Inositol Monophosphatase: A Bifunctional Enzyme in Mycobacterium smegmatis

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ABSTRACT: Inositol monophosphatase (IMPase) is a crucial enzyme for the biosynthesis of phosphatidylinositol, an essential component in mycobacterial cell walls. IMPase A (ImpA) from Mycobacterium smegmatis is a bifunctional enzyme that also functions as a fructose-1,6-bisphosphatase (FBPase). To better understand the bifunctional nature of this enzyme, point mutagenesis was conducted on several key residues and their enzyme activity was tested. Our results along with active site models support the fact that ImpA is a bifunctional enzyme with residues Gly94, Thr95 hypothesized to be contributing to the FBPase activity and residues Trp220, Asp221 hypothesized to be contributing to the IMPase activity. Double mutants, W220A + D221A reduced both FBPase and IMPase activity drastically while the double mutant G94A + T95A surprisingly partially restored the IMPase activity compared to the single mutants. This study establishes the foundation toward obtaining a better understanding of the bifunctional nature of this enzyme.

INTRODUCTION

Most enzymes are specific for the substrates they act upon. However, many enzymes are found to have broad substrate specificity. Diversity of an enzyme may be important for survival of an organism and could be the product of evolution. This would explain why a secondary function of one enzyme could share the primary function in other members of the same family. Enzymes that are multifunctional are present in many organisms and in many different enzyme families. The broad specificity classically involves the same systematic approach to multiple substrates or can use an altered catalytic mechanism within the active site.

The enzyme inositol monophosphatase (IMPase) is crucial for the biosynthesis of phosphatidylinositol (PI) and is an essential component of mycobacterial cell wall. IMPase belongs to the family of enzymes which hydrolyses phosphate groups of sugar phosphates. Unlike other sugar phosphatases, it is a bifunctional enzyme which hydrolyses two substrates, fructose-1,6-bisphosphate and inositol-1-phosphate. IMPase requires Mg2+ for its activity but does not require any coenzymes. Previous studies have shown that the Rv2131c gene product of Mycobacterium tuberculosis displays the IMPase activity with a broad substrate specificity compared to other known IMPases.

Inositol is not specific for mycobacteria; however, the major immunomodulatory components of the mycobacterial cell wall are phosphatidyl-based lipids such as PI mannosides (PIM). Glucose-6-phosphate is converted to inositol-1-phosphate and is dephosphorylated by IMPase to form myo-inositol. Inositol is biosynthesized as shown below (Figure 1). Inositol is a component of lipoarabinomannan, which in turn is found in high levels in the mycobacterial cell envelope, anchored by a PI moiety. Lipomannan and PIM are also found in the cell envelope. PI-containing molecules have shown to be essential for Mycobacterium smegmatis. Inositol is also involved in the production of mycothiol, which helps maintain the redox state of the cell.

Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), a key enzyme of gluconeogenesis, catalyzes the hydrolysis of fructose-1,6-bisphosphate to form fructose-6-phosphate and orthophosphate. Fructose-6-phosphate is an important precursor in various biosynthetic pathways generating important structural components of the cell wall and glycolipids in mycobacteria.
Thus, IMPase is an important enzyme that produces two different important precursors for synthesizing other cell components. Although the bifunctional nature of IMPase is known, it is not clear whether both activities involve a common active site. In this paper, we describe the nature of IMPase duality through point mutations of residues known to have catalytic significance. We show that in *M. smegmatis*, conserved residues from both putative active sites regulate both IMPase and FBPase activities.

### RESULTS AND DISCUSSION

The enzyme IMPase is crucial for the biosynthesis of PI, an essential component of mycobacteria. IMPase is responsible for dephosphorylating inositol-1-phosphate to inositol. The FBPase enzyme dephosphorylates fructose-1,6-bisphosphate to fructose-6-phosphate. Earlier studies have shown that the Rv2131c gene product of *M. tuberculosis* (CysQ), displays the IMPase activity alongside the FBPase activity.3,4 This study focuses on the enzyme IMPase A (ImpA) from *M. smegmatis*, which is homologous to CysQ and carries the same dual functionality.

