.32 mM, for free *Mt*SAT.

In the fourth chapter, we have discussed the purification of *Mt*C.S complex and its characterization to uncover its possible role in the regulation of cysteine biosynthesis. While investigating the catalytic properties of the *Mt*Cysteine synthase complex, we discover that as concentrations of acetyl CoA > 0.15 mM become inhibitory for the complex, it keeps a check both on the production of cysteine and the utilisation of acetyl CoA for cysteine biosynthesis. Further, on the revival of favourable conditions like nutrient availability, the cell channels more acetyl CoA for cysteine biosynthesis and the enzymes SAT and OASS are dissociated from the complex to their free forms. Now, the uptake of acetyl CoA by *Mt*SAT increases as it is not inhibitory till concentration of > 0.32 mM. Consequently, the enzyme activity increases to almost ~4 folds as compared to the activity of the complex form. Hence, the *Mt*Cysteine synthase complex formation in cell seems to be a method of regulation of cysteine biosynthesis (Figure 6.1).

The substrate acetyl CoA is a key player in this pathway which may inhibit or activate the pathway, depending on its concentration and its functional preferences in *Mtb*. As acetyl CoA is produced mainly during glucose metabolism, there is a high probability that during nutrient limiting conditions in the macrophage, the sulfide dependant pathway may become strongly inhibited due to non- availability of acetyl CoA, the substrate of *Mt*SAT. In such nutrient limiting conditions, host

lipids are a primary source of nutrients which are broken down by fatty acid oxidation to provide energy to the bacteria, hence acetyl CoA although synthesized, has role in other vital pathways. Henceforth, we propose a hypothesis that, the alternative cysteine synthesis pathway, CysM-CysO may be more important for the *Mtb* cell in the granuloma stage, as it does not use acetyl CoA as its substrate and moreover its substrates are much more resistant to oxidation than substrates of the sulfide dependant pathway (Figure 6.2).

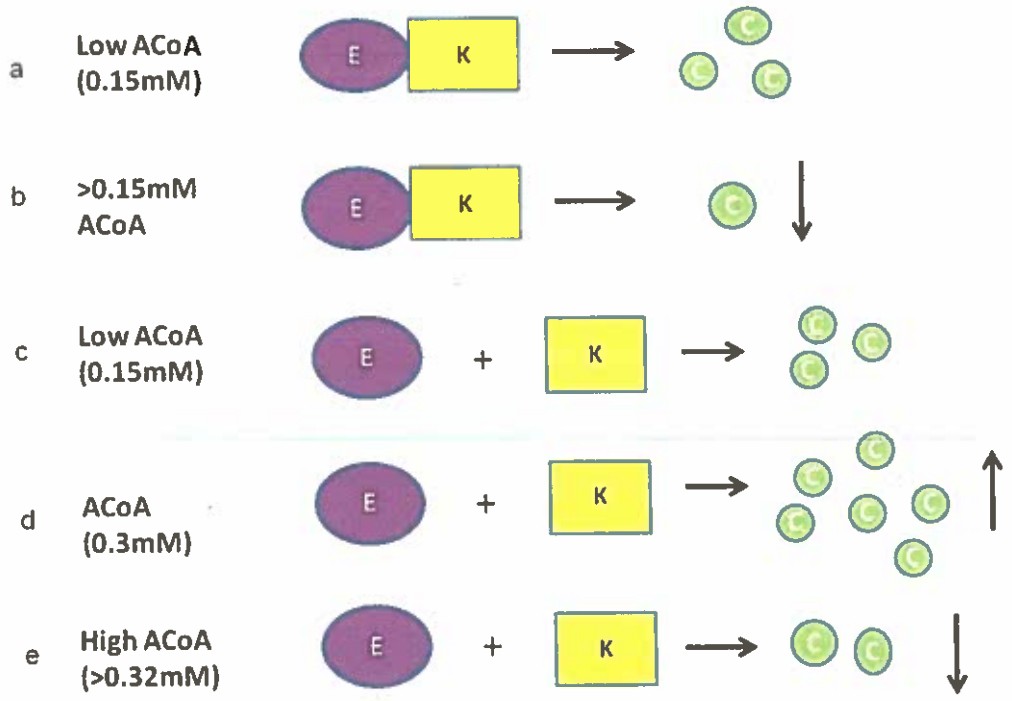

Figure 6.1. Relationship beween acetyl CoA concentration and synthesis of cysteine (C) in *Mtb* when the enzymes SAT (E) and OASS (K) form the cysteine synthase complex (a,b) and when both the enzymes are in their free state (c,d ,e).

Meanwhile the product cysteine activates its own production by binding to yet uncharacterized allosteric site in the enzyme *Mt*SAT and reducing the K_m of its substrates, possibly via conformational changes. This activation continues till the levels of thiol in the cytoplasm reach ~5 mM, when products like mycothiol or CoA etc, start inhibiting the activity of *Mt*SAT. The abundant production of cysteine is helpful for the pathogen which stores it for use during unfavourable conditions, when the synthesis of cysteine might be very low. Hence, *Mycobacteria* have huge

reserves of mycothiol in their cytoplasm as a 'storehouse for intracellular cysteine'. This requirement for cysteine coupled with the limited availability of acetyl CoA in *Mtb* may be the reason for the presence of such unique features in the cysteine biosynthetic pathway in *Mycobacteria*.

