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Kinetic and mutational studies of the adenosine diphosphate ribose hydrolase from *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis represents one of the world's most devastating infectious agents – with one third of the world's population infected and 1.5 million people dying each year from this deadly pathogen. As part of an effort to identify targets for therapeutic intervention, we carried out the kinetic characterization of the product of gene *rv1700* of *M. tuberculosis*. Based on its sequence and its structure, the protein had been tentatively identified as a pyrophosphohydrolase specific for adenosine diphosphate ribose (ADPR), a compound involved in various pathways including oxidative stress response and tellurite resistance. In this work we carry out a kinetic, mutational and structural investigation of the enzyme, which provides a full characterization of this *Mt*-ADPRase. Optimal catalytic rates were achieved at alkaline pH (7.5–8.5) with either 0.5–1 mM Mg^{2+} or 0.02–1 mM Mn^{2+} . K_m and k_{cat} values for hydrolysis of ADPR with Mg^{2+} ions are $200 \pm 19 \mu M$ and $14.4 \pm 0.4 s^{-1}$, and with Mn^{2+} ions are $554 \pm 64 \mu M$ and $28.9 \pm 1.4 s^{-1}$. Four residues proposed to be important in the catalytic mechanism of the enzyme were individually mutated and the kinetics of the mutant enzymes were characterized. In the four cases, the K_m increased only slightly (2- to 3-fold) but the k_{cat} decreased significantly (300- to 1900-fold), confirming the participation of these residues in catalysis. An analysis of the sequence and structure conservation patterns in Nudix ADPRases permits an unambiguous identification of members of the family and provides insight into residues involved in catalysis and their participation in substrate recognition in the *Mt*-ADPRase.

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Keywords

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Tuberculosis (TB) remains one of the world's major health threats with 9.6 million people reported falling ill with TB in 2014 and an estimated one third of the world's population infected with latent TB (WHO 2016). Strains of TB that are multidrug-, extensively drug-, and totally drug-resistant further increase TB's disease burden worldwide – even in high-income countries with lower incident rates (WHO 2016).

Nudix hydrolases are widely-distributed enzymes characterized by the Nudix signature sequence, a highly conserved consensus sequence

$G_1^N[5X]E_7^N[7X]R_{15}^NE_{16}^NXXE_{20}^NXG_{22}^NU_{23}^N$ where U represents an I, L or V amino acid and the subscript denotes the position of the residue in the sequence of the Nudix motif denoted by superscript N (Bessman et al. 1996; Boto et al. 2011; Wolff et al. 2015). Over one hundred thousand putative sequences encoding open reading frames containing the Nudix motif have been found in the genomes of organisms ranging in complexity from viruses to humans, with about ~87,000 in eubacteria and 15,000 in eukaryotes, accounting for repetition due to similar strains (Interpro). Biochemical characterization of some of these proteins shows that they hydrolyze the diphosphate bond of substrates including nucleoside triphosphates, coenzymes, sugar nucleotides, dinucleoside polyphosphates, and capped RNA.

The common feature of the Nudix substrates is a **nucleoside diphosphate** bound to another moiety, **X**. Known substrates include potentially toxic, deleterious compounds as well as important cell signaling molecules, regulators, and metabolic intermediates whose concentrations require careful control during different states of the cell (O'Handley et al. 1998). Because these Nudix hydrolases eliminate and/or control the cellular level of metabolically-important molecules such as ADP-ribose (ADPR), CoA, NADH, Ap_nA, and UDP-glucose, the function of Nudix enzymes has been described as “housecleaning” or “sanitizing” (Bessman et al. 1996; McLennan 2006; Mildvan et al. 2005).

Analysis of the *M. tuberculosis* genome reveals the presence of eleven putative proteins that contain the Nudix motif. We have partially characterized one of these proteins, the product of the *rv1700* gene (Kang et al. 2003). The structure of the protein and the sequence flanking the Nudix motif suggested that this enzyme is probably an ADPR hydrolase (*Mt*-ADPRase; Kang et al. 2003) similar to the *E. coli* ADPRase (*Ec*-ADPRase) (O'Handley et al. 1998), the *Thermus thermophilus* ADPRase (Ooga et al. 2005), and the *Homo sapiens* NUDT5 (*h*NUDT5) (Song et al. 2005).

The substrate of these enzymes, ADPR, is produced by the pyridine nucleotide cycle, by cytosolic and ecto-NAD glycohydrolases, and by cyclic ADPR breakdown (Perraud et al. 2001). Several lines of evidence suggest that high levels of ADPR may function as a cellular signal and be deleterious to the cell (Tong et al. 2009). It is known that free ADPR is a highly reactive molecule that causes non-enzymatic mono-ADP-ribosylation of proteins. Since proteins that are ADP-ribosylated by bacterial toxins lead to immediate cytotoxicity

(McDonald LJ et al. 1992; Moss 1994a; Moss 1994b), nonenzymatic ADP-ribosylation is thought to be similarly deleterious.

