Chapter 6

CLONING, OVEREXPRESSION & STRUCTURE SOLUTION OF ALANINE DEHYDROGENASE
6.1 Introduction

*Mycobacterium tuberculosis* exhibits significant changes in gene expression during the latent/persistent stage of infection. Proteomic analysis of the nutrient starved/latent phase bacteria suggested a decreased expression of proteins involved in energy metabolism, lipid biosynthesis and cell division in addition to induction of stringent response and several other genes that may play a role in long-term survival within the host (Betts *et al*., 2002 & Stewart *et al*., 2003). Expression of *ald* is upregulated in *M. tuberculosis* upon nutrient starvation and in *M. marinum* during persistence within the granulomas of infected frogs (Chan *et al*., 2002). L-Alanine dehydrogenase production and activity were also increased when *M. tuberculosis* and *M. smegmatis* when shifted from aerobic to anaerobic growth (Rosenkrands *et al*., 2002 & Usha *et al*., 2002). The enzyme has been rated among the top 3 targets against persistence in an analysis (Hasan *et al*., 2006). Western blotting has failed to detect L-alanine dehydrogenase in BCG, even though the *ald* gene has been identified by DNA hybridization, this absence of functional protein is due to a single nucleotide deletion within the *ald* gene that causes a frame shift mutation and disrupts the full-length protein. L-alanine dehydrogenase, encoded by *ald*, catalyzes the oxidative deamination of L-alanine or, in the reverse reaction, the reductive amination of pyruvate. This enzyme is known to be required for normal sporulation in *Bacillus subtilis* (Siranosian *et al*., 1993) and for normal development of *Myxococcus xanthus* (Ward *et al*., 2000). In mycobacteria, L-alanine dehydrogenase is secretory antigen associated with bacterial persistence during infection.

In mycobacteria, the gene product is a secretory antigen associated with bacterial persistence during infection suggests its role in virulence of *M. tuberculosis*. Its involvement in nitrogen metabolism, releasing ammonia from alanine, second most abundant amino acid in extra cellular fluid of host would also suggest a role of ALD in inhibition of phagosome acidification. *Rv2780* which encodes L-alanine dehydrogenase (MtAlaDH) found to be over-expressed under hypoxic (Rosenkrands *et al*., 2002 & Starck *et al*., 2004) and nutrient starvation regimes (Betts *et al*., 2002). Increased levels of this enzyme have been linked to the generation of alanine for peptidoglycan biosynthesis (Starck *et al*., 2004 & Hutter *et al*., 1999) and the maintenance of the NAD+ pool under conditions when the terminal electron acceptor oxygen becomes limiting (Betts *et al*., 2002 & Hutter *et al*., 1998). This enzyme plays an important role in carbon and nitrogen metabolism in the various microorganisms and is key factor in assimilation of L-alanine as
an energy source through the tricarboxylic acid cycle during sporulation in several *Bacillus* spp (Grimshaw *et al.*, 1981a).

L-alanine dehydrogenases purified from sources like *Mycobacterium tuberculosis* (Andersen *et al.*, 1992), *Bacillus sphaericus* (Ohshima *et al.*, 1990) *Phormadium lapideum* (Sawa *et al.*, 1994) and *S. phaeochromogenes* (Itoh, 1983) exist as homohexamers, with subunit molecular weight ~40,000 Da, while octamer and tetrameric oligomeric associations are reported from *S. aurifaciens* (Vancurova *et al.*, 1989) and *S. fradiae* (Vancura *et al.*, 1989) respectively. Earlier work involving kinetics has resulted in the understanding of several aspects of the enzyme mechanism. These studies have suggested that the reaction is ordered with NAD\(^+\) binding before alanine in the forward reaction involving oxidative deamination while NADH binds first in the reverse reaction involving reductive amination (Grimshaw *et al.*, 1981a; Grimshaw *et al.*, 1981b & Ohashima *et al.*, 1979). However the order of release/binding of pyruvate and ammonia apparently varies between the enzymes from different species (Grimshaw *et al.*, 1981a & Grimshaw *et al.*, 1981b). Work on the *B. subtilis* enzyme suggested that imino acid and carbinolamine intermediates are formed during the catalytic cycle (Grimshaw *et al.*, 1981) (Scheme 6.1).