To investigate the nature of duality of action, we successfully expressed and purified ImpA from *M. smegmatis*. We determined the conditions for the optimal enzyme activity which included temperature, time, enzyme and substrate concentrations, and buffer conditions (data not shown). We also determined the $K_m$ values for both the functionalities of ImpA and found that both activities show very similar $K_m$ values (Figure 2). The $K_m$ values are in agreement with $K_m$ values for CysQ IMPase.

Our next goal was to understand whether the two active sites are independent of each other. To do this, we conducted mutational analysis of key residues that we hypothesized to be involved in the two activities. The residues were selected based on sequence analysis with other known FBPase and IMPase enzymes.

Known structures and amino acid sequences of close homologs of IMPases in both *M. smegmatis* and *M. tuberculosis* were examined. In *M. tuberculosis* the homologs are ImpA, SubB (or ImpB), CysQ, and ImpC (Rv3137). Only ImpC is shown to be essential for growth and probably in the early synthesis of mycothiol.4 Crystal structures of SubB (PDB ID 2Q74) and CysQ (PDB ID SDJG) were found to align well with root mean squared deviation (rmsd) of only 1.6 Å. The SubB structure has an ADP in its active site. The conserved region predicted by PROSITE was shown to be the active site of the IMPase activity *M. tuberculosis*. This site was structurally conserved in these two enzymes.9 The start of the conserved sites $^{220}WD^{221}$ was highly conserved among ImpA, SubB, CysQ, ImpC, and ImpB in *M. tuberculosis* and is shown in Figure 3. Sequence comparisons with select other IMPases show that in the ImpA protein, these two residues are in close proximity of ADP in SubB.10

It appears that the two conserved amino acids W220 and D221 may play a role in the IMPase activity. To confirm this possibility, we created single mutants, where W220 or D221 were separately mutated to alanine, or double mutants where both residues were mutated to alanine. The IMPase and FBPase activity was studied in each of these mutants (Figure 4). Our results show that both FBPase and IMPase activities were impacted by these mutations, with approximately 60–70% loss of activity in each of the single mutants, whereas double mutation completely abolishes both IMPase and FBPase activity. These results indicate that the enzyme might use the same active site residues for both activities or it may be possible that the active sites are separate but could be in close proximity in the tertiary structure. Enzymes with broad specificity often have the same active site. Several studies have reported enzymes in nucleotide synthesis, amylases, and

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**Figure 1.** Suggested inositol metabolism pathway.5

**Figure 2.** Lineweaver–Burke plots of wild type ImpA protein tested for both IMPase activities with the substrate inositol-1-phosphate and the FBPase activity, with fructose-1,6-bisphosphate as the substrate. The $K_m$ is 11.5 μM for the IMPase activity and 11.1 μM for the FBPase activity with $p < 0.001$. 

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several glycolytic hydrolases use the same active site for bifunctionality.\textsuperscript{13,14}

To better understand how these two activities are connected double mutants of both FBPase and IMPase putative active sites were created. Activities were measured and the results indicated that the IMPase double mutant W220A + D221A drastically reduced both FBPase and IMPase activities (Figure 4). These mutational studies implied that both activities may be connected either by sharing active sites or by the active sites being in close proximity.

The sequence of ImpA was compared with other known FBPase and IMPase sequences (Figure 3). IMPase shares active site for the FBPase activity, which is similar to other phosphatases,\textsuperscript{15} specifically sharing the motif DPIDGT, as previously cited among other phosphatases.\textsuperscript{16,17} We mutated T95 in the highly conserved FBPase motif to Ala and Ser separately and measured both FBPase and IMPase activities in these mutants. Unexpectedly, both T95A and T95S mutants not only affected the FBPase activity but also affected IMPase activity (Figure 5). The serine mutation (with the more highly conserved amino acid change) showed only 35% loss of activity, which could be due to the similarity in size and structure of serine and threonine. However, mutation to alanine resulted in a 60% loss of FBPase activity and 85% loss of IMPase activity (Figure 5), lending further support to the possibility that the two active sites are in close proximity in the tertiary structure.