ADPRases have been associated with at least two phenotypes (McDonald LJ et al. 1992; Moss 1994a; Moss 1994b). In *Rhodobacteria spheroides*, the *trgB* gene codes for an ADPRase that has been shown to confer resistance to tellurite – an antibiotic adjuvant (O'Gara et al. 1997). Moreover, transformation with a plasmid containing the ADPRase gene of *M. jannaschii* dramatically increased tellurite resistance of *E.coli* (Dunn et al. 1999). In *E. coli*, an ADPRase (*orf209*) prevents glycogen biosynthesis by hydrolyzing its synthetic precursor, ADP-glucose (Moreno-Bruna et al. 2001).

In this paper we describe the characterization and mutational analysis of the *Mt*-ADPRase, focusing on the catalytic mechanism, substrate specificity and metal ion requirements. The 3D structures of *Mt*-ADPRase that we previously reported (Kang et al. 2003) and the structures of the three homologs mentioned above (ADPRases from *E. coli*, *T. thermophilus*, and *Homo sapiens*), strongly suggested that four residues - E93Q, E97Q, R64A, and E142Q- may be involved in catalysis and substrate recognition. To probe their participation, we expressed four mutants - E93Q, E97Q, R64A, and E142Q-, determined the structure of the E142Q mutant, and characterized kinetically the four mutants. The information, combined with data from the other Nudix ADPRases, suggests a common mechanism for this family of Nudix enzymes.

Results and Discussion

Sequence similarity and enzymatic activity suggests Rv1700 is an ADPRase

A search for Nudix hydrolases in the *Mycobacterium tuberculosis* genome identified eleven sequences, one of which was *rv1700*. More detailed analysis based on the presence of a proline residue 16 amino acids past the terminal glycine ($G_{22+\sim 15}^N$) of the Nudix motif and additional sequence identities suggested that the gene product of *rv1700* is a potential ADPR hydrolase (Kang et al. 2003) (Fig. 1).

Induction of gene expression at 37 °C of *E. coli* BLR(DE3) transformed with a pET11b plasmid containing the *rv1700* construct (*pET-rv1700*) resulted in the expression of a 20.5 kDa protein (22.5 kDa expected molecular weight) not present in the un-induced cell extract. The lysate from this culture showed a 10-fold increase in ADPRase activity compared to that of a culture transformed with an empty pET11b plasmid. An additional 10-fold increase in ADPRase was obtained when the culture containing pET-*rv1700* was induced and grown at 15°C as described in Methods. After gel filtration and anion exchange chromatography, SDS-PAGE gels stained with Coomassie Blue showed no contaminating bands (Fig. 2a). Further evidence of the purity of the protein was provided by the ability of the sample to produce well-diffracting crystals of the wild type and the mutants (Kang et al. 2003).

rv1700 preferentially hydrolyzes ADPR at pH 8.0 in the presence of Mg²⁺ or Mn²⁺

Maximal activity was observed at pH 8.0 (Fig. 2b), and thus metal ion studies were carried at this pH. The enzyme requires either Mn²⁺ or Mg²⁺ for optimal activity; Zn²⁺ and Co²⁺

support up to 37% and 8.6% maximal activity, respectively, but little activity was detected with either Ca^{2+} or Li^+ (Fig. 2c).

Substrate preference was determined by testing known Nudix substrates in a Tris-HCl buffer, pH 8.0, containing 0.5 mM Mg^{2+} and 2 mM substrate using the spectrophotometric assay described in Methods. Significant levels of activity were found with ADP-glucose and ADP-mannose; however, the activity was two-fold higher with ADPR as a substrate (Table 1; Fig. 3b) consistent with our prediction based on sequence comparison. There was activity with NAD, GDP-sugars and UDP-sugars, though less than with ADP-sugars (Table 1). Little to no activity was found with the common Nudix substrates NADH, Ap3A, CoA or FAD (Table 1).

Kinetic parameters for ADPR hydrolysis, estimated by non-linear least-squares analysis of initial velocities, show the enzyme follows Michaelis-Menten kinetics with Mg^{2+} and Mn^{2+} with K_m s and turnover numbers of $200 \pm 19 \mu\text{M}$ and $14.4 \pm 0.4 \text{ s}^{-1}$ with 0.5 mM Mg^{2+} and $554 \pm 64 \mu\text{M}$ and $28.9 \pm 1.4 \text{ s}^{-1}$ with 0.5 mM Mn^{2+} (Fig. 3c). Maximal activity at pH 8.0 was obtained with 0.5-1 mM Mg^{2+} or 0.02-1 mM Mn^{2+} (Fig. 3d). Turnover number toward ADPR with Mn^{2+} is nearly twice the turnover number with Mg^{2+} . This difference is even larger for the hydrolysis of ADPG that is at least five times slower when assayed with Mg^{2+} than when assayed with Mn^{2+} (Fig. 3b). These parameters are in agreement with what we previously published (Kang et al. 2003). Patil et al. measured the K_m and k_{cat} of Rv1700 for the hydrolysis of 8-oxo-2'-deoxyguanosine -5'-diphosphate (8-oxo-dGDP), producing 8-oxo-dGMP and a free phosphate. Interestingly, the catalytic efficiency (k_{cat}/K_m) of Rv1700 is much higher for the hydrolysis of ADPR than 8-oxo-dGDP, suggesting a more relevant biological role of Rv1700 in hydrolysis of ADPR (The k_{cat}/K_m of ADPR is $14.4 \text{ s}^{-1}/200 \mu\text{M} = 0.072$ in the presence of Mg^{2+} ; k_{cat}/K_m of 8-oxo-dGTP is $0.0164 \text{ s}^{-1}/9.5 \mu\text{M} = 0.0017$) (Eisenthal et al. 2007; Patil et al. 2013).