Scheme 6.1: Reaction mechanism of L-alanine dehydrogenase. Alanine reacts as a monoanion and a hydride transfer takes place between Co of Alanine and C4 of the nicotinamide ring resulting in an iminopyruvate intermediate. The next step involves attack by a water molecule in the presence of the catalytic histidine (base) to form a carbinolamine intermediate. Proton removal from the hydroxyl group of the carbinolamine results in elimination of ammonia and formation of pyruvate. (Adopted from Grimshaw *et al.*, 1981).
The crystal structure of alanine dehydrogenase from *Phormadium lapideum* (cynobacteria) (Baker et al., 1998) and *Archaeoglobus fulgidis* (Hyperthermophilic archaean) (Gallagher et al., 2004) have been reported. The level of sequence similarity between ALD from *P. lapideum* and other eubacteria is high. These enzyme comprise a C-terminal NAD binding Rossmann fold domain, and a catalytic domain. Both domains are separated by cleft. Bacterial ALD are far away, in sequence and in structure from other aminoacid dehydrogenases that include phenylalanine dehydrogenase, leucine dehydrogenase and glutamate dehydrogenase. Alanine dehydrogenase is first secretory antigen reported in virulent strain of Mycobacteria and absence of its functional copy in vaccine strain suggests its role in virulence of *M. tuberculosis*. Alanine dehydrogenase (ALD) from Mycobacterium (MtAlaDH) is hexamer of 40kDa chain which catalyze the NAD-dependent interconversion of alanine and pyruvate.

### 6.2 Experimental Methods

#### 6.2.1 Cloning, over-expression and purification

* Ald gene (Rv2780) was amplified from *Mycobacterium tuberculosis* H37Rv genomic DNA using the following primers, sense primer 5'-ATGCGCTAGCATGCGCGTCGGTATTTCCG-3' and antisense primer 5'-TATAAAGCTTGCCCCACGCACGCTGG-3' containing NheI and HindIII restriction sites (underlined nucleotides). The amplified PCR product was digested with NheI and HindIII and ligated in *pET21d* (Novagen) digested with same enzyme and ensures a his-tag at the C-terminal end. Clones were screened by restriction digestion and the resulting construct was transformed into *E.coli* C41 (DE3). The integrity of the insert was verified by sequencing.

A single colony was inoculated into 50 ml of YT medium containing 50 µg/ml ampicillin and grown overnight at 37°C. These cells were used to inoculate 1 liter YT medium containing 50 µg/ml ampicillin and expression was induced by 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at an optical density of 0.6 at 600 nm. Cells were allowed to grow at 30°C for 6 hrs after induction with IPTG. The IPTG induced cells were harvested resuspended in ice cold buffer 50 mM Tris-HCl, pH 7.2, 300 mM NaCl, and 10 mM Imidazole (Buffer A) and sonicated. The crude lysate was centrifuged at 27,000g for 30 min. The supernatant was applied to a Ni⁺⁺-IDA column pre-equilibrated with Buffer
A. Protein was eluted using the same buffer supplemented with 500 mM Imidazole. Fractions containing the protein were pooled and precipitated using ammonium sulfate (60%) saturation. The pellet was resuspended in 50 mM Tris-HCl pH 7.2, 50 mM NaCl, 5 mM EDTA, 2 mM β-mercaptoethanol (Buffer B) and further purified on a Superdex S-200 (GE Healthcare) gel filtration column equilibrated with buffer B. The protein was pooled and concentrated to 12 mg/ml using a 30-kDa cutoff centicon (Amicon). The purified protein was assayed for activity and protein concentrations were determined with the Bradford reagent (Bradford, 1976) using Bovine serum albumin as a standard. MtAlaDH remained stable at 4°C without degradation up to one week. Purity of the protein was confirmed using 12% SDS-PAGE. Photometric determination of enzyme activity was accomplished by measuring the rate of the production of NADH at 340 nm that accompanies the conversion of alanine into pyruvate in the oxidative deamination (Hutter et al., 1998).

