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Identification and characterization of novel adenosine cleavage enzymes in mycobacteria

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IDENTIFICATION AND CHARACTERIZATION OF NOVEL ADENOSINE CLEAVAGE ENZYMES IN MYCOBACTERIA

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PURIFICATION AND CHARACTERIZATION OF NOVEL ADENOSINE CLEAVAGE ENZYMES IN MYCOBACTERIA

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PHARMACOLOGY AND TOXICOLOGY

ABSTRACT

Tuberculosis (TB) is one of the leading infectious diseases in the world. An estimated one third of the world's population is infected with *Mycobacterium tuberculosis*, the causative pathogen of TB. With the emergence of drug resistant strains of the mycobacterium, and the HIV-TB coinfection epidemic, TB remains a global health emergency. Purine metabolism is an essential cellular component to all living cells. Previous studies have shown that differences exist between mycobacterial and human purine metabolism. One of the differences was in the metabolism of adenosine (Ado), whose cleavage was observed in mycobacterial cells whereas Ado cleavage is inefficient in human cells. While Ado cleavage activity had been observed in mycobacteria, the enzyme(s) responsible for this activity was unknown. The identification and understanding of the biochemical properties of Ado cleavage enzyme(s) could aid in the development of Ado analogs for the treatment of TB. There are several candidate enzymes in nature that can cleave Ado, and therefore the identification of endogenous Ado cleavage activity from *M. smegmatis* was preferred over a genetic approach. This strategy enabled the discovery of two Ado cleavage activities. The first enzyme was

named adenosine-purine nucleoside phosphorylase (Ado-PNP), and could cleave Ado, inosine, and guanosine. Because of its ability to cleave Ado, Ado-PNP is different from the trimeric mycobacterial PNP that accepts 6-oxopurines but not 6-aminopurines as substrates. The second enzyme identified could cleave Ado, but 5'-methylthioadenosine (MTA) was the preferred substrate. MTA cleavage was phosphate dependent, and therefore the second Ado-cleaving enzyme identified in *M. smegmatis* was an MTA phosphorylase. This marked the identification of the first bacterial MTAP. Based on sequence homology, Rv0535 had been annotated as a probable MTAP in *M. tuberculosis*. Recombinant Rv0535 was expressed, purified, and characterized. While MTA was the preferred substrate, Rv0535 could also cleave Ado and S-adenosyl-L-homocysteine at 2% and 0.8% of the MTA cleavage activity, respectively. To identify other substrates of Rv0535, a structure-activity relationship study was conducted and the initial results are presented. This work has identified two mycobacterial Ado cleavage enzymes that could be exploited for the development of new anti-TB drugs.

Keywords: *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, purine metabolism, adenosine, 5'-methylthioadenosine phosphorylase, Rv0535

DEDICATION

To my parents, whose knowledge, wisdom, support, guidance, and love have been key to my success in life.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure *Page*

INTRODUCTION

IDENTIFICATION AND CHARACTERIZATION OF TWO ADENOSINE PHOSPHORYLASE ACTIVITIES IN *MYCOBACTERIUM SMEGMATIS*

IDENTIFICATION OF RV0535 AS 5'-METHYLTHIOADENOSINE PHOSPHORYLASE FROM *MYCOBACTERIUM TUBERCULOSIS*

STRUCTURE-ACTIVITY RELATIONSHIP OF 5'-METHYLTHIOADENOSINE PHOSPHORYLASE FROM *MYCOBACTERIUM TUBERCULOSIS*

SUMMARY AND CONCLUSIONS

LISTS OF ABBREVIATIONS

Ade	adenine
Ado	adenosine, or 9-[β-D-ribofuranosyl]-adenine
AdoP	adenosine phosphorylase
AK	adenosine kinase
AraA	9-[β-D-arabinofuranosyl]-adenine
BCG	Bacille Calmette Guerin
ddA	2',3'-dideoxyadenosine
F-Ado	2-fluoro-adenosine
FPLC	fast protein liquid chromatography
Gua	guanine
Guo	guanosine
HIC	hydrophobic interaction column
Hx	hypoxanthine
HPLC	high pressure liquid chromatography
IMP	inosine monophosphate
Ino	inosine
MDR	multiple drug resistant
MeP-dR	6-methylpurine-2'-deoxyriboside
Methyl-Ado	2-methyl-adenosine
MTA	5'-methylthioadenosine
MTAP	5'-methylthioadenosine phosphorylase

MTAN	5’-methylthioadenosine nucleosidase
MTI	5’-methylthioinosine
MTIP	5’-methylthioinosine phosphorylase
OADC	oleic acid, albumin, dextrose, and catalase
PNP	purine nucleoside phosphorylase
PRPP	5-phospho-α-D-ribosyl-1-diphosphate
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SAR	structure-activity relationship
TB	tuberculosis
WHO	World Health Organization
XDR	extensively drug resistant

INTRODUCTION

Tuberculosis as a global health concern.

Tuberculosis (TB) is the second leading infectious disease in the world. According to the World Health Organization (WHO), there were 8.8 million new TB cases reported in 2010 and TB caused 1.1 million deaths that year (59). In developed countries, TB incidence rates are kept low largely because of adequate public health systems. Sadly, most of the high-burden TB countries are developing nations, which account for more than 80% of active TB cases and over 98% of TB deaths worldwide (42, 57).

A TB infection starts when an individual inhales the causative pathogen, *Mycobacterium tuberculosis*, in doses as low as a single bacterium. In 10% of infections, active TB disease develops and medically presents with bloody sputum, cough, chest pains, night sweats, weight loss, and general malaise. TB diagnosis is confirmed by microbiological analysis of sputum and other body fluids. Chest X-rays that reveal cavitary lesions and a positive tuberculin skin test reaction are also used as diagnostic tools. While TB mostly presents as a pulmonary disease, extrapulmonary TB has also been reported. However, most infected individuals often remain asymptomatic, and are considered to have a latent TB infection. It is estimated that 2 billion individuals are latently infected. About 10% of these individuals will develop active TB in their lifetime (57), with higher reactivation rate seen in immunocompromised patients and older individuals.

The human immunodeficiency virus (HIV) epidemic is one of the obstacles in the eradication of TB. Infection with HIV leads to a weakened immune system, which makes

HIV positive individuals more susceptible to contract active TB or develop reactivation TB. It is estimated that 13% of TB cases occur in HIV infected individuals (59). TB is the leading cause of death in the HIV infected population.

Anti-tuberculosis drugs and problems with current therapy.