***Mt*-ADPRase catalyzes the hydrolysis of the diphosphate bond in ADPR**

The products of the hydrolysis of ADPR by *Mt*-ADPRase were analyzed to determine what reaction is catalyzed by the enzyme. HPLC analysis of the products of a standard reaction mixture without calf intestinal alkaline phosphatase showed the presence of AMP and ribose-5-phosphate (Fig. 3a). No contaminating AMP or ADP was detected in the ADPR substrate; neither ADP nor free phosphate was generated by the hydrolysis. These data indicate that the reaction catalyzed by the enzyme is the following:

Mutations E93Q, E97Q, E142Q, and R64A decrease catalytic efficiency of *Mt*-ADPRase

Mt-ADPRase is a dimer with a quaternary structure that displays domain swapping (Gabelli et al. 2001; Kang et al. 2003) (Fig. 4a). Each monomer of the ADPRase has 2 domains: a 3-stranded β -sheet N-Terminal (residues 1-42) domain followed by the Nudix domain (residues 46-204). ADPRases are functional dimers that display domain swapping of the β -sheet N-terminal domains, meaning that the β -sheet N-terminal domain of one monomer

extends to interact with the Nudix domain of the other monomer (Gabelli et al. 2001; Zha et al. 2006); in particular, each active site is formed by residues of both monomers (Gabelli et al. 2001; Kang et al. 2003; Liu and Eisenberg 2002).

Structural alignment of the coordinates of *Mt*-ADPRase (PDB ID: 1MQW) and *Ec*-ADPRase (PDB ID: 1KHZ) shows that residue *Mt*-Glu93 (E_{16}^N) of the Nudix domain is equivalent to *Ec*-Glu112, the residue closest to the metal site, and *Mt*-Glu97 (E_{20}^N) is equivalent to *Ec*-Glu116, a residue required for metal coordination. In *Ec*-ADPRase, two other glutamate residues – *Ec*-Glu162 and *Ec*-Glu164 – become ordered in the presence of a metal cation and participate in the conformational change that most likely positions a water molecule as the base for catalysis. The alignment shows that only one of the two *E. coli* residues, *Ec*-Glu164, has a counterpart in *Mt*-ADPRase (*Mt*-Glu142; Fig. 4b). A conserved arginine residue (*Mt*-Arg64; *Ec*-Arg79; *Tt*-Arg54; *Hs*-Arg84) is found in the active site at hydrogen bonding distance of the α -phosphate of the substrate and the product.

To shed light on the mechanism, four different mutants of *Mt*-ADPRase – E93Q (E_{16}^N), E97Q (E_{20}^N), E142Q, and R64A – were expressed and purified. For each of these mutants, K_m and k_{cat} values were determined at pH 8.0 using 0.5 mM Mg^{2+} as the metal cation (Table 2). The decrease in k_{cat} for E93Q and E97Q confirms the role of *Mt*-Glu93 and *Mt*-Glu97 as the metal-ion ligands conserved in the Nudix motif. The 3-fold increase in K_m of R64A suggests of a role in stabilizing the negatively-charged phosphate groups in ADPR. The 303-fold decrease in k_{cat} of the E142Q mutant points to *Mt*-Glu142 as the catalytic base for hydrolysis.

Loops L2 and L9 of *Mt*-E142Q adopt a new conformation away from the active site

To gain insight into the structural basis of the mechanism of catalysis by *Mt*-ADPRase, we determined the structure of the *Mt*-E142Q mutant (PDB ID: 5I8U) (Table 3). The asymmetric unit contains three dimers and one monomer. This monomer forms a dimer with that of another asymmetric unit related by a crystallographic 2-fold axis. The overall fold and quaternary structure of the *Mt*-E142Q is similar to that of the native enzyme (PDB ID: 1MQW) with RMS deviations between 0.7 -1.4 depending on the monomer. As in the other structures, the dimers display domain swapping in which the N-terminal domain of each monomer (residues 1 – 42) interacts more closely with the Nudix domain of the other monomer than with the Nudix domain of its own chain (Fig. 4a). The main differences between mutant and wild-type *Mt*-ADPRase are in loops L2 of the N-terminal domain (residues 29-38) and L9 of the Nudix domain (residues 133-145, Fig. 4c). In the absence of metal and substrate analog, loop L9 and L2 of the mutant enzyme adopt an open conformation away from the active site. *Mt*-ADPRase in the presence of metal and substrate analog loops L2 and L9 moves towards the active site (PDB ID: 1MQW, Fig. 4c, d).

