CHAPTER 5

SUMMARY
Importance of basic metabolic pathways in the development of a microbial disease became apparent with the discovery of involvement of fatty acid metabolic pathways and glyoxylate pathway in virulence in many pathogens (De voss et al, 2000; McKinney et al, 2000). This implies that if a pathogen is unable to synthesize the precursors of important metabolites required for growth, it is unlikely to proliferate or persist in host. Several adaptive alterations in house keeping metabolic pathways have been observed in diverse group of organism in response to internalization by macrophages or contact with host immune system. One of the prominent adaptations found amongst varied organisms from commensals to phytopathogens (McKinney et al, 2000; Manabe et al, 1999; Goldstein et al, 2001; Lorenz et al, 2002) is adjustments in physiology in response to nutrition starvation inside phagolysosome in macrophages. The microenvironment is usually devoid of complex carbon sources like glucose and certain essential amino acids. Pathogen in turn perceives simple carbon sources like acetyl Co-A or C2 sources or deficiency in essential metabolites like magnesium and iron in their environment and responds accordingly. Whole genome sequencing of Mycobacterium tuberculosis and microarray technology has presented a global picture of these adaptations in terms of controlled expression of selected genes. Often an additional, altered or unusual function of a gene has been noted under such conditions.

The work presented in this thesis is an extended investigation of the basic energy pathway enzymes, isocitrate dehydrogenase and aconitase and understanding their distinctive role in immune response and iron metabolism respectively as additional properties.

Two isoforms of isocitrate dehydrogenase, M. tb icd-1 and M. tb icd-2, have been identified in the M. tb genome. This study, for the first time, biochemically characterizes M. tb ICD-1 and ICD-2 in terms of their substrate and coenzyme specificity, metal ion requirement, pH, temperature, salt tolerance and oligomeric assembly. Differences in coenzyme affinity, oligomeric state, pH tolerance and phylogenetic affiliation of the two isoforms of M. tb ICD were evident. While M. tb
ICD-1 also accepts Zn\(^{+2}\) ion as metal ion apart from Mg\(^{+2}\) for catalytic reactions. *M. tb* ICD-2 did not show any activity in presence of Zn\(^{+2}\). *M. tb* ICD-1 could tolerate a wider pH range than *M. tb* ICD-2 and showed 33-35% activity in an acidic pH upto 5. The optimum temperature for the enzyme activities was 37°C - 45°C. Both the enzymes required NADP\(^{+}\) as the coenzyme, but remained inactive in presence of NAD\(^{+}\). Km [isocitrate] for *M. tb* ICD-1 in presence of Mg\(^{+2}\) or Zn\(^{+2}\) was 10\(\mu\)M ± 5 and 22\(\mu\)M ± 7, respectively while that for *M. tb* ICD-2 was 20\(\mu\)M ± 1 in presence of Mg\(^{+2}\). *M. tb* ICD-1 is a homodimeric NADP\(^{+}\) dependent isocitrate dehydrogenase, whereas, *M. tb* ICD-2, originally annotated as a monomeric protein, was found to exist in a dimeric state. Phylogenetic analysis revealed that unlike *M. tb* ICD-2 which groups with bacterial ICDs, *M. tb* ICD-1 exhibits a closer lineage to eukaryotic NADP\(^{+}\) dependent ICDs.

A differential expression of *M. tb* ICD-1 and ICD-2 under different stages of growth of *Mycobacterium tuberculosis* in in vitro culture was further demonstrated. The experiment pointed to a transient expression of the two ICDs. ICD-1 was expressed during early log phase and reappeared along with ICD-2 during late log and stationary phase. However, ICD-1 was less expressed than ICD-2 on 12\(^{th}\) and 18\(^{th}\) day quantitatively. The tolerance to a broader range of pH by ICD-1 as shown by biochemical assays may be a logical explanation to its reappearance with ICD-2 expression during late log or stationary phases when pH inside the cell is much more variable than log phase.