Several TB drugs exist and are grouped as either first line drugs or second line drugs (Table 1). Standard treatment consists of 2 months with all four first line drugs (isoniazid, rifampin, pyrazinamide, and ethambutol) taken daily, followed by 4 additional months of isoniazid and rifampin administration. Because these drugs have toxic side effects, blood tests are performed to monitor liver and kidney toxicity while taking TB drugs. Thus, lengthy treatment and additional medical tests often lead to financial burden. Further, high TB incidence is often found in remote and rural areas of third-world countries, where access to TB drugs and medical facilities are limited. While TB can be cured, treatment is plagued by poor patient compliance, limited access to treatment, and financial burden of treatment.

Drug resistant TB is also a concern. In the 1940s, the discovery of streptomycin and *p*-aminosalicylic acid provided optimism for the treatment of TB. However, this was short lived as less than 20 years later, TB strains that were resistant to these two agents appeared. Resistance to isoniazid also became prevalent and treatment with a single agent was discontinued. However, this did not stop the emergence of new strains of drug resistant TB. Multiple drug resistant tuberculosis (MDR-TB) is resistant to at least isoniazid and rifampicin (two of the first line drugs), and extensively drug resistant tuberculosis (XDR-TB) is resistant to isoniazid, rifampicin, any of the quinolone based drugs, and amikacin, kanamycin or capreomycin. Both MDR-TB and XDR-TB are

deadlier forms of TB and are on the rise. According to the March 2010 estimates of the WHO, one in four new cases of TB in northwest Russia was MDR-TB, and 58 countries reported at least one incidence of XDR-TB (58). Further, treating MDR-TB and XDR-TB is more complicated, involving surgery, isolation, longer treatment periods, and chemotherapy with second line drugs, which are more expensive and toxic.

Table 1. Current tuberculosis drugs

Drug	Year of discovery	Mechanism of action
First line drug		
Isoniazid	1952	Inhibition of cell wall mycolic acid synthesis and other multiple effects on DNA, lipids, carbohydrates, and NAD metabolism
Rifampin	1966	Inhibition of RNA synthesis
Pyrazinamide	1952	Disruption of membrane transport and energy depletion
Ethambutol	1961	Inhibition of cell wall arabinogalactan synthesis
Second line drug		
Streptomycin	1944	Inhibition of protein synthesis
Kanamycin	1957	Inhibition of protein synthesis
Quinolones	1963	Inhibition of DNA synthesis
Ethionamide	1956	Inhibition of mycolic acid synthesis
Para-aminosalicylic acid	1946	Inhibition of folic acid and iron metabolism
Cycloserine	1952	Inhibition of peptidoglycan synthesis

The lack of an effective TB vaccine hampers efforts to prevent new TB cases. The BCG (Bacille Calmette-Guerin) vaccine is the only TB vaccine on the market. It has been suggested that the vaccine offers most benefit to children and that there is a geographical component to the efficacy of the vaccine (42). The vaccine provides effective TB protection in some countries in the United Kingdom but has been shown to offer no benefit in India (12, 42). One of the main hypotheses for the geographical efficacy of the

vaccine is the exposure of children to different environmental mycobacteria. While the BCG vaccine is commonly administered to children who live in close proximity to infected adults, it is not a recommended vaccine in the US.

Because of the high number of infected individual, the increase in TB-HIV co-infections, the emergence and spread of drug-resistant TB, and the ineffective protection of the BCG vaccine, the WHO has declared TB a global health emergency. There is an urgent need to develop new anti-TB regimens, which are less toxic and administered for a shorter period of time. Additionally, new TB drugs are needed to treat latent and drug-resistant TB.

Purine metabolism and nucleoside analogs.

The first step of any drug discovery effort is the identification of suitable targets, such as enzymes in specific pathways, which can lead to the identification of compounds with selective toxicity (18). Thus, an enhanced basic understanding of the enzymes involved in metabolic processes in mycobacteria could lead to identification of molecular targets for drug discovery. Purine metabolism is an essential process of all living cells as it generates macromolecules necessary for DNA, RNA, and energy production. Extensive studies of human purine metabolism have led to the development of nucleoside analogs that are currently used to treat cancer. Moreover, differences in purine metabolism between human cells and infectious agents have been exploited to develop nucleoside analogs against parasitic and viral infections (27). In contrast, purine metabolism has not been well studied in mycobacteria and nucleoside analogs are not used against bacterial infections, mostly because of the success of traditional antibiotics.

However, because the efficacy of currently available antibiotics is decreasing, it is crucial to develop new agents that have alternate mechanism of action than current drugs.

In 1998, the genome of the *M. tuberculosis* H37Rv strain was published (17), and it became apparent that *M. tuberculosis* contained functional *de novo* and salvage purine pathways (29, 31). However, only a few mycobacterial purine enzymes have been studied, including purine nucleoside phosphorylase (PNP, Rv3307, E.C. 2.4.2.1) (6, 22), adenosine kinase (AK, Rv2202c, E.C. 2.7.1.20) (28), S-adenosyl-L-homocysteine hydrolase (SAHH, Rv3248c, E.C. 3.3.1.1) (41), and hypoxanthine-guanine phosphoribosyl transferase (HGPRT, Rv3624c, E.C. 2.4.2.8) (11). In 2003, *M. tuberculosis* AK became the first bacterial homolog to be biochemically characterized, and it was found to be considerably different from the human form (28). Further studies of AK revealed that 2-methyl-adenosine (methyl-Ado), an analog of adenosine (Ado), was highly toxic against active and latent mycobacteria (5, 28, 35), thereby providing proof of concept for the additional investigation of nucleoside analogs against TB. Thus, it is possible that the differences in purine metabolic enzymes between human and mycobacterial cells could be exploited to design a nucleoside pro-drug that can only be converted to its active cytotoxic form by mycobacterial enzymes (28, 35). Moreover, because purine metabolism is not a target of current TB drugs, it is likely that nucleoside analogs would be active against drug-resistant TB and latent TB infections.

Exploiting Ado metabolism for drug discovery.

Previous studies have shown the conversion of Ado to adenine (Ade) in *M. tuberculosis* and *M. smegmatis* cell cultures (16, 34), without evidence of the enzyme responsible for this activity. Ado is a purine nucleoside that can undergo four general

reactions: it can be deaminated to inosine (Ino) by Ado deaminase, it can be phosphorylated to AMP by AK, it can be hydrolyzed to Ade by purine nucleosidase, or it can be phosphorolytically cleaved to Ade by PNP (Figure 1). Only two of these reactions are supported by either sequence homology (in the case of Ado deaminase) or at the protein level (in the case of AK) in mycobacteria. The existence of purine nucleosidase has not been shown but Ducati et al., suggested that IunH, a probable inosine-uridine preferring nucleoside hydrolase, could cleave Ado to Ade (21), whereas unpublished results from the Parker lab indicated that IunH cannot cleave Ado (14).