ADPRases contain a domain-swapped N-terminus and a similar substrate recognition site

Alignment of the known structures of ADPRases (*H. sapiens* NudT5 (PDB ID: 2DSC) ; (Zha et al. 2006); *E. coli* (PDB ID: 1HKZ) (Gabelli et al. 2001); *M. tuberculosis* (PDB ID: 1MQW) (Kang et al. 2003); *T. thermophilus* (PDB ID: 1V8T) (Abdelghany et al. 2003);

Ooga et al. 2005; Yoshiba et al. 2004)} shows that they share a common fold, including the domain swapping involved in dimer formation with RMS deviations among these structures between 1.9 and 2.4 Å (Fig. 4a, 5a). In addition, they have identical residues at critical positions, suggesting that they share a common catalytic mechanism (Fig. 1). Overall sequence identity in these proteins ranges between 20.4% (*Ec* to *Hs*) to 32.0% (*Tt* to *Mt*), suggesting that there is significant homology.

In the N-terminal domain, conserved residues involved in domain swapping (*Mt*-Leu14 and *Mt*-Leu22; *Ec*-Leu31; *Tt*-Leu22; *Hs*-Leu31) form a hydrophobic patch that contributes to the interactions of the swapped domain of one monomer and the main domain of the other. Also conserved is a domain-swapped glutamate (*Mt*-Glu38; *Ec*-Glu52; *Tt*-Glu29; *Hs*-Glu47) whose backbone carbonyl is at hydrogen bonding distance with the primary amino group of the substrate's adenine base (6'-NH₂) (Fig. 5b). Its side chain is at hydrogen bonding distance of the amide of *Mt*-Ala18 (*Ec*-Phe28, *Tt*-Arg18, or *Hs*-Trp28) at the tip of loop L1 of the opposite monomer. Residues *Mt*-Ile19, *Ec*-Phe28, *Tt*-Ile19, and *Hs*-Trp28 on the L1 loop, in conjunction with residues *Mt*-Arg37, *Ec*-Arg51, *Tt*-Tyr28, and *Hs*-Trp46, on the opposite monomer, contribute to stacking with the adenine base of the substrate (Fig. 5c). This suggests a dual role of *Mt*-Glu38 (*Ec*-Glu52, *Tt*-Glu29, and *Hs*-Glu47) in substrate recognition and further stabilization of a domain-swapped L1 loop that is involved in base stacking.

Mt-Glu41 (*Ec*-Glu55; *Tt*-Glu32) connects the N-terminal of the protein to the Nudix fold by hydrogen bonding to a polar residue (*Mt*-Asp119; *Ec*-Ser138; *Tt*-Asp107). In the human enzyme, although this residue is not conserved, *Hs*-Lys50 performs a similar role, hydrogen bonding to *Hs*-Asn138.

Mt-Gln62 (*Ec*-Gln77; *Tt*-Gln52; *Hs*-Gln82) is at hydrogen bonding distance of *Mt*-Glu73 (*Ec*-Glu93; *Tt*-Glu63; *Hs*-Glu93) connecting strands β5 and β6. *Mt*-Glu73 and its equivalents form a salt bridge with *Mt*-Arg64 (*Ec*-Arg79; *Tt*-Arg54; *Hs*-Arg84). This arginine, found in the active site, is in hydrogen bonding distance of the β-phosphate of the substrate or the phosphate of the product, stabilizing the negative charge that develops on the leaving group upon hydrolysis (Fig. 5c). Loop L8 (β8-β9) of one monomer and L5 (β5-β6) of the opposite monomer have complementary shapes and interact tightly with each other.

Mt-Pro115 ($G_{22+\sim 15}^N$), *Ec*-Pro134; *Tt*-Pro103; *Hs*-Pro134) in loop L8 defines the end of the binding site, limiting the size of the terminal sugar to that of ribose or glucose (Fig. 5b). The fact that the following residue is a glycine (*Mt*-Gly116; *Ec*-Gly135; *Tt*-Gly104; *Hs*-Gly135) is most probably a requirement for the turn that the chain forms at this position. The carbonyl of this glycine is involved in adenine recognition by forming a hydrogen bond with the 6'-NH₂ and the N7 of the adenine base (Fig. 5b).

A glutamate in loop L8, conserved in only the bacterial ADPRases (*Mt*-Glu120; *Ec*-Glu139; *Tt*-Glu108), is in hydrogen bonding distance of a hydroxyl group of the terminal sugar. At this same position the human ADPRase contains a cysteine residue (*Hs*-Cys139). However, this residue is too far from the terminal sugar to form a hydrogen bond (Fig. 5d). Upstream in the same loop, polar residues *Ec*-Ser133, *Tt*-Ser102, and *Hs*-Asp133 are at hydrogen bonding distance of another hydroxyl group of the terminal sugar. This hydrogen bond is not

possible in the *Mt*-ADPRase since there is a nonpolar alanine (*Mt*-Ala114) present at this position (Fig. 5d).