6.2.2 Crystallization

MtAlaDH was concentrated to ~12 mg/ml in a buffer comprising 50 mM Tris (7.2), 50 mM NaCl, 5 mM EDTA and 2 mM β-Mercaptoethanol. All crystallization experiments were carried out by the hanging-drop vapour-diffusion method in 24-well Linbro tissue-culture plates (ICN Inc.). The experiments were setup exploiting two different sparse matrix screening strategies (Majeed et al., 2003; Jancarik et al., 1991 & Cudney et al., 1994) at 295K and 277K. Each hanging drop was prepared using 2μl protein sample with 1 μl reservoir solution and was equilibrated over 500μl of reservoir solution. These screens gave an unexpected high yield with about 6% of 96 screen produce crystals. Although the purified protein crystallized readily under many conditions but most of the crystals diffracted very weakly or not at all. Optimization of these conditions gave a few reasonably sized crystals, but they were generally not suitable for data collection as most of them are cracked or twinned. Single crystals (1.4 x 0.7 x 0.4 mm) from various conditions were screened for diffraction, but it is found that crystals of Rv2780 were highly sensitive to X-ray and temperature and crystal death would occur only as after five frames at room temperature. After screening of various condition for stability and diffraction crystal grown in presence of ammonium sulfate was found to be most suitable for data collection. Best diffraction quality crystals of MtAlaDH were obtained by reservoir solution consisting of 2M Ammonium sulfate solution. The single rod shape
crystals started appearing within 3-4 days and grew to maximum size in two week. Increasing the protein concentration and temperature and time variation led to a clustering of crystals as well as reduction in size.

6.2.3 Data collection and analysis

A single crystal obtained from the ammonium sulfate condition was mounted in a capillary. Diffraction data for native crystal was collected over RIGAKU MICROMAX, X-ray generator using MAR345DTB image plate detector. A crystal to detector distance of 200 mm and an exposure time of 60 s were used for data collection. The crystal remained stable up to 97% data completion. Diffraction images were indexed, integrated and scaled using the HKL-2000 package (Otwinowski et al., 1997). The best native crystals diffracted up to 2.6 Å resolution were monoclinic with dimensions a=173.89, b=127.07, c=135.95 Å and belong to space group C2. The data collection statistics are summarized in Table 6.1.

Size exclusion chromatography experiments had earlier indicated that the protein exists as a hexamer in solution. Calculation of the Matthews coefficient (Matthews, 1968) suggests that the asymmetric unit contains six molecules. Assuming six molecules in asymmetric unit corresponds to a Matthews’s coefficient of 2.83 Å³/Da or solvent content of about 56.56%.

6.2.4 Structure solution and refinement

Amino acid sequence alignment shows an identity of 45% with alanine dehydrogenase from Phormidium lapidem (pdb code 1PJB), (Baker et al., 1998) Therefore, a model consisting of the atomic co-ordinates of ALD from P. lapideum with non-identical residues converted to alanine was used as search model for structure solution of MtAlaDH by molecular replacement program with program AMoRe (Navaza, 1994) implemented in the CCP4 package (Collaborative computing projects No.4 1994). The correct solution gave a correlation coefficient of 56.2% and R-factor 47.8% using data between 15 to 3 Å. The transformed model was subjected to restrained refinement using REFMAC5 (Murusudov et al., 1997). The phases were improved and extended to 2.6 Å incrementally. 2Fo–Fc and Fo–Fc electron density maps were visualized using the program TURBO-FRODO (Roussel et al., 1989 ) and COOT (Emsley et al., 2004) which were used for model building. The crystallographic R-factor and R-free (Brünger et al., 1989) were monitored at each stage to prevent model bias. Water molecules are added by
automatic water-picking algorithm of COOT. The positions of these automatically picked waters were manually checked, and a few more waters were manually identified on the basis of electron density contoured at 1.0 $\sigma$ in the $2Fo-Fc$ and 3.0 $\sigma$ in the $Fo-Fc$ map. The six subunits of MtAlaDH in the asymmetric unit is labeled as A-F forming A-D, B-E, and C-F dimers. During the last stage of model building loop region from 234-245 in each subunit shows very weak electron density. This is manually built after close examination of each subunit. The final model of MtAlaDH contains six subunits of ALD, 224 water molecules. The final refinement statistics are given in Table 6.1.