PNP is an essential component of the purine salvage pathway, and cleaves the glycosidic bond between the ribose moiety and the purine nucleobase, using phosphate as the attacking group. PNPs from different organisms have been characterized and it was shown that PNPs from various sources have different substrate specificity. Thus, it is common for PNPs to be categorized according to substrate specificity and quaternary structure (8, 26). In general, trimeric PNPs exist in mammals and are specific for 6-oxo-purines. For example, human PNP is a trimer that can cleave Ino and guanosine (Guo) but not Ado. Hexameric PNPs are expressed by bacteria and can cleave both 6-oxo-purines and 6-amino-purine. Some organisms express both trimeric and hexameric PNPs (26). *Escherichia coli* is known to express a trimeric PNP in addition to the hexameric enzyme. The trimeric form, also known as xanthosine phosphorylase, cleaves all purine nucleosides except for Ado and 2'-deoxy-Ado (20, 44). There are also some PNPs that do not fit the above description. Adenosine phosphorylase (AdoP) is another member of the PNP family and *Bacillus cereus* AdoP is specific for Ado and 2'-deoxy-Ado (48).

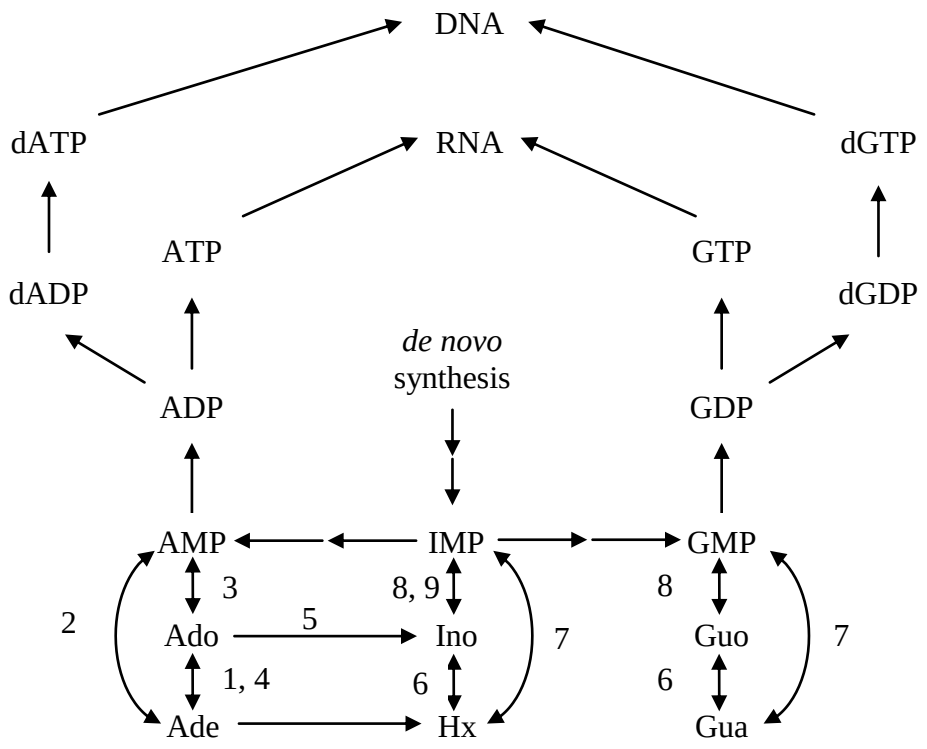

Figure 1. Overview of enzymes involved in purine metabolism

Enzymes: (1) 5'-Methylthioadenosine/S-adenosylhomocysteine nucleosidase (not present in human cells) or 5'-Methylthioadenosine phosphorylase; (2) Adenine phosphoribosyl transferase; (3) Adenosine kinase; (4) Adenosine phosphorylases (not present in human cells) and most bacterial purine nucleoside phosphorylases; (5) Adenosine deaminase; (6) Purine nucleoside phosphorylase (7) Hypoxanthine/guanine phosphoribosyl transferase; (8) Guanosine/inosine kinase (not present in human cells); (9) 5'-nucleotidase

The high specificity for 6-oxopurines by human PNP is attributed to Asn243 and Glu201 (49), which are also present in *M. tuberculosis* homolog. It was therefore predicted that similar to human PNP, *M. tuberculosis* PNP would not accept Ado as substrate. When *M. tuberculosis* PNP was expressed and characterized, it was shown that

unlike other bacterial PNPs, *M. tuberculosis* PNP was a trimer that could cleave Ino and Guo, but not Ado (15, 22). Therefore, in humans and *M. tuberculosis*, Ado is not a readily cleaved to Ade by PNP but primarily metabolized by Ado deaminase and AK. However, since Ado to Ade cleavage has been observed in *M. tuberculosis* cell extracts, it is possible that the metabolism of Ado between human and mycobacterial cells is different. Identification of the enzyme(s) responsible for Ado cleavage in mycobacteria could result in target identification and validation, which are important steps in a drug discovery effort.

Candidate enzymes for Ado cleavage in *M. tuberculosis.*

The observed Ado cleavage in *M. tuberculosis* could be due to a novel enzyme that has yet to be characterized or a known enzyme whose substrate specificity has not been studied. As mentioned previously, Ado can be cleaved by some bacterial PNPs, including AdoP. Because the sequence of AdoP is not known, the identification of an AdoP gene in *M. tuberculosis* by sequence homology is not possible. Another category of PNP that has to be considered is 5'-methylthioadenosine phosphorylase (MTAP, E.C. 2.4.2.28) which is primarily found in Eukarya and Archaea (1, 26). MTAP catalyzes the cleavage of 5'-methylthioadenosine (MTA), a natural purine that is generated as a by-product of polyamine synthesis. MTA cleavage by MTAP yields Ade, which participates in purine metabolism, and 5'-methylthioribose-1-phosphate, which can be further recycled to methionine. In bacteria, MTA is catabolized by 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN or MTA/SAH nucleosidase, E.C 3.2.2.16), producing Ade and 5'-methylthioribose. Cleavage of Ado by parasitic MTAP has been reported (25, 30), whereas human MTAP cleaves Ado very inefficiently (23, 25, 53).

According to the UniProt Consortium (52), *M. tuberculosis* encodes for MTAN (Rv0091) and a probable MTAP (Rv0535). Thus, either MTAN or MTAP could be responsible for the Ado cleavage observed in *M. tuberculosis*.

Preliminary studies.

As mentioned previously, Ado cleavage in crude cell extracts from *M. smegmatis* and *M. tuberculosis* has been reported (16, 34). However, the enzyme(s) responsible for this activity had not been identified. Therefore, the goal of this project is to identify and characterize the Ado cleavage enzyme(s) in mycobacteria. Since there are a number of candidate enzymes that may cleave Ado in nature, preliminary studies were conducted to purify the endogenous Ado cleavage activity from *M. tuberculosis* crude extract.