We further mutated two residues G94 and T95 of putative FBPase active site to alanine and measured both activities. With the FBPase double mutant G94A + T95A, the results were unexpected as this mutant did not significantly impact either FBPase or IMPase enzyme activity (Figure 5). Glycine is a unique amino acid in that it contains hydrogen as its side chain. Glycine can reside in parts of protein structures that are conserved and substitution with any other amino acids (e.g. tight turns in structures) will affect the function of a protein.\textsuperscript{14} We observed that single Gly to Ala (G94A) mutation decreased both activities, which was not surprising. Only about 30–40% activity was lost compared to other mutants (Figure 5). If both residues play a role in the active site of several glycolytic hydrolases use the same active site for bifunctionality.\textsuperscript{13,14}

Figure 3. The primary sequence alignment of different IMPases and FBPases. All proteins share the motif DPIDGT as the key active site for the phosphatase activity and are indicated on the alignment. All IMPases also share a conserved motif, which in ImpA is 220WDHAAGVALVRAGG\textsuperscript{23-25}, that is not present in the class II FBPases. The enzymes listed, in order, are ImpA from \textit{M. smegmatis} (accession ID A0QX68), ImpA from \textit{M. tuberculosis} (accession ID OS3907), SubH (accession ID P9WJK9), CysQ (accession ID P9WJKJ), and ImpC (accession ID PSV189) from \textit{M. tuberculosis}, GlpX from \textit{Francisella tularensis} (accession ID A0A02ERP3) and GlpX from \textit{M. tuberculosis} (accession ID P9WN21). Sequence alignment created with Clustal Omega.\textsuperscript{11,12} Asterisks (*) below the aligned residues indicate a fully conserved residue. Colons (:) and periods (.) indicates conservation among residues with strongly similar and weakly similar properties, respectively.
FBPase then a total loss in activity should have been apparent. Our results indicated that the double mutants G94A + T95A partially restored FBPase and IMPase activities when compared to the single mutants.\textsuperscript{14,18,19} The partial restoration of the activity by the double mutant (G94A + T95A) was thought to be the result of change in the active site structure.

To understand how these mutants could alter the putative active site structure, we used PEP-FOLD3 to analyze the predicted structure of these mutants.\textsuperscript{20,21} The G94A + T95A mutants showed an interesting change in the structure. In the native enzyme, the $^{94}$GT\textsuperscript{55} region showed an open loop structure, and in the T95S mutant, there was no appreciable change in the loop-like structure. The G94A mutant on the other hand displayed a shorter ribbon-like structure compared to the wildtype enzyme. In the T95A mutant, according to the PEP-FOLD3 prediction, the loop structure was altered to a helical structure, probably causing reduced activity. The double mutant, G94A + T95A (Figure 6) also showed a helical structure with a ribbon-like extension at the end of helix. However, both catalytic activities were not reduced drastically, suggesting that the helical structure and ribbon-like extension may bring other residues in close proximity of the IMPase site and make a bigger loop to accommodate the substrate comfortably. A similar mechanism has been proposed for a bifunctional IMPase/FPBse enzyme in \textit{Archaeoglobus fulgidus}.\textsuperscript{9}

The IMPase active site double mutant (W220A + D221A) showed a drastic change in the structure, which possibly resulted in the loss of both FBPase and IMPase activities. The PEP-FOLD3 predictions of the helix in the IMPase site showed that the two residues, Trp220 and Asp221 were exposed on the same side, whereas in the double mutant a tighter helical structure was formed (Figure 7).

In summary, the IMPase active site double mutant involving W220A + D221A, reduced both IMPase and FBPase activities significantly, whereas the FBPase active site double mutant, G94A + T95A did not significantly impact both catalytic activities. Our results, along with active site models, imply that W220 and D221 are critical for IMPase and FBPase activity. G94 and T95 play a role in catalysis for both activities but do not significantly impact catalytic rates. This indicates that it is possible that the highly conserved residues of Gly94, Thr95, Trp220, and Asp221 are a part of the same active site. Crystallographic investigation of the ImpA protein is currently underway and these structures will provide further insight into the nature of the active site and the possible mechanism of action.