In the experiments presented here, replacement of *Mt*-Glu142 in loop L9 had a large decrease in k_{cat} , suggesting a role of this residue in the catalytic mechanism. Mutations in loop L9 of the *T. thermophilus* ADPRase, *Tt*-D126N, *Tt*-E127Q, *Tt*-D128N and *Tt*-E129Q, in which the aspartate or glutamate is mutated to asparagine or glutamine, respectively, do not change K_m or k_{cat} significantly (for example, *Tt*-E129Q mutant has an 8.1-fold decrease in k_{cat} , a 2.5-fold decrease in K_m) (Ooga et al. 2005; Yoshiba et al. 2004). In the case of *Tt*-Glu129 (corresponding to *Mt*-Glu142, *Ec*-Glu164, and *Hs*-Glu166) the aligned structures show that this residue participates in metal coordination. It is possible other residues in the same loop, particularly *Tt*-Asp126 (*Mt*-His139) and *Tt*-Glu127 (*Mt*-His140), compensate the effects of the E129Q mutation in *T. thermophilus* but not in *Mt*-E142Q. There is a noticeable difference between the use of divalent cations in *Mt*-ADPRase versus *Tt*-ADPRase. While three cations were observed in the structure of the *Mt*-ADPRase only two were present in *Tt*-ADPRase (Furuike et al. 2016). These differences may be due to differences in the experimental procedures; in *Mt*-ADPRase the ADPR analog was soaked in the presence of high metal concentration while in *Tt*-ADPRase the metal was diffused for a short time in crystals of the preformed *Tt*-ADPRase/ADPR complex (Furuike et al. 2016).

Comparison of the conserved motif of the ADPRase family and that of nucleoside diphosphate sugar hydrolases (NDPSase) family (i.e., *Bd*-NDPSase from *Bdellovibrio bacteriovorus*) reinforces the idea of a common role of important catalytic residues while allowing diversity in substrate recognition. In *Bd*-NDPSase, the residue equivalent to *Mt*-Glu142 is *Bd*-Glu140, which acts as a catalytic base and, upon mutation (*Bd*-E140Q), results in a significant reduction in k_{cat} (de la Peña et al. 2015). There is an aspartate-X-lysine (DXK) motif present in NDPSases that determines sugar preference in the substrate binding site (de la Peña et al. 2015) that *Mt*- and other bacterial ADPRases lack, which is replaced in *Mt*-ADPRase by an asparagine-serine-isoleucine sequence (Fig. 6).

Conclusions

In this paper, we characterize the product of the gene *rv1700* from the *M. tuberculosis* genome, an enzyme that was previously predicted by our group to be a Nudix ADPRase (Kang et al. 2003). Following subcloning, expression, and purification of Rv1700 in *E. coli*, the enzyme was found to hydrolyze ADPR into AMP and ribose-5-phosphate and requires divalent cations, either Mg^{2+} or Mn^{2+} , for activity. Its activity is highest at pH 8.0. The enzyme shows a strong preference for compounds with an adenine base and a small moiety – such as a simple sugar – at the end of the diphosphate.

Kinetic studies of the mutants E93Q, E97Q, R64A, and E142Q provide strong evidence for the involvement of these residues in the mechanism of the catalyzed reaction. Analysis of the structure of wild-type *Mt*-ADPRase indicates that while *Mt*-Glu93 (E_{16}^N), *Mt*-Glu97 (E_{20}^N), and *Mt*-Glu142 are metal ligands, *Mt*-Arg64 is in hydrogen-bonding distance of the β -phosphate group of the substrate, and thus plays a role in stabilizing the leaving group.

In *Ec*- and *Tt*-ADPRases there are two residues in loop L8 whose side chains make hydrogen bonds to the terminal moiety of the substrate and contribute to its recognition; these residues are neither compensated for nor conserved in *Mt*- and *Hs*-ADPRases.

Comparison of the 3D structures of *Mt*-, *Ec*-, *Tt*-, and *Hs*-ADPRase, however, suggest that these enzymes have similar catalytic mechanisms and identical positioning of many key residues, as predicted by protein sequence alignment. Basic knowledge of the function and the mechanism of Nudix hydrolases from pathogenic organisms may aid the development of bacteriostatic and bactericidal strategies.

Methods

Materials

Oligodeoxynucleotides were obtained from Integrated DNA Technologies. The plasmid pET11b was from Novagen. The BAC-based genomic DNA library from *M. tuberculosis* H37RV was kindly provided by Stewart Cole from Institute Pasteur (Cole et al. 1998). Competent cells of *E. coli* DH5 α were obtained from Life Technologies and *E. coli* BLR(DE3) competent cells were from Novagen. The FailSafeTM PCR system was from Epicentre, the restriction enzymes, NdeI and BamHI, and calf intestinal alkaline phosphatase were from New England Biolabs, the IPTG was from Research Organics, and the GeneClean[®] Kit was from Bio101.