M. tuberculosis H37Ra was cultured in Middlebrook 7H9 medium supplemented with oleic acid, albumin, dextrose, and catalase (OADC), and 0.05% Tween 80 at 37 ˚C in a shaking incubator until the OD_{600} was 0.4 – 0.6. Cells (from a 2 L culture) were pelleted by centrifugation, and the cell pellet was washed three times in Buffer A (50 mM Tris HCl, 1 mM dithiothreitol, and 20% glycerol, adjusted to pH 7.6). The cell pellet was resuspended in Buffer A containing protease inhibitor cocktail (Roche), and the cells were lysed using a French pressure cell. The resulting suspension was centrifuged at 4 ˚C for 1 hour at 100,000 × *g*. The supernatant was dialyzed overnight against two changes of 1 L of Buffer A plus 150 mM NaCl at 4 ˚C (Spectra/Por 4 Membrane Tubing, 12,000 to 14,000 Dalton MWCO, Fisher Scientific) and filtered using a 0.2 µm pore – size filter. The resulting crude extract was applied to a HiTrap Q anion exchange column and eluted using a linear salt gradient of 0 to 1 M NaCl in Buffer A.

Crude cell extracts contain Ado deaminase, which can interfere with the correct evaluation of Ado cleavage. 2-Fluoro-adenosine (F-Ado) is not a substrate for Ado deaminase, but as shown in Figure 2, F-Ado and Ado were comparable substrates for Ado cleavage enzyme in a partially purified sample from *M. tuberculosis*.

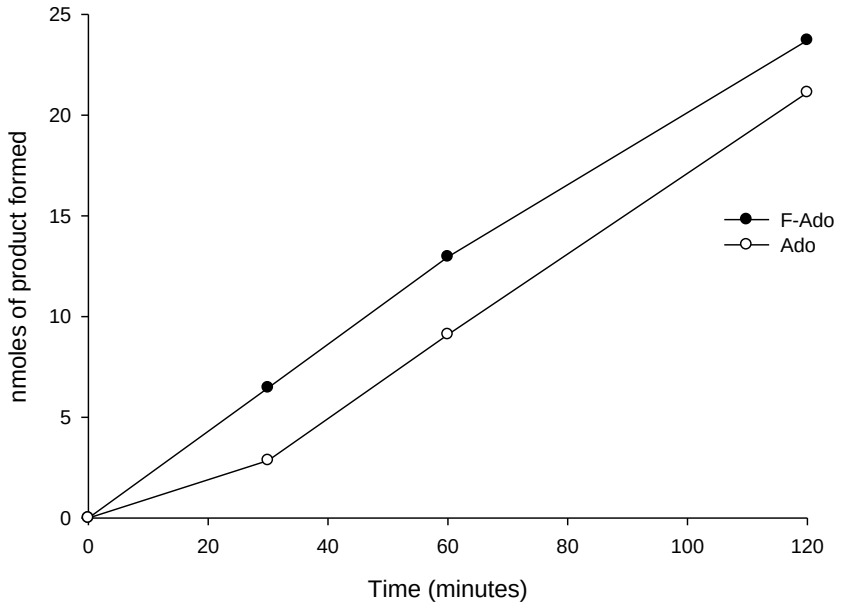

Figure 2. Comparison of 2-fluoro-adenosine and Ado cleavage.

The cleavage of F-Ado and Ado in a partially purified sample of *M. tuberculosis* was measured by reverse-phase HPLC.

Since F-Ado and Ado were comparable substrates, fractions eluting from the anion exchange column were evaluated for their ability to cleave F-Ado using reverse-phase HPLC. Since *M. tuberculosis* PNP can cleave Ino but not Ado, and could interfere with the biochemical characterization of the Ado cleavage enzyme, it was important to

separate the two enzymes. Therefore, fractions were also monitored for their ability to cleave Ino by reverse-phase HPLC.

As shown in Figure 3, a miscorrelation between Ino and F-Ado cleavage activities was observed, indicating that the Ado cleavage enzyme was distinct from PNP.

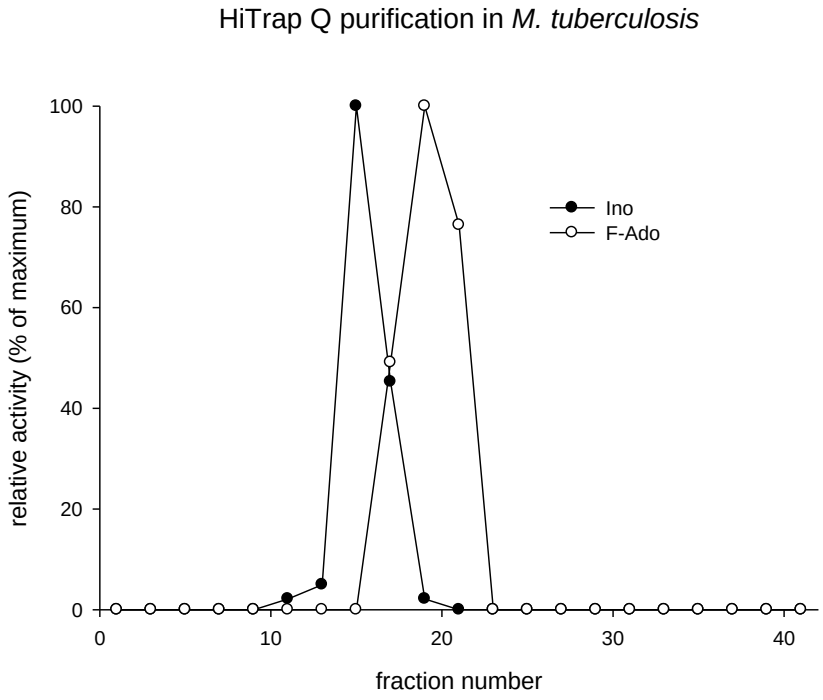

Figure 3. Elution profile of *M. tuberculosis* Ado cleavage activity from the HiTrap Q column.

M. tuberculosis crude cell extract was applied to a HiTrap Q anion exchange column and eluted with a linear gradient of 0-1 M NaCl in buffer A. Fractions eluting from the column were tested for their ability to cleave Ino and F-Ado using reverse-phase HPLC.

Fractions containing the most F-Ado cleavage activity were pooled and applied to a gel filtration column. Proteins were eluted using an isocratic buffered solution of 10 mM KCl. Despite the use of two purification columns, a single band responsible for the Ado cleavage activity could not be visualized on an SDS-PAGE gel stained with either

Coomassie or Silver Stain reagents. However, the initial biochemical characterization of this enzyme could be performed.

Since Ado can be cleaved by phosphorylases or hydrolases, it was important to evaluate whether the Ado cleavage enzyme was a phosphorylase or a hydrolase. A phosphorylase can only cleave its substrate in the presence of phosphate, whereas a hydrolase does not require phosphate for activity.