The fifth chapter of this dissertation, is regarding the ligand interaction studies in *Mtb* and a comparison with other pathogenic bacterial strains. We have studied the thermodynamics of OASS from *Salmonella typhimurium* (*St*OASS), *Haemophilus influenza* (*Hi*OASS), and *Mycobacterium tuberculosis* (*Mt*OASS) binding to their substrate O-acetylserine (OAS), substrate analogue (methionine), and product (cysteine). We observe that, OASS from all the three different pathogenic bacteria bind substrate and product through two different mechanisms; a predominantly entropically driven substrate binding and both entropically and enthalpically driven product binding. The binding of the substrate (OAS and methionine) to OASS is dominated by a favorable entropy change, with minor contribution from enthalpy change, whereas cysteine binding to OASS shows that both enthalpy and entropy contribute significantly to the binding free energy at all temperatures (10-30°C) examined. Further experimental verifications imply that, this binding is not mediated through classical hydrophobic binding, and instead, may involve desolvation of the polar active site. Hence, we speculate that OASS in general, may exhibit two different binding mechanisms for recognizing substrates and products. This differential mode of binding of substrates and products was further verified by the ligand aggregation studies for *Mt*OASS, where we observe that the substrates and products vary in their aggregation profiles. While substrates tend to aggregate *Mt*OASS irreversibly, most likely due to major conformational changes in the structure of the enzyme, the products show reversible aggregation even at high concentrations (> 30 mM). We have also crystallized the apo form structure of *Hi*OASS (PDB 4H01).

6.2. Future Scope of the study.

Mycobacterium has evolved to be a highly successful human pathogen. Its long term residence inside the host is facilitated by metabolic adaptation to the hostile environment in the macrophages. The identification of new antibacterial targets is essential to address MDR (Multi drug resistant) and latent-TB infection. Numerous

studies have validated amino acid biosynthetic pathways and downstream metabolites as antimicrobial targets The lage number of genes of the sulphate assimilation pathway upregulated during its survival reveal that this pathway is crucial for its existence henceforth major efforts are been targeted towards inhibitor discovery of mycobacterial sulphur metabolic pathways. Cysteine is incorporated into proteins, coenzymes and mycothiol. Mycothiol regulates cellular redox status in all actinomycetes including mycobacteria and has great significance in this pathogen. Coenzyme A (CoA) is heavily utilized for lipid metabolism which is a process central to mycobacterial cell wall maintenance and remodelling. Small molecule inhibitors represent valuable chemical tools that can be used to investigate the role of sulphur metabolism in *M.tuberculosis* and represent new leads for drug development. The study of the transcriptional and biochemical mechanism of sulphur metabolism regulation in Mtb and potential small molecule regulators will have a strong impact on designing candidates for therapeutic invention.

The presence of OASS and SAT on the same operon gives reason to believe that the pathway may be under transcriptional control by some regulators, which may switch the genes on or off according to the conditions prevailing in the cell. Infact, interestingly OASS and SAT lie in the same operon in almost all *Mycobacteria*, except *M.smegmatis* which further supports the fact of existence of regulation at the level of transcription. This dissertation is an effort made to comprehend some basic concepts of the sulfide dependant cysteine biosynthetic pathway in *M.tuberculosis*. The research may be further extended to a better understanding of the regulation of cysteine biosynthesis in this unique pathogen.

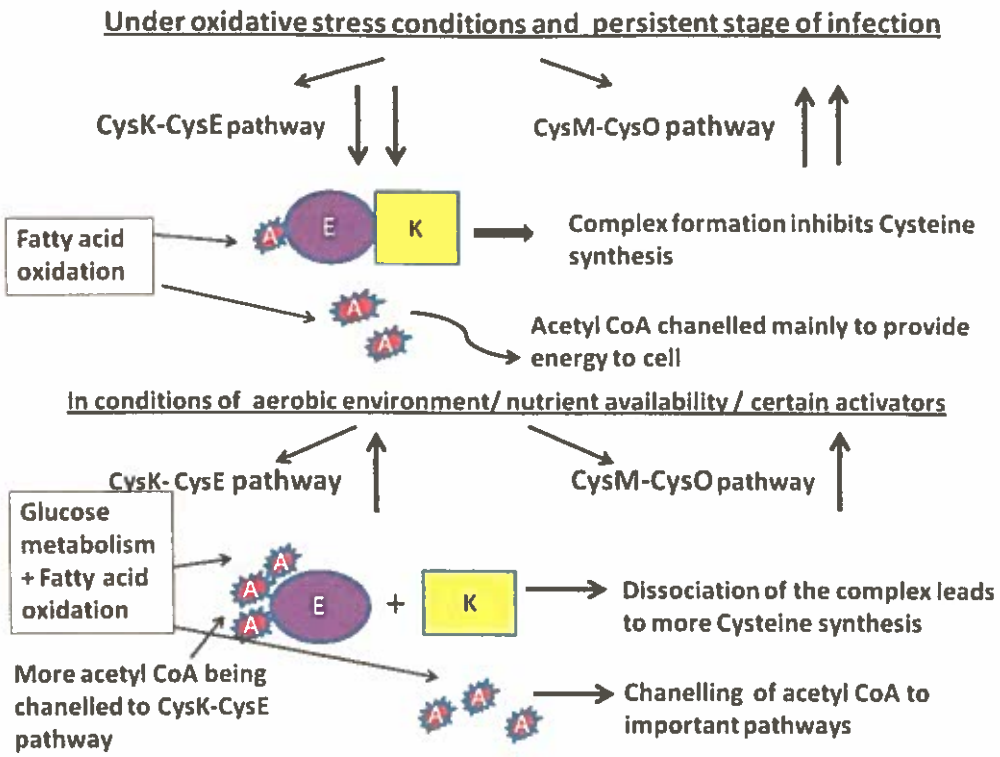

Figure 6.2. Hypothetical mechanism of Cysteine Biosynthesis in the *Mtb* cell