6.2.5 Structure analysis

The geometry of final models was checked using PROCHECK (Laskowski, 1993) and WHAT-IF (Vriend, 1990). All the structural superimposition and r.m.s.deviation for ALD and other protein were determined using the program ALIGN (Hubbard et al 1993) and PROFIT (http://www.bioinf.org.uk/software/profit). Average B-factors for the protein atoms and water molecule were carried out by using the BAVERAGE program from CCP4 package (Collaborative computing projects No.4 1994). Interactions were calculated using program CONTACT module of CCP4 suite. NACCESS (Cohen, 1997) was used for surface area calculations. The figure was prepared by using the program CCP4MG (Potterton et al., 2004), PYMOL (DeLano, 2002), and InsightII (Accelrys, 2000).

6.2.6 Fluorescence titration of NAD and NADH binding

Binding of NAD and NADH was monitored by change in tyrosine fluorescence fluorescence upon their ligand binding. Measurement were made on Perkin-Elmer flourimeter ($\lambda_{\text{excitation}}$ = 276 nm and $\lambda_{\text{emission}}$ = 309 nm; slit widths = 8 & 6 nm). Titration were performed at 25°C by addition of NAD (500 mM stock) or NADH (10 mM stock) to 0.6 ml of 50 mM Tris (7.2), 50 mM NaCl, 5 mM EDTA and 2 mM $\beta$-mercaptoethanol containing 100 $\mu$g protein. To avoid dilution effects, volume change during titration was limited to only 3% of total volume. Control titration with buffer alone did not produce any change in emission signal.
6.3 Results and Discussion

6.3.1 Cloning of Rv2780

The gene of *Rv2780c* of *M. tuberculosis* H37Rv is 1116 bp long and was amplified by PCR by using sense and antisense primer set for C-terminal his-tagged protein. The PCR product corresponds to 1116 bp was eluted using PCR gel extraction kit and cloned into T7 RNA polymerase-based bacterial expression vectors, *pET21d* between *NheI* and *HindIII*. Clones were confirmed through restriction digestion (Fig. 6.1A) and PCR.

6.3.2 Over-expression and purification

The expression plasmids were introduced into *E.coli* C41 (DE3), a strain that contains T7 RNA polymerase gene under the control of *lacUV5* promoter. MtAlaDH were optimized for growth and induction at 30°C for 6 hrs by inducing with 0.2 mM IPTG. MtAlaDH were very less soluble (10%) and over-expressed under these conditions and a prominent ~ 40 kDa polypeptide was detected in case of MtAlaDH by SDS-PAGE in whole cell extracts of IPTG induced bacteria (Fig 6.1B). These polypeptides were not present in uninduced samples or when bacteria containing the *pET21d* alone were induced with IPTG. A Ni++-IDA column was used in affinity chromatography to purify the protein with 6x histidine tag at C-terminal. Protein fractions containing MtAlaDH were pooled, precipitated using ammonium sulfate (60%) and resuspended in 50 mM Tris-HCl pH 7.2, 50 mM NaCl, 5 mM EDTA, 2 mM β-mercaptoethanol. After gel filtration using Superdex S-200 (*GE Healthcare*) column, elute fraction showed that the preparation was essentially homogenous with respect to the ~ 40 kDa MtAlaDH polypeptide (Fig 6.1B).
Fig 6.1: Cloning and purification of MtAlaDH.

(A) Cloning of MtLat as his-tagged protein. Mr, 1 kilobase DNA ladder; Lane 1, Restriction digestion of vector pET21d; Lane 2, PCR product of MtAlaDH; Lane 3 and 4, restriction digestion of pET21d-MtAlaDH.