Subcloning

For subcloning into an expression vector, the *rv1700* gene was amplified from lysed whole cells of *E. coli* DH10 β containing *rv1700* on a BAC plasmid using the polymerase chain reaction. Oligonucleotide primers were used to incorporate an NdeI restriction site at the beginning of the gene (forward primer) and a site for BamHI at the end (reverse primer) of the gene. The amplified DNA was purified, digested with NdeI and BamHI, and ligated into pET11b under the control of a T7 lac promoter. The resultant plasmid pET-*rv1700* was used to transform *E. coli* DH5 α for storage and *E. coli* BLR(DE3) for expression or mutagenesis. The sequence of the subcloned gene was confirmed through dideoxy sequencing.

Site-Directed Mutagenesis

Mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Pairs of complementary oligonucleotides 35-41 bases long, with a 'melting' temperature of 75-78°C and containing the desired mutation were used to prime the replication of circular plasmid DNA by Pfu or Pfu turboDNA polymerase. The PCR reaction (50 μ l) contained the following: 50-150 ng plasmid DNA, 11 pmol of each mutagenic oligonucleotide primer, 200 μ M dNTPs, 10 mM KCl, 6 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.0, 2 mM MgCl₂, 0.1 % Triton X-100, 10 μ g/mL BSA and 2.5 units of Pfu DNA polymerase. The reaction was subjected to an initial heating step of 30 s at 95°C, and 20 rounds of 95°C for 30 s, 55°C for 1 min, and 68°C for 19 min in a GeneAmp[®] PCR System 2400 (PerkinElmer). Parental DNA was digested with 10 units of DpnI endonuclease to remove the methylated parent strands, and the synthesized plasmid DNA was used to

transform *E. coli* XL1-Blue cells. The identity of all mutants was confirmed by complete sequencing of both DNA strands.

Expression and Purification

E. coli BLR(DE3) containing pET-*rv1700* or its mutants were grown in three stages. A single colony, picked from an LB-agar plate, was inoculated into 3 mL of minimum M9 medium containing 100 µg/mL ampicillin and grown overnight. The cells were transferred to 100 mL of LB medium and grown to saturation. The expression and purification were modified from the protocol of Kang et al. (Kang et al. 2003) The 100 mL of culture were transferred to 2 L of LB medium and grown to an A_{600} of 0.5; cells were cooled overnight to 4 °C. The culture was placed in a 15 °C bath, induced with the addition of 0.5 mM IPTG and grown at 15 °C for an additional 16 hours (Duong-Ly and Gabelli 2014a; Duong-Ly and Gabelli 2014b).

The induced cells were harvested, washed by suspension, resuspended in 25 mL buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA), sonicated, centrifuged, and the precipitate discarded. A typical preparation yielded approximately 200 mg of protein from 3 g of cells. The protein concentration of the supernatant was adjusted to 10 mg/mL with buffer A (Fraction I).

To Fraction I, 0.1 volumes of 10% streptomycin sulfate (w/v) was added and, after 15 minutes on ice, the precipitate was discarded. The supernatant was collected and solid ammonium sulfate was added to 30% saturation. The supernatant was collected and ammonium sulfate was added to 45% saturation. The precipitate was dissolved in 10 mL buffer A and dialyzed overnight against 2 L of buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 300 mM NaCl). The solution was then loaded at 0.5 mL/min onto a HiPrep 26/60 Sephacryl S-100 high resolution gel filtration column (Amersham Pharmacia Biotech) pre-equilibrated with buffer B at 4 °C. The eluted fractions were dialyzed against 2 L of buffer A and loaded onto a Mono-Q anion exchange column. Elution was carried out using a continuous NaCl gradient (0 to 0.5 M) in buffer A at a rate of 1 mL/min, collecting 2 mL fractions. The *Mt*-ADPRase elutes as a symmetrical peak at a NaCl concentration of 0.2 M. Protein concentration was determined by absorbance at 280 nm. During the purification, the *Mt*-ADPRase was identified by running aliquots of the different fractions in SDS-PAGE. The purified enzyme was stable for months at -80°C.

Biochemical Characterization

In the standard assay, the kinetics of hydrolysis of ADPR to AMP and ribosyl phosphate was determined by measuring the phosphate liberated from the products by calf intestinal phosphatase. The reaction mixture containing 2 mM ADPR, 50 mM Tris-HCl, pH 8.0, 0.5 mM MgCl₂, 0.5 units of calf intestinal phosphatase, and 0-1 milliunit of ADPRase enzyme in 50 µL was incubated at 37°C for 30 min. The reaction was terminated by the addition of 250 µL of 10 mM EDTA plus 700 µL of Ames solution. The phosphate produced was measured by the colorimetric method of Dubin and Ames (Ames and Dubin 1960). For negative controls ADPRase was replaced with reaction buffer. To detect ADPRase activity in the lysate, a small aliquot (2 µL) of either the lysate of the cells expressing Rv1700 or of the

control cells (same *E.coli* strain with the same plasmid without RV1700 gene, pET11b) were each added to a large volume (25 μ L) of 2 mM of ADPR and 2mM MgCl₂ and the amount of reaction was measured after 30 minutes using the assay described above. The greater amount of reaction in the lysate from the RV1700 gene containing cells was used as the indication of the presence in the lysate of the product of the Rv1700 gene.