Isocitrate dehydrogenase (ICD) was found to be amongst proteins that are released from *Mycobacterium tuberculosis* during late logarithmic growth phase. In addition to biochemical characterization, the immunogenic properties of the two isoforms of *M. tuberculosis* ICDs were evaluated and compared the same with the control antigen – HSP 60 as well as purified protein derivative (PPD). PPD lacks the sensitivity to distinguish between BCG vaccinated and TB-infected populations and therefore epidemiological relevance of PPD in BCG vaccinated regions is debatable. It was shown that *M.tb*
ICDs elicit a strong B-cell response in TB-infected population and can differentiate between healthy-BCG vaccinated population and those with tuberculosis. The study population (n=215) was categorized into different groups, namely, patients with fresh infection (n=42), relapsed TB cases (n=32), patients with extrapulmonary tuberculosis (n=35), 44 clinically healthy donors, 30 NTMs and 32 non-TB patients (culture negative for acid fast bacteria but carrying other infections). The M.tb ICDs showed statistically significant antigenic distinction between healthy-BCG vaccinated controls and tuberculosis patients (p<0.0001) and those with other infections. Surprisingly, extrapulmonary infections could not be discriminated from healthy controls by HSP 60 (p=0.2177) which M. tb ICDs could do significantly (p<0.0001). These results highlight the immunodominant, immunosensitive and immunospecific nature of M.tb ICDs and point to a novel and an unusual property of this TCA cycle enzyme. A combinatorial study of biochemical and immunological properties, differential expression and phylogenetic analysis while reflecting an attempt to trace the evolution of intracellular pathogenicity, may additionally help understanding the adaptive role of isocitrate dehydrogenase in intracellular persistence of this pathogen.

Aconitase is the common enzyme between both TCA cycle and glyoxylate shunt. Also, M. tb Acn is a Fe-S cluster containing protein. Such proteins are known to act as iron regulatory and sensor proteins. Cellular iron levels are one of the critical nutritional alteration inside infected macrophages and hence is closely monitored by Mycobacterium tuberculosis for survival. Amongst the sensor proteins reported in eukaryotic systems and a few prokaryotes are iron responsive proteins (IRPs) that bind to iron responsive elements (IREs) and regulate the translation or stability of mRNAs encoding proteins involved in iron homeostasis. Sequence comparison at amino acid level showed that it carries the conserved residues of IRPs. Both the enzymatic and the IRP activity of M. tb Acn were evaluated in this study. M. tb Acn functionally existed as a monomer and was enzymatically active in converting isocitrate to cis-
aconitate at a broad pH range of 7-10 (optimum pH 8) with a single interconvertible transition state. Along with being enzymatically active, it was observed that *M. tb* Acn could successfully bind to IRE-like sequences of eukaryotes taken as control and also to predicted IRE-like sequences found in *M. tb* genome. *M. tb* genome was scanned for IRE-like elements and the selected sequences were subjected to RNA secondary structure prediction. Three such regions were selected which are found in either 3' or 5' untranslated regions of mRNAs of *trxC, ideR* and *acn* that have probable role in iron metabolism. IRE – binding activity of *M. tb* Acn was checked by gel retardation assays. It was observed that availability of iron affected both the IRP and the enzymatic activity of *M. tb* Acn in-vitro. *M. tb* Acn when reactivated with Fe**+** acted as a TCA cycle enzyme, while when depleted of iron by specific iron chelator acted like an IRP binding to the selected IREs. It was also shown that both the activities of *M. tb* Acn are mutually independent. Factor Xa protection assay show site specific binding of IREs to *M. tb* aconitase. The importance of Cysteine residues for both enzymatic and IRP activity was demonstrated. These results support that *M. tb* Acn is a bifunctional protein and points to its role in iron homeostasis.

Summarizing the above work, I have biochemically characterized Isocitrate dehydrogenases and Aconitase of *Mycobacterium tuberculosis* and have pointed out the additional properties of these two proteins apart from them being TCA cycle enzymes. The results reveal that *M. tb* ICDs have additional property to elicit high B-cell response in TB-patients and can differentiate healthy BCG vaccinated population from TB infected population. This study also points to post transcriptional regulatory role of *M. tb* Aconitase as a RNA binding protein. Lineage of these two proteins was studied in detail in an attempt to trace the evolution of intracellular pathogenicity.