Hydrolase: Adenosine + H_2O ←→ Adenine + ribose

Phosphorylase: Adenosine + PO_4^- ←→ Adenine + ribose-1-phosphate

The partially purified *M. tuberculosis* Ado cleavage enzyme was incubated in the presence or absence of phosphate. F-Ado cleavage was only observed in the presence of phosphate, indicating that the enzyme was a phosphorylase (Figure 4).

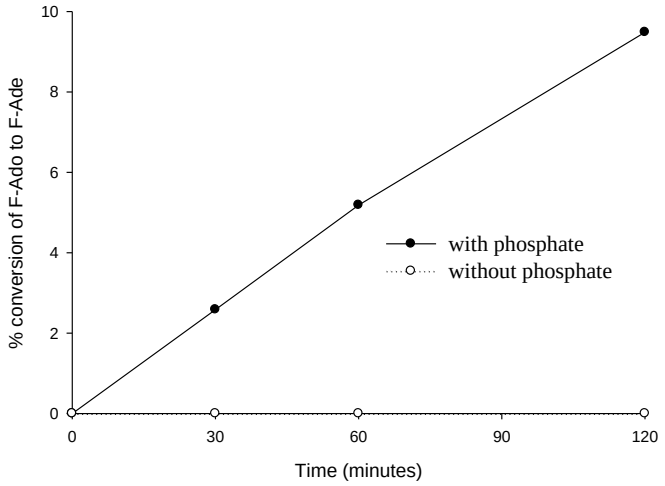

Figure 4. 2-Fluoro-adenosine cleavage in the presence or absence of phosphate. Partially purified *M. tuberculosis* Ado cleavage enzyme was incubated with F-Ado in the presence or absence of 50 mM phosphate and the formation of 2-fluoro-adenine was measured by reverse-phase HPLC.

In crude cell extract from *M. tuberculosis*, cleavage of F-Ado, Ino, Ado, and MTA was detected. Moreover, the MTA cleavage was phosphate-dependent, indicating that *M. tuberculosis* expressed MTAP. This experiment was repeated in the partially purified sample. Cleavage of Ado, F-Ado and Ino was detected, but no activity was seen with MTA, indicating that the Ado cleavage enzyme was different from MTAP (Figure 5). Since a partially purified sample was used, the Ino cleavage could be due to PNP, Ado cleavage enzyme, or both.

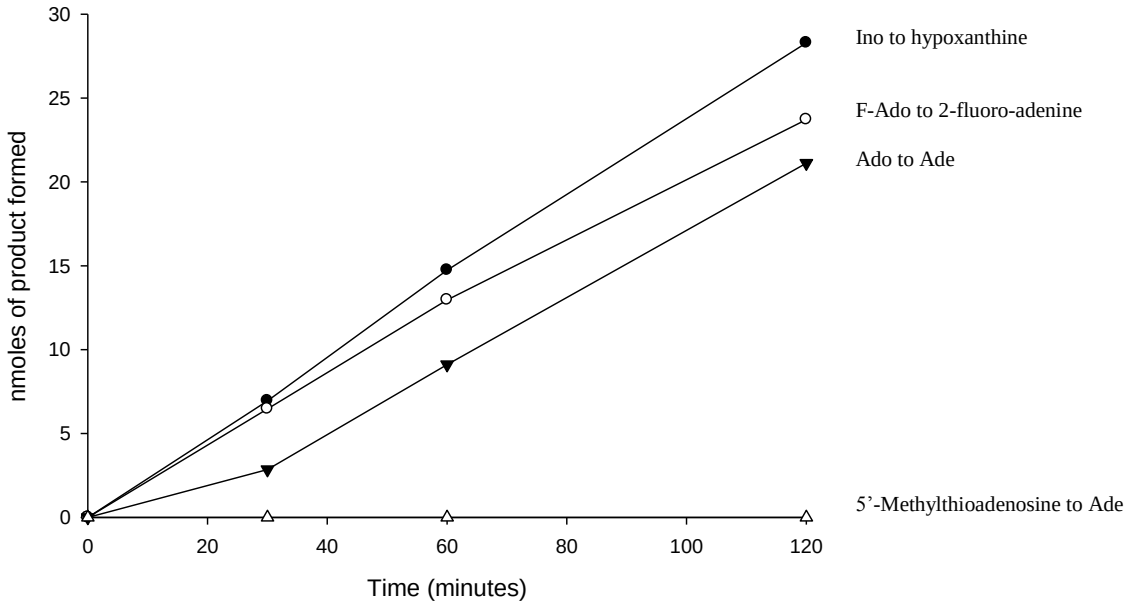

Figure 5. Substrate specificity of the *M. tuberculosis* Ado cleavage enzyme.
Cleavage of Ino, F-Ado, Ado, and MTA in a partially purified sample of *M. tuberculosis* Ado cleavage enzyme was measured by reverse-phase HPLC.

The preliminary results suggested that the Ado cleavage enzyme was a phosphorylase that was different from *M. tuberculosis* PNP or MTAP. While the two purification columns were useful, the identification of a single band responsible for this activity was not possible. After using two purification steps, the Ado activity decreased considerably, which prevented the subsequent use of other purification columns. One strategy would be to scale up the culture volume to obtain a larger cell pellet. However, this was not possible due to limits imposed by the internal biosafety committee at Southern Research Institute.

The approach was modified to use *M. smegmatis*, a fast-growing and non-pathogenic mycobacterium. Initial studies were conducted to verify whether *M. smegmatis* also expressed Ado cleavage enzyme(s). As shown in Figure 6 (A-C), F-Ado cleavage was observed suggesting the presence of Ado cleavage enzyme. In the first purification step (Figure 6A), there was a miscorrelation between Ino and F-Ado cleavage activities, suggesting the presence of two enzymes, namely PNP and Ado-cleavage enzyme. In fraction 26, Ino cleavage was three times higher than F-Ado activity. This could be due to contaminating PNP in that fraction or the fact that Ado cleavage enzyme could cleave both Ado and Ino. Fraction 15 from the Mono Q column (Figure 6B) contained mostly Ino activity, indicating the presence of PNP. This indicates that this fraction contains mostly PNP and that F-Ado is not a substrate for PNP. Fraction 17 from that same purification step had identical Ino and F-Ado cleavage. In the third purification step, no distinct Ino and F-Ado peaks were observed. Fraction 20 from the Superose gel filtration column was run on a denaturing SDS-PAGE gel which was then silver stained (data not shown). Since many protein bands were seen, it was concluded that the final

fraction was not pure enough for protein identification by mass spectrometry. The preliminary studies performed in *M. smegmatis* showed that similar to *M. tuberculosis*, *M. smegmatis* also expressed an Ado-cleavage enzyme.

