3.A.7.c Functional study 3

Aim: *Elucidate the role and function of the Mycobacterium tuberculosis virulence-associated protein Rv0577*

Background: *Mycobacterium tuberculosis* is the etiological agent responsible for tuberculosis (TB), a chronic infectious disease that killed approximately 1.6 million people and infected another 8-9 million in 2008. Due to the emergence of multi-drug and extremely drug-resistant *M. tuberculosis* strains, there is an urgency in developing a new generation of intervention strategies [16]. One approach is to focus on better understanding the molecular biology of the *M. tuberculosis* gene products, especially with regards to the interaction of virulence factors in the microenvironment in the host [140]. *Rv0577* encodes a 261-residue protein, which has no annotated function, but has been associated with the methylglyoxal detoxification pathway [141]. More importantly, Rv0577 has been shown to be the component responsible for neutral red staining of virulent strains of *M. tuberculosis* [142] and Rv0577 protein has been isolated from *M. tuberculosis* culture filtrates. Taken together, these data suggest that Rv0577 may be a virulence factor. In a recent collaborative effort between SSGCID and the TB Structural Genomics Consortium (TBSGC), the X-ray crystal structure for Rv0577 (SSGCID ID: MytuD.17269.a) was determined and deposited in the PDB (3OXH) and the NMR chemical shifts were assigned and deposited into the BMRB (17597) [50]. In pursuit of understanding the function of Rv0577, SSGCID collaborator Dr. C-Y Kim at Los Alamos National Laboratory (LANL) showed that two adenosine nucleoside analogues, 2-methy-adenosine (2MeAdo) and 6-methyl-adenosine (6MeAdo), bind to Rv0577. Interestingly, 2MeAdo expresses anti-mycobacterial activity *in vitro* [143]. Although our attempts to co-crystallize Rv0577 with either of these two ligands have not been successful, it has been possible to map the location of the binding onto the three-dimensional structure of the protein using NMR chemical shift perturbation experiments [144], because protein-ligand interactions produce changes in the chemical environment of the nuclei at the interface of ligand binding which are accompanied by perturbations in the chemicals shifts of the backbone ^{1}H and ^{15}N resonances [145]. **Figure 1** shows the results of a chemical shift perturbation experiment with Rv0577 and the ligand 2MeAdo. Cross-peaks in the ^{1}H-^{15}N HSQC spectrum of Rv0577 that shifted in the presence of 2MeAdo were mapped onto the crystal structure of Rv0577. All these residues aggregate in one region of the protein, suggesting that this is the ligand binding surface. Identical results were obtained for the ligand 6MeAdo (data not shown). We propose to use a suite of solution phase biophysical techniques to characterize, in detail, the specificity, affinity, and structure of small molecule ligand binding to Rv0577. Insights from these studies will give a better understanding of the biological role and function of Rv0577 and may point the way toward the development of novel antibiotics.

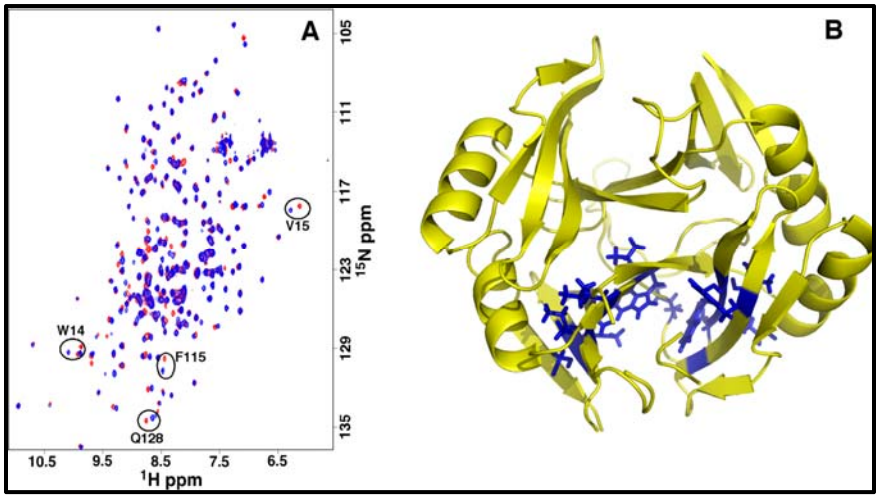

Figure 1. Chemical shift perturbation of Rv0577 and 2MeAdo

A. Overlay of the ^{1}H-^{15}N HSQC spectra of Rv0577 in the absence (red) and presence (blue) of the nucleotide analogue 2MeAdo. The four cross-peaks with the largest chemical shift perturbations are circled and assigned. **B**. The side-chains of the amide resonances most perturbed in the ^{1}H-

^{15}N HSQC spectrum of Rv0577 upon the addition of 2MeAdo are highlighted in blue on the structure of X-ray Rv0577 (3OHX), identifying the likely substrate-binding pocket.