(B) Purification of MtAlaDH. Mr, Molecular weight marker; Lane 1, Uninduced cell lysate; Lane 2, Induced cell lysate with 0.5 mM IPTG; Lane 3, Supernatant; Lane 4, Sample washed with 10 mM Imidazole; Lane 5, Sample washed with 80 mM Imidazole; Lane 6, Purified protein after Ni²⁺-IDA; Lane 7, Purified protein after size exclusion chromatography.
6.3.3 Gel filtration analysis

Gel filtration chromatography was used to ascertain the oligomeric state of *M. tuberculosis* alanine dehydrogenase under standard experimental conditions. MtAlaDH shows hexameric behaviour. We used pre-packed Superdex 200 high-resolution column in all the purifications to remove the trace contamination ensuring high level of purification. The column used to ascertain the molecular weight was calibrated with known molecular weight marker proteins and calibration graph of $V_e/V_o$ was plotted against the logarithm of mol. wt. of the corresponding proteins. $V_e$ and $V_o$ represent elution volume of the protein and void volume of the column respectively. Blue dextran (2000 kDa) was used to determine the void volume. MtAlaDH eluted at 12.56 ml (Fig 6.2) corresponding to 240 kDa which is in good agreement with theoretical values 240 kDa.

![Gel filtration profile of wild type MtAlaDH](image)

**Fig 6.2:** Gel filtration profile of wild type MtAlaDH. Protein was eluted at 12.56 ml showing its hexameric in nature.
6.3.4 Biochemical studies

Each subunit of MtAlaDH consists of 371 residues and the purified enzyme ran as a 40 kDa protein consistently in SDS-PAGE gels. Its oligomeric state from size exclusion chromatography was found to be hexameric as observed in many members of this family of enzymes as also reported earlier. Binding of NAD and NADH to MtAlaDH was probed by fluorescence titration assays (Fig 6.3A, B, C &D). Addition of NAD or NADH quenches the fluorescence emission signal of MtAlaDH. Titration of MtAlaDH with NAD and NADH shows that NADH has very strong binding in comparison to NAD with $K_d$ value $12.81 \pm 2.5 \mu M$ and $276.0 \pm 17.0 \mu M$ for NADH and NAD respectively at neutral pH. The lower $K_d$ value for NADH at physiological pH assumes that MtAlaDH catalyses exclusively the formation of L-alanine in vivo.

6.3.5 Crystallization and data collection

Good diffraction quality crystals of MtAlaDH were obtained from a reservoir solution consisting of 2M ammonium sulfate solution. The single rod shape crystals for the *apo* form (Fig 6.4) started appearing within 3-4 days and grew to maximum respective sizes in about two weeks. The best native crystals diffracted up to 2.6 Å resolution were monoclinic with dimensions $a=173.89$, $b=127.07$, $c=135.95$ Å and belong to C2 space group. A total of 275297 measured reflections were merged into 46867 unique reflections with an $R_{merge}$ 12%. Calculation of the Matthews coefficient (Matthews, 1968) suggests that the asymmetric unit contains six molecules. Assuming six molecules in asymmetric unit corresponds to a Matthews’s coefficient of $2.8 \text{Å}^3$/Da and a solvent content of 56.3 %. 
Fig 6.3: **Fluorescence titration of NAD and NADH binding.** (A) Titration of MtAlaDH with increasing concentration of NAD up to 10 mM. (B) Emission spectra of MtAlaDH and the enzyme presence of increasing concentration of NADH up to 80 μM.
6.3.6 Structure solution and refinement

Data in the resolution range 20.0-3.0 Å were used for molecular replacement calculation. Calculations were carried out in space group C2. BLAST of protein sequence of MtAlaDH against RCSB database showed nearest homology with alanine dehydrogenase from *P. lapideum*. The coordinates of alanine dehydrogenase from *P. lapideum* was used as search model during molecular replacement. Calculation of position of MtAlaDH using AMoRe in space group C2 shows a very good solution. The correct solution gave a correlation coefficient of 56.2% and R-factor 47.8% using data between 20 to 3 Å. Data collection and refinement statistics of wild type MtAlaDH crystal are described in Table 6.1. The suggested solutions and the packing were examined carefully. An examination of the crystal packing corresponding to this solution showed that there were no steric clashes between symmetry generated molecules. Rigid body refinement was carried out using REFMAC5 (Murshudov et al., 1997) implemented in CCP4 by defining each subunit as a rigid molecule. An independent set of 5% reflections were set aside to monitor the R-free value during the course of refinements. Subsequently, transformed model was subjected to cycles of stereo-chemically restrained (Engh et al., 1991) refinement was carried out.