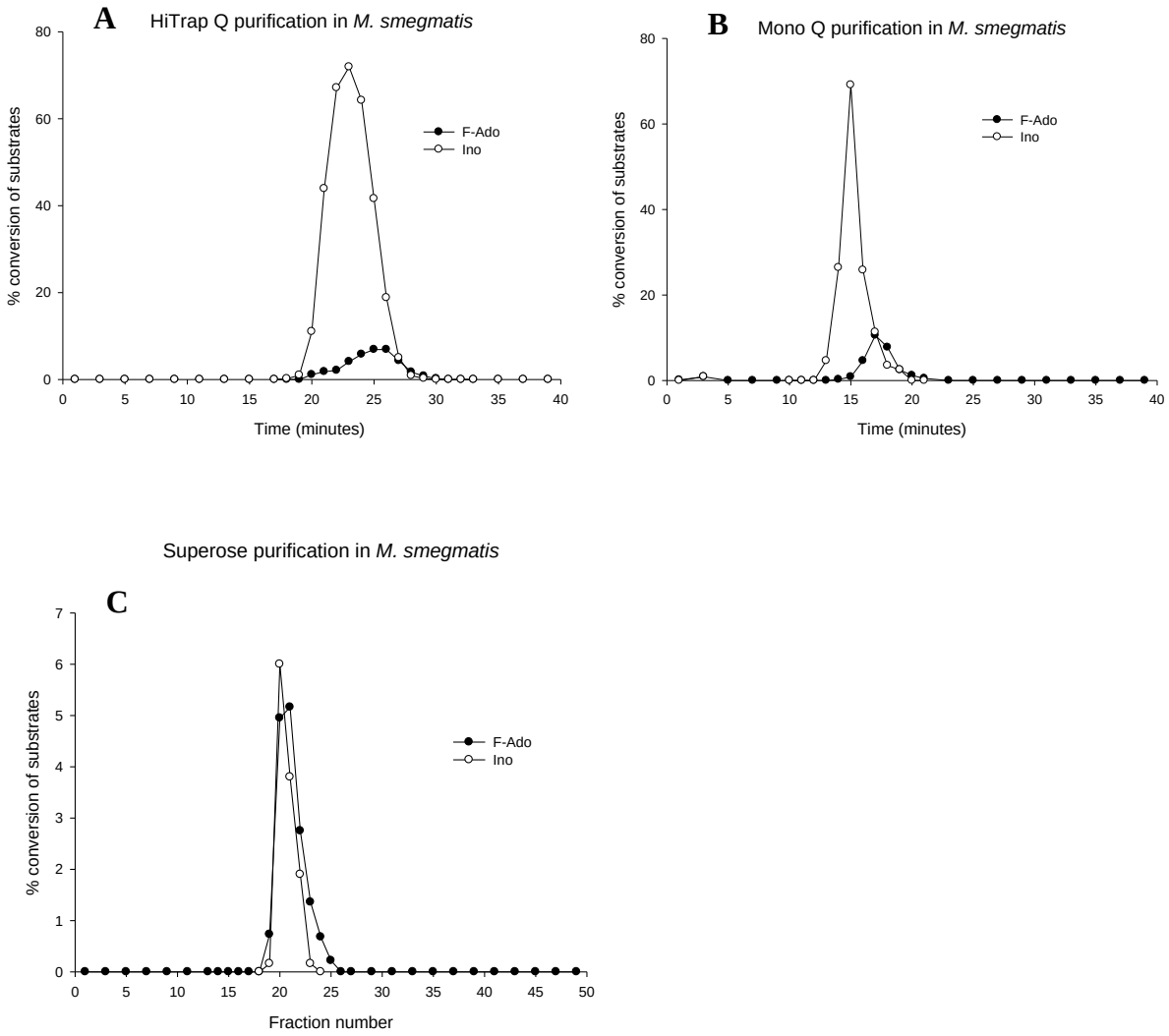

Figure 6. Purification of Ado cleavage activity from *M. smegmatis* crude cell extract. Elution profile of proteins from HiTrap Q anion exchange column (A) and Mono Q anion exchange column (B) using a linear salt gradient of 0-1M NaCl; elution profile of proteins from Superose gel filtration column using an isocratic gradient of 10 mM KCl (C).

After a three-step purification, the final active sample of Ado-cleavage enzyme still contained other proteins, indicating that the purification scheme needed to be modified. The main selection criterion for column selection was the ability to separate Ino cleavage from F-Ado cleavage activity. The column that had the most separation was chosen. Different hydrophobic interaction columns (HIC) were tested for their ability to separate F-Ado cleavage from Ino cleavage. The Phenyl HP HIC (GE Lifesciences) produced the best separation. The HiTrap Q and Mono Q columns are both anion exchange columns with similar resin. Since the HiTrap Q can bind to more protein and therefore allow the use of greater starting sample volume than the Mono Q column, the Mono Q column was retired. Buffers with various pH were also evaluated with the HiTrap Q anion exchange column and it was found that the optimal pH for this column was 7.6. A different gel filtration column, Superdex 200PG (GE Lifesciences), was calibrated and added to the new purification scheme (Figure 7).

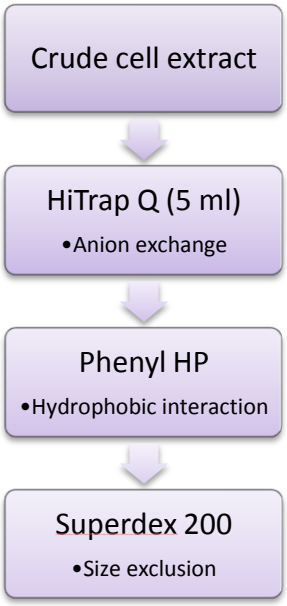

Figure 7. Purification scheme developed for the purification of Ado cleavage enzyme from *M. smegmatis* crude cell extract.

Aims of dissertation.

There are differences in the metabolism of Ado between human and mycobacterial cells. Previous studies have shown the cleavage of Ado in *M. tuberculosis*, whereas human cells do not readily cleave Ado. The identification of Ado cleavage enzyme(s) is important since this enzyme could be exploited in the drug development process. Thus, the overall aim of this dissertation is to identify and characterize the enzyme(s) responsible for the cleavage of Ado in mycobacteria.

Since there are a number of candidate enzymes that may cleave Ado in nature, purification of the endogenous enzyme was preferred over a genetic approach. Preliminary results showed that both *M. tuberculosis* and *M. smegmatis* expressed an Ado-cleavage activity. Therefore *M. smegmatis*, which is non-pathogenic and fast-growing, was used as the model to purify and identify the enzyme activity. This strategy identified two adenosine phosphorylases, one of which was MTAP, and the other was Ado-PNP, an Ado cleavage enzyme that was different from PNP (MSMEG_1701), MTAP (MSMEG_0990), and MTAN (MSMEG_1753). The biochemical properties of both endogenous *M. smegmatis* enzymes are presented.