Objective 1: Characterization of ligand-binding by Rv0577 using Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments provide biophysical information on ligand binding including stoichiometry (n), the binding affinity (K_a), the enthalpy change (ΔH), the Gibb's free energy (ΔG), and entropy change (ΔS). Such information has proven useful for drug discovery, function discovery, enzyme assay development, ligand binding identification, and ligand optimization.

Approach: ITC will be used to biophysically characterize the biomolecular interactions of the native Rv0577 protein with the two nucleoside analogues, 2MeAdo and 6MeAdo, and adenosine. These experiments will be performed on a MicroCal iTC$_{200}$ instrument (PNNL) using freshly purified protein and commercially purchased ligands. This objective will be performed at PNNL.

Objective 2: Identify important residues in the Rv0577 substrate-binding pocket

The chemical shift perturbation data show that residues W_{14}, V_{15}, F_{115}, and Q_{128} are most perturbed upon the addition of 2MeAdo. Consequently, these residues may be directly involved in ligand binding. To verify this, each site will be point-mutated to alter the chemical nature of side chains and tested to determine whether the mutations disturb the binding of 2MeAdo.

Approach: The first set of point mutations will be W_{14}A, V_{15}A, F_{115}A, and Q_{128}A substitutions, introduced using Strategene's commercially available kits. These mutants will be generated by Dr. Kim (LANL) at no extra cost to the project and shipped to PNNL. ^{15}N-labeled protein will be prepared at PNNL for each point mutant and an ^{1}H-^{15}N HSQC spectrum collected. These ^{1}H-^{15}N HSQC spectra will be compared to the ^{1}H-^{15}N HSQC spectrum of wild-type Rv0577 to determine if these point mutations introduced any structural perturbations to the protein (generally, the more cross peaks that shift due to the mutation the greater the structural perturbations). The mutant proteins will also be tested for ligand binding using a dye-based affinity chromatography assay (LANL) and NMR chemical shift perturbation experiments (PNNL). If warranted, ITC data will also be collected with these proteins as described in Objective1 (the ITC experiments will not be necessary if the chromatography and NMR experiments show no ligand binding). This objective will be performed at LANL and PNNL.

Objective 3: Characterization of the backbone dynamics using ^{15}N NMR relaxation experiments

Motion for a subset of individual residues that differ substantially from the rest of the protein may signal sites important for ligand binding or enzyme catalysis.

Approach: Using isotopically ^{15}N-enriched proteins and a suite of NMR experiments, the motions of individual protein residues on the picosecond to second (or longer) time-scale will be probed [20, 144, 146]. NMR experiments to measure backbone dynamics for Rv0577 (T1, T2, and steady-state {^{1}H}-^{15}N heteronuclear NOEs) will be collected for free Rv0577 and in the presence of 1:1 molar ratio of both methylated adenosine ligands. This objective will be performed at PNNL.

Objective 4. Determination of a NMR-based solution structure for a Rv0577: ligand complex

Attempts to co-crystallize Rv0577 bound to a ligand have not been successful, but preliminary NMR data show that both 2MeAdo and 6MeAdo bind to Rv0577 with tractable perturbations to the ^{1}H-^{15}N HSQC spectrum. Hence, solving the structure of Rv0577 bound to a ligand is possible using NMR-based methods, but has been avoided because of the substantial amount of labor required (analyzing the NOE data for all 261 residues is time-consuming). However, as part of a special functional study, it will be possible to make this time investment to obtain a ligand-bound structure.

Approach: The first effort will be made to determine the structure using the complete NOE data set for the complex. This will be facilitated by having already assigned the ^{1}H, ^{13}C, and ^{15}N chemical shifts [50]. If difficulties arise, an alternative approach will be to identify the ligand-protein contacts from the NOE data and use these restraints to model a ligand bound structure using the available crystal structure of ligand-free Rv0577 (3OXH) and programs such as Rosetta-ligand. These computation-heavy calculations will be done in the EMSL Supercomputing Center (Chinook) at PNNL/Battelle.

Time-line:

Months 1-3	ITC experiments with wild type Rv0577 (PNNL) Clone the four Rv0577 point mutations (LANL) Prepare ^{15}N-labeled mutant protein and collect ^{1}H-^{15}N HSQC spectra (PNNL)
Months 4-6	Collect relaxation data for wild-type Rv0577 with and without ligand bound (PNNL) Chromatography (LANL) and chemical shift mapping perturbation (PNNL) of mutants Collect NOE data on ^{13}C-,^{15}N-labelled Rv0577-ligand complex (PNNL) Analyze complex NOE data, structure calculations (PNNL)
Months 7-9	Collect ITC data on mutant proteins if warranted (PNNL) Analyze relaxation data (PNNL)
Months 10-12	Tie up any loose-ends and pursue any interesting leads (PNNL) Prepare and submit manuscript(s) for publication (PNNL)