The refinements were interspersed with rounds of model building. 2Fo – Fc and Fo – Fc electron density maps were calculated using fast Fourier transformation and converted into TURBO-FRODO format by MAPPAGE and visualized using the program TURBO-FRODO (Roussel et al., 1989) and COOT (Emsley et al., 2004) which was used for model building. The crystallographic R-factor and R-free (Brünger et al., 1989) were monitored at each stage to prevent model bias. Examination of Fourier maps continued till a stage where no significant density was left unaccounted for in the maps. Water molecules were added into the final model by using the program COOT. Rounds of model building and restrained refinement were continued till R-factor and R-free values converged to 19.56 and 25.10% respectively. The final model consists of six chains, and 224 water molecules.
Fig 6.4: Crystals of *M. tuberculosis* Alanine dehydrogenase.
Crystallization of MtAlaDH in 1.6 M ammonium sulfate. Crystals grew at 22-24°C within couple of days and increased to maximum sizes within a week.
6.3.7 Model quality

The MtAlaDH crystal structure shows that the overall fold is conserved and is similar to those already described in detail in Alanine dehydrogenase from *P. lapideum* (Baker *et al.*, 1998). The final models consist of residues between 1 and 371 (Fig 6.5A). The polypeptide chain folds into two domains which are separated by a cleft. Electron density is of good quality throughout the polypeptide chain except for a loop from 237-245. All residue except C-terminal hexa-histidine tag, are modeled in final structure. Final structure consists of six chains of protein, and 224 water molecules. The most favored and additionally allowed regions of the Ramachandran plot of native protein contained 98 % of non-glycine and non-proline residues.

6.3.8 Subunit structure

The subunit of MtAlaDH folds in to two compact domains which are separated by a cleft. The two domains share structural homology with *r.m.s. deviations* between them of 4.47 Å for 181 Ca atoms. The NAD binding domain consists of residues 130-310 while the catalytic domain consists of residues 1 to 129 and 311 to 371. Both domain1 (residue 1 to 129 and 311 to 371) and domain2 (129 to 298) are constructed from a mainly parallel central β-sheet flanked by helices. Secondary structure were identified by using PROCHECK (Laskowski, 1993), there are total 13 α-helices, 15 β-sheets. Like other dehydrogenase MtAlaDH has two domains, the dinucleotide binding domain which is mainly conserved among other members of dehydrogenases and substrate binding domain.

The sequences of ALD from *M. tuberculosis*, *B. sterothemophilus*, *B. sphaericus*, *B. subtilis* and *P. lapideum* have been aligned with respect to each other. Within these five sequences there are total of 120 invariant residues (33%). Many of these residues cluster around the cleft between the two domains in the subunit, indicating the active is likely to occur in this area. Six subunits of MtAlaDH pack together to form a hexamer with almost cylindrical appearance.
## Table 6.1: Data Collection and refinement statistics of MtAlaDH.

<table>
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<td><strong>Cell dimensions</strong></td>
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<tr>
<td>$a, b, c$ (Å)</td>
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<td>Resolution (Å)</td>
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<tr>
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(*Highest resolution shell statistics are in parenthesis).
Fig 6.5: Crystal structure of alanine dehydrogenase.
(A) Cα trace of a subunit of MtAlaDH with marked C and N-terminal. NAD and catalytic domain marked respectively.
(B) Ribbon diagram of homodimeric MtAlaDH, with each subunit colored differently.
(C) Hexameric association in MtAlaDH. The hexamer is formed as a trimer of dimers. The individual subunits are colored distinctly.
6.3.9 Oligomeric association