Having identified MTAP in *M. smegmatis*, the subsequent aim was to characterize the *M. tuberculosis* homolog. Rv0535 had been annotated as a probable MTAP in *M. tuberculosis*. Therefore, Rv0535 was expressed in *E. coli* BL21 (DE3) cells, and the recombinant protein was purified and characterized.

Since *M. smegmatis* and *M. tuberculosis* MTAPs were the first bacterial MTAPs to have been studied, the substrate specificity of these enzymes was not known.

Therefore, a structure-activity relationship (SAR) study was carried out on purified Rv0535, to determine the effects of modifications to the ribose and/or purine base moieties of Ado and MTA on enzyme activity. It is hoped that the SAR will also enable the identification of differences between human and mycobacterial MTAP, and that these differences could be exploited in the discovery of new anti-TB drugs.

IDENTIFICATION AND CHARACTERIZATION OF TWO ADENOSINE PHOSPHORYLASE ACTIVITIES IN MYCOBACTERIUM SMEGMATIS

by

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Format adapted for dissertation

Abstract

Purine nucleoside phosphorylase (PNP) is an important enzyme in purine metabolism and cleaves purine nucleosides to their respective bases. Mycobacterial PNP is specific for 6-oxopurines and cannot account for the adenosine (Ado) cleavage activity that has been detected in *M. tuberculosis* and *M. smegmatis* cultures. In the current work, two Ado cleavage activities were identified from *M. smegmatis* cell extracts. The first activity was biochemically determined to be a phosphorylase that could reversibly catalyze adenosine + phosphate ↔ adenine + alpha-D-ribose-1-phosphate. Our purification scheme led to a 30-fold purification of this activity, with the removal of more than 99.9% of total protein. While Ado was the preferred substrate, inosine and guanosine were also cleaved, with 43% and 32% of the Ado activity, respectively. Our data suggest that *M. smegmatis* expresses two PNPs: a previously described trimeric PNP that can cleave inosine and guanosine only, and a second novel PNP (Ado-PNP) that can cleave Ado, inosine, and guanosine. Ado-PNP had a $K_{m,\ app}$ of 98 ± 6 μM (with Ado) and a native molecular mass of 125 ± 7 kDa. The second Ado cleavage activity was identified as 5'-methylthioadenosine phosphorylase (MTAP) based on its biochemical properties and mass spectrometry analysis. Our study marks the first report of the existence of MTAP in any bacterium. Since human cells do not readily convert Ado to Ade, an understanding of the substrate preferences of these enzymes could lead to the identification of Ado analogs that could be selectively activated to toxic products in mycobacteria.

Introduction

Mycobacterium tuberculosis is the etiological agent of tuberculosis (TB), an infectious disease that was diagnosed in more than 9 million individuals and claimed nearly 2 million lives in 2007 (34). An estimated one third of the world's population is infected with the latent form of the disease, and 10% of these people will develop active TB in their lifetime. In 2007, TB caused 23% of the estimated HIV deaths (34), and thus, as the global HIV burden and TB-HIV co-infections increase, TB remains a growing health concern. *M. tuberculosis* strains that are resistant to first and second line drugs are also on the rise. According to the March 2010 estimates of the World Health Organization, one in four new cases of TB in northwest Russia was Multiple Drug Resistant TB, and 58 countries reported at least one incidence of Extensively Drug Resistant TB (35). As drug resistant TB becomes more prevalent, the array of drugs available to treat this deadly bacterial infection decreases. Thus, there is an urgent need to develop new anti-tuberculosis drugs that have alternate mechanisms of action than current drugs.

An enhanced basic understanding of the enzymes involved in metabolic processes in mycobacteria could lead to identification of molecular targets for drug discovery. Purine metabolism is an essential process of all living cells as it generates macromolecules necessary for DNA, RNA, and energy production. Extensive studies of human purine metabolism have led to the development of nucleoside analogs that are currently used to treat cancer. Moreover, differences in purine metabolism between human cells and various infectious agents have been exploited to develop nucleoside analogs used in the treatment of parasitic and viral infections. Since differences in purine

metabolism between human and mycobacterial cells exist (20, 24), purine enzymes could be potential targets for the development of nucleoside analogs against TB. Moreover, because purine metabolism is not a target of current TB drugs, nucleoside analogs would likely be active against TB that is resistant to current agents. Furthermore, it is possible that nucleoside analogs could disrupt basic metabolic processes and thus be useful against latent TB.

Previous studies have shown the conversion of adenosine (Ado) to adenine (Ade) in *M. tuberculosis* and *M. smegmatis* cell cultures (9, 23). In human cells, Ado is primarily catalyzed by Ado kinase and Ado deaminase (Figure 1) and is not readily cleaved to Ade. Therefore, the ability of mycobacteria to cleave Ado indicates a difference in the metabolism of Ado in mycobacteria that could be exploited for drug discovery. There are numerous enzymes known in nature that can cleave Ado. Although most bacterial purine nucleoside phosphorylases (PNP, E.C 2.4.2.1) can cleave Ado to Ade, it is known that mycobacterial PNP does not accept Ado as a substrate (5, 11, 19). Some bacteria such as *Bacillus subtilis* and *Bacillus cereus*, express an Ado phosphorylase (AdoP) that selectively cleaves Ado (15, 29, 30). A few parasites can also salvage Ado through hydrolases such as Ado nucleosidase (E.C. 3.2.2.7) or purine nucleosidase (E.C. 3.2.2.1). Cleavage of Ado by parasitic 5'-methylthioadenosine phosphorylase (MTAP, E.C. 2.4.2.28) has been reported (13, 21). MTAP is primarily found in Eukarya including trypanosomes and Archaea (1, 14). Although *Pseudomonas* were thought to possess MTAP (1), recent work in *P. aeruginosa* showed that the enzyme was a methylthioinosine phosphorylase instead of MTAP (14). The bacterial equivalent of MTAP is 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN or

MTA/SAH nucleosidase, E.C 3.2.2.16). According to the UniProt Consortium (31), *M. tuberculosis* encodes for MTAN (Rv0091) and a probable MTAP (Rv0535). Thus, either MTAN or MTAP could be responsible for the Ado cleavage observed in *M. tuberculosis*.