\section*{MATERIALS AND METHODS}

Materials were purchased from Fisher Scientific (Waltham, MA), unless otherwise noted. Primers were purchased from IDT DNA Inc. through the UIC core facility.
Table 1. Primer List for Mutations

| FBPase site mutations | GACCATGCGGCCGGGGTCG-3
|-----------------------|------------------------|
| original Sequence     | ∆CTTCAACTACGCGGCGGGCTCACC-3
| T95A                  | −GCTCGATCGAGCGGCGGGCTCACC-3
| T95S                  | −GCTCGATCGAGCGGCGGGCTCACC-3
| G94A                  | −GCTCGATCGAGCGGCGGGCTCACC-3
| G94A and T95A         | −GCTCGATCGAGCGGCGGGCTCACC-3

| IMPase site mutations |
|-----------------------|
| original sequence     |
| W220A                 |
| D221A                 |
| W220A and D221A       |

Cloning and Purification. In order to express recombinant ImpA, the coding sequence of *M. smegmatis* ImpA was amplified by PCR using primers ImpA-forward (in which the start codon has been changed from GTG to ATG; 5′-GGAATTCCATATGACGGTGGTGGG-3′) and ImpA-reverse (CGAGGATCCACTCACAGGTAATCC) (accession ID: M. smegmatis, ImpA, A0QX68). These primers introduced an NdeI site at the 5′ end and a BamHI site at the 3′ end to allow the gene to be cloned in-frame into the expression vector pET-15b. PCR was carried out using the Invitrogen Platinum Taq DNA Polymerase High Fidelity with *M. smegmatis* DNA as the template. The PCR product was cleaved with NdeI and BamHI and cloned into the vector pET-15b to give a construct that expresses a protein with a 21 residue amino-terminal extension containing a histidine tag. The plasmid was then transformed into BL21 DE3 PlysS *Escherichia coli* cells for protein purification.

Purification and expression of the ImpA protein was performed via a Ni-NTA column according to previously established protocols for purification of other phosphatase enzymes.17,22 Bacterial strains were grown on media containing a histidine tag. The plasmid was then transformed containing 200 mM KCl, 20 mM tricine at pH 8.0, 8 mM MgCl2, and 15 nM enzyme. The malachite green solution was added after 6 min and then incubated at room temperature for 10 min. Absorbance levels were read at 630 nm.

The results confirmed the FBPase activity via a coupled assay by measuring production of NADPH at 340 nm, was used to determine enzymatic parameters and analyzed with a nonlinear fit of Michaelis–Menten equation using Excel. Using the coupled assay, the following optimal conditions were found where FBPaseII is the limiting reagent: 5 units/mL of phosphoglucoisomerase, 2 units/mL of glucose-6-phosphate dehydrogenase, 0.3 mM NADPH, 15 μM F16BP, and 50 nM FBPaseII. The reaction was monitored for 3.5 min. The residual activity after heating was assessed by incubating protein samples for 30 min at various temperatures in a water bath (10–80 °C), returning to ice for 15 min and assaying against a sample on ice for the same length of time.

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R.G. and J.M.G.B. are co-first authors. R.G., J.M.G.B. and F.M. have made equal contributions. R.G. and J.M.G.B. contributed equally in performing experiments and interpreting data. P.R.W., A.J. and T.G. helped in experimental procedures. S.M. and F.M. designed the experiments, and F.M. oversaw the project. R.G., J.M.G.B., S.M. and F.M. contributed in writing the manuscript. All authors have approved the final version of manuscript.

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**Notes**

The authors declare no competing financial interest.

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ABBREVIATIONS

IMPase, inositol monophosphatase
ImPα, inositol monophosphatase protein
FBPase/GLpx, fructose-1,6-phosphatase
rmsd, root mean squared deviation

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