Specific activities for different substrates, metal ions, or pH values were determined using the same reaction mixture except for the substitution of the relevant component. Maximal ADPR pyrophosphatase activity in the presence of 0.5-5 mM Mg²⁺ was measured at pH 8.0. To determine metal preference, assays were repeated with 0.5-100 μ M Mn²⁺, Co²⁺, Ca²⁺, and Li⁺. K_m and turnover number for ADPR and ADP-glucose were determined using seven different substrate concentrations (0, 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 mM); at least five independent measurements were carried out at each substrate concentration.

Product Determination

The products of the hydrolysis of ADP-ribose with *Mt*-ADPRase were determined by HPLC analysis. The reaction mixture, containing 2 mM ADPR, 50 mM Tris-HCl pH 8.0, 0.5 mM MgCl₂, and 0-1 milliunit of *Mt*-ADPRase in 50 μ L was incubated at 37°C for 15 minutes, quenched with 250 μ L of 10 mM EDTA, filtered through a microcon-10 filter, and analyzed by HPLC. The HPLC was carried out on an YMC ODS-AQ column with an isocratic mobile phase consisting of 12.5 mM citric acid, 25 mM sodium acetate, and 10 mM acetic acid, adjusted to pH 6.3 with sodium hydroxide. The substrate and nucleotide product were detected at 254 nm and identified by comparison to standards of ADP-ribose, AMP, and ADP.

Crystallization and Structure Determination

Crystals of E142Q *Mt*-ADPRase were grown by hanging drop vapor diffusion with a 1 mL reservoir consisting of 0.1 M Tris-HCl, pH 8.0, and 3.7 - 4.4 M sodium formate. One μ L of reservoir solution was added to 1 μ L of about 10 mg/mL E142Q *Mt*-ADPRase in TED buffer. Data were collected with a copper rotating anode RU-H3R generator (Rigaku) as the source of X-rays on a RAXIS IV image plate detector at the X-ray facility of the Department of Biophysics and Biophysical Chemistry of the Johns Hopkins University. Indexing and data reduction was carried out using HKL2000 (Otwinowski 1997) (Table 3). The structure was determined by molecular replacement using the program AMoRe. The wild type enzyme in complex with ADPR was used as the search model (PDB ID: 1MK1) (Navaza 1994). Model building was completed through iterative cycles of manual rebuilding with the program COOt (Emsley et al.) and refinement with REFMAC5 CCP4 (Murshudov et al. 2011).

Sequence and Structure Alignment

Sequence identity and homology between ADPRases were determined through MOE (MOE 2016). Sequence and structure alignment figures for ADPRase structures: *H. sapiens* NudT5 (PDB ID: 2DSC), *E. coli* (PDB ID: 1HKZ), *M. tuberculosis* (PDB ID: 1MQW), and *T. thermophilus* (PDB ID: 1V8T) was carried out with the program ESPript (Robert and Gouet 2014) and SSM (Krissinel and Henrick 2004), respectively.

Protein Structure Accession Numbers

Atomic coordinates and structure factors of the *Mt*-ADPRase E142Q (PDB ID: 5I8U) were deposited in the Protein Data Bank.

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Figure 1.

Structural alignment of some of the ADPRases from the NUDT5 subfamily with known structure (1MQW, *M. tuberculosis*; 1V8T, *T. thermophilus*; 1KHZ, *E. coli*; 2DSC, *H. sapiens*). Secondary structure elements are shown above the sequences. The 3 β -sheet N-Terminal domain is marked with striped, gray arrows and the Nudix domain with a bar on top. Magenta background highlights identical residues in the bacterial and the human enzymes; green background shows residues identical in bacterial enzymes that are not conserved in the human enzyme. *Tt*-Glu70 is marked with an orange background. Sequence similarities are in blue and boxed in magenta with a white background. Structural alignment was carried out with the program SSM (Krissinel and Henrick 2004) and the figure drawn with the program ESPript (Gouet et al. 1999).

Figure 2.

a) Purification of the *Mt*-ADPRase. Polyacrylamide gel (12.5%) stained with Coomassie blue: molecular weight standards ladder (lane 1); *Mt*-ADPRase (wt) (lane 2); *Mt*-ADPRase (E142Q) (lane 3); molecular weight standards ladder (lane 4). *Mt*-ADPRase activity dependence on **b)** pH and **c)** metal cation.

Figure 3.

Rv1700 products of hydrolysis and kinetic characterization. **a)** HPLC Chromatogram of the Rv1700 ADP-ribose hydrolase with ADP-ribose after 15 minutes shows AMP at 12.3 minutes and ADP-ribose at 13.2 minutes indicating that the reaction of Rv1700 with ADP-ribose yields AMP and ribose-5-phosphate as the products (black trace). Standards of ADP (8.5 minutes), AMP (12.3 minutes), and ADP-ribose (13.2 minutes) are in a light gray trace. The reaction mixture containing ADP-ribose and Rv1700 did not give a peak for ADP. **b), c), and d)** Turnover number of *Mt*-ADPRase activity using Mg²⁺ (gray) or Mn²⁺ (gold) depending on **b)** substrate preference of ADPR versus ADP-glucose (ADPG), **c)** increasing concentrations of ADPR (0 - 2 mM), or **d)** increasing concentrations of Mg²⁺ or Mn²⁺ (0 - 1 mM).