The purified protein was consistent with a hexameric association as observed in the size exclusion chromatography experiments. The crystal structure of MtAlaDH revealed an interface of characteristic of a tight dimer. Extensive complementarity exists in surface feature of the two subunits at the dimeric interface. Residues that mainly contribute to interface are 139, 147-161 and 304-308. The dimeric interface is extensive and predominantly non-polar. Each subunit associates to form a dimer which exhibits extensive interactions (Fig 6.5B). The interactions largely involve the NAD binding domain with the respective catalytic domains situated at either end of the dimer. The total buried surface area upon dimerisation is 3631 Å² in the native enzyme out of which about 30% is polar. Three such dimers associate into a hexamer which can also be described as a ‘trimer of dimers’ (Fig 6.5C). Approximately 20825 Å² surface area is buried upon hexamer formation out of which about 35% is polar in nature in wild type enzyme.

6.3.10 Similarity with other alanine dehydrogenase

The three-dimensional structure of MtAlaDH was compared to those in the Protein Data Bank by submitting the coordinates to the Dali server at the EMBL-EBI. The resulting list of structural neighbors showed that the structure of alanine dehydrogenase from the cyanobacteria P. lapideaum (PDB; 1PJB) (Baker et al., 1998) was most closely related (Z-score of 51.9). The crystal structure of wild type MtAlaDH is similar to the structures of AlaDH from P. lapideaum (Pl) and T. thermophilus (Tt) [RIKEN Structural Genomics Initiative, Protein Data Bank (PDB; 2EEZ)]. Superposition results in r.m.s. deviations of 0.94 Å (348 equivalent Cα atoms) for PlALD (PDB; 1PJB) and 0.85 Å (336 equivalent Cα atoms) for TtALD (PDB; 2EEZ). These crystal structures of the homologues represent the open form of the enzyme and, thus, do not reveal all features of the active enzyme. The structure-based alignment reveals that there is ~45% sequence identity between alanine dehydrogenase from P. lapideaum and MtAlaDH, and that the folds are very similar (Fig 6.6).

Comparison of MtAlaDH with the alanine dehydrogenase from Archaeoglobus fulgidus (AfALD) structure shows that, although the catalytic domains are in the same region with respect to the common NAD domain, they are folded differently (Gallagher et al., 2004). Moreover, superposition of the entire oligomers indicates that the quaternary contacts utilize completely different surfaces of the protomers while in MtAlaDH the
NAD domain forms most of the quaternary interactions, in AfALD dimerization is entirely the function of the catalytic domain. Structural superimposition of MtAlaDH with AfALD results in *r.m.s. deviation* of 2.18 Å for 82 residues.

**Fig 6.6:** Superposition of respective subunits of Alanine dehydrogenase from *Mycobacterium tuberculosis* (MtAlaDH), *Phormedium lapideum* (PIALD) and *Thermus thermophilus* (TtALD). The cartoon representation of the MtAlaDH, PIALD and TtALD are shown in blue, cyan and pink respectively.
6.4 Summary

Each subunit of MtAlaDH consists of 371 residues and the purified enzyme ran as a 40 kDa protein consistently in SDS-PAGE gels. Its oligomeric state from size exclusion chromatography was found to be hexameric as observed in many members of this family of enzymes as also reported earlier. Binding of NAD or NADH to MtAlaDH was probed by fluorescence titration assays. Addition of NAD or NADH quenches the fluorescence emission signal of MtAlaDH.

Each subunit of MtAlaDH folds into two compact domains that are separated by a cleft. The two domains share structural homology with r.m.s. deviations between them of 4.47 Å for 181 Cα atoms. Both domains consist of mainly a parallel central β-sheet, flanked by helices. The NAD binding domain consists of residues 130-310 while the catalytic domain consists of residues 1 to 129 and 311 to 371. There are a total of 13 α-helices and 15 β-sheets in the subunit. The two domains are linked by two α-helices. Most of the active site residues are clustered around a cleft between the two domains. The individual subunits associate into a hexamer with the catalytic domains on the outside. Future work involving a ternary complex with NAD and pyruvate should allow for a better understanding of the enzyme mechanism.