Given that the literature indicates that a number of bacterial, parasitic, and mammalian enzymes can cleave Ado to Ade, we investigated Ado cleavage from cell extracts from *M. smegmatis*, a closely related, fast growing model of *M. tuberculosis*. Ado cleavage activity was assayed in all the fractions eluting from our first purification column. This step indicated that two enzymes could cleave Ado. One of the enzymes was identified as MTAP and the other was an Ado cleavage enzyme that was different from PNP (MSMEG_1701), MTAP (MSMEG_0990), and MTAN (MSMEG_1753). Hence, our work marks the first investigation of two previously uncharacterized Ado cleavage activities in mycobacteria and further illustrates differences in purine metabolism between mycobacterial and human cells. These differences could be exploited for the development of Ado analogs that could be selectively activated to toxic products, thereby exhibiting selective toxicity against *M. tuberculosis* and forming the basis of a new class of anti-TB drugs.

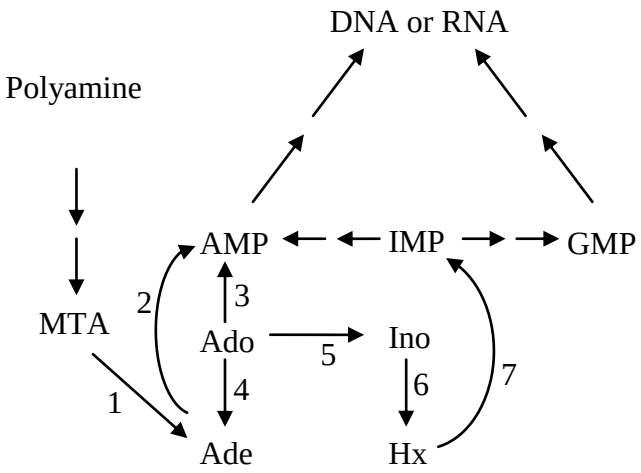

Figure 1. Enzymes involved in adenosine metabolism

Enzymes: (1) 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (not present in human cells) or 5'-methylthioadenosine phosphorylase; (2) adenine phosphoribosyl transferase; (3) adenosine kinase; (4) adenosine phosphorylases (not present in human cells) and most bacterial purine nucleoside phosphorylase; (5) adenosine deaminase; (6) purine nucleoside phosphorylase; (7) hypoxanthine/guanine phosphoribosyl transferase.

Abbreviations used are: Ade, adenine; Ado, adenosine; Hx, hypoxanthine; Ino, inosine; MTA, 5'-methylthioadenosine.

Materials and Methods

Reagents. The natural nucleosides, nucleobases, and ribose-1-phosphate were purchased from Sigma-Aldrich. 2-Fluoroadenosine, 2-methyladenosine (methyl-Ado) and 9-benzyl-9-deazaguanine (BDG) were synthesized at Southern Research Institute. Protease inhibitor cocktail was obtained from Sigma and Roche. Sodium dodecyl sulfate – 12% polyacrylamide gel electrophoresis (SDS-12% PAGE) minigels, Tris/Glycine/SDS running buffer, Coomassie stain, silver stain reagents, low range molecular weight standards, and Bradford dye reagent were purchased from Bio-Rad Laboratories. EZ-Run Pre-stained protein marker was purchased from Fisher Scientific. Xanthine Oxidase (from bovine milk) was purchased from Sigma-Aldrich. Purification columns and molecular weight standards for gel filtration chromatography were purchased from GE Healthcare.

Bacterial strain and growth conditions. *M. smegmatis* ATCC 700084 was cultured in Middlebrook 7H9 medium supplemented with oleic acid, albumin, dextrose, and catalase (OADC), and 0.05% Tween 80 at 37 ˚C in a shaking incubator until the OD_{600} was 0.4 – 0.6.

Preparation of crude protein extract. *M. smegmatis* cells (from a 5 L culture) were pelleted by centrifugation, and the cell pellet was washed three times in Buffer A (50 mM Tris HCl, 1 mM dithiothreitol, and 20% glycerol, adjusted to pH 7.6). The cell pellet was resuspended in Buffer A containing protease inhibitor cocktail (Sigma/Roche), and the cells were lysed using a French pressure cell. The resulting suspension was centrifuged at 4 ˚C for 1 hour at 100,000 × *g*. The supernatant was dialyzed overnight against two

changes of 1 L of Buffer A plus 150 mM NaCl at 4 ˚C (Spectra/Por 4 Membrane Tubing, 12,000 to 14,000 Dalton MWCO, Fisher Scientific) and filtered using a 0.2 µm pore – size filter.

Purification of Ado cleavage activity. A 17-ml aliquot of crude extract was applied to an anion exchange column (HiTrap Q) and eluted using a linear salt gradient of 150 to 750 mM NaCl in Buffer A. Fractions with the most Ado-PNP activity were pooled, and 10 ml of this solution was applied to a hydrophobic interaction column (HiTrap Phenyl). The activity was eluted using a reverse linear gradient from 1 to 0 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.5). Fractions with the most Ado-PNP activity were pooled and concentrated to 300 µl, 200 µl of which were loaded onto a size exclusion column (Superdex 200 PG) and eluted with an isocratic run of 150 mM NaCl in Buffer A. Similarly, MTAP or PNP activity eluting from the HiTrap™ Q column was pooled and purified as described above. Protein concentrations of each pooled sample and crude extract were obtained by the Bradford method (7) using bovine serum albumin as standard.

Activity Assays. Enzyme activity was followed by measuring the formation of product using reverse phase high performance liquid chromatography (HPLC). A reaction mix (50 µl) consisting of 50 mM potassium phosphate buffer (pH 7.5), 50 mM HEPES (pH 7.3), 100 µM substrate, and enzyme was prepared. The reaction was started by the addition of the enzyme. After incubation at 37 ˚C, the reaction was stopped at specific time points by the addition of 50 µl 1 M perchloric acid, and the mixture was neutralized and buffered to pH 7 with a solution containing 3 M KOH plus 0.6 M potassium phosphate buffer. The potassium perchlorate precipitate was removed by

centrifugation. For acid labile substrates such as 2'-deoxyadenosine and thymidine, the reaction was stopped by boiling for 5 minutes. For the reverse reaction, the reaction mix consisted of 200 µM ribose-1-phosphate, 50 mM HEPES (pH 7.3), 200 µM Ade, and enzyme. The substrates were separated from the products using reverse-phase HPLC with a BDS Hypersil C-18 column (Keystone Scientific) and a mobile phase consisting of ammonium dihydrogen phosphate and acetonitrile buffer as described previously (23). The substrates and products were detected as they eluted from the column by their absorbance at 260 nm.

The xanthine oxidase coupled spectrophotometric method of Savarese et al., (27) and Jensen and Nygaard (16) was modified to a 96-well plate format and was used to rapidly detect Ado-PNP, PNP, and MTAP in fractions eluting from purification columns. Ado and MTA cleavage produce Ade, which is then converted to 2, 8-dihydroxyadenine by xanthine oxidase, leading to an increase in absorbance at 305 nm. Similarly, mycobacterial PNP converts inosine (Ino