Figure 4. Structural alignment of ADPRases. **a)** Scheme of the quaternary structure of the dimer of ADPRase with swapped domains; one monomer shown in orange, the other monomer in yellow. **b)** Close up of Loop L9 in the structures of the *M. tuberculosis* in gray (PDB ID: 1MQW), *E. coli* in green (PDB ID: 1KHZ), and *H. sapiens* in purple (PDB ID: 2DSC). Shown as sticks are *Ec*-Glu162, *Ec*-Glu164, and *Mt*-Glu142, as well as AMPCPR (thin lines) from *Mt*- and *Ec*-ADPRase complex structures. **c)** One monomer of wild-type *Mt*-ADPRase (gray) and *Mt*-E142Q shown in yellow (boxes indicate loops L2 and L9) and orange to emphasize domain swapping. Loop L2 and L9 display a conformational change upon substrate binding. **d)** Zoom-in of the area of loop L9 of wild-type *Mt*-ADPRase (gray) and *Mt*-E142Q (yellow).

Figure 5.

a) Structural alignment of *Mt*- (gray), *Ec*- (green), *Tt*- (blue), and *Hs*- ADPRases (purple). Shown in black is the other subunit of the *Mt*-ADPRase. The Nudix domain of one subunit or monomer interacts with the β -sheet N-terminal domain of the other one (black in the case of *Mt*-ADPRase). **b)** Adenine recognition by *Mt*-Glu38 and *Mt*-Gly116, as well as pocket closure by *Mt*-Pro115. **c)** Adenine stacking by *Mt*-Arg37 and *Mt*-Ile19, as well as β -phosphate recognition by *Mt*-Arg64. The label for *Mt*-Glu38 indicates the direction of the chain but the side chain is clipped in this view. **d)** Ribose recognition by *Mt*-Glu120 (*Ec*-Glu139; *Tt*-Glu108) and *Ec*-Ser133, *Tt*-Ser102, and *Hs*-Asp133. *Hs*-Cys139 and *Mt*-Ala114, present variation and differences in polarity of these positions.

Figure 6. Amino acids involved in ribose recognition in *Mt*-ADPRase vs *Bd*-NDPSase. **a)** Ribose recognition by the wild type (gray) and E142Q (yellow) *Mt*-ADPRases (PDB ID: 1MQW and 5I8U). **b)** Ribose recognition by the DXK motif in *Bd*-NDPSase (lime green) (PDB ID: 5CT7).

Table 1

Substrate specificity of Rv1700. Substrates were present at a concentration of 2 mM and were assayed using the colorimetric procedure described in “*Methods*”, except for CoA which was assayed using HPLC to monitor the reaction.

Substrate	Relative activity (%)
ADP-ribose	100
ADP-glucose	54
ADP-mannose	59
GDP-glucose	15
GDP-mannose	33
GDP-fructose	2
UDP-glucose	5
UDP-mannose	14
UDP-galactose	2
NAD	34
NADH	<1
Ap3A	3
CoA	<1

^a A unit of enzyme hydrolyzes 1 μ mol of substrate per min.

Table 2Effect of mutations on *Mt*-ADPRase catalytic activity for ADP-ribose.

	K_m (μM)	k_{cat} (s^{-1})
Wild type	200 ± 19	14.4 ± 0.4
E93Q(E_{16}^N)	424 ± 88	0.0074 ± 0.0005
E97Q(E_{20}^N)	452 ± 281	0.0125 ± 0.002
R64A($E_{+50\text{ish}}^N$)	592 ± 125	0.0157 ± 0.001
E142Q	544 ± 293	0.0475 ± 0.009

Table 3Data collection and refinement statistics for *Mt*-ADPRase (E142Q).

5I8U	
Data collection	
Space group	C121
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	152.7, 124.8, 142.9
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90.0, 98.5, 90.0
Resolution (Å)	50.0-1.99 (2.06-1.99) *
Wavelength (Å)	1.542
<i>R</i> _{sym} (%)	0.08 (0.91)
Total reflections	500,316
Unique reflections	159,897
<i>I</i> /σ	10.7 (1.0)
Completeness (%)	88.2 (56.2)
Redundancy	3.2 (1.8)
Refinement	
Resolution (Å)	50.00-1.99 (2.05-1.99)
No. reflections	148,859
<i>R</i> _{work} / <i>R</i> _{free}	0.22/0.26 (0.40/0.41)
No. atoms	11,364
Protein	10,759
Ligand/ion	-
Water	609
B-factors	
Protein	63.2
Ligand/ion	-
Water	57.1
RMS deviations	
Bond lengths (Å)	0.018
Bond angles (°)	1.990

* Highest resolution shell is shown in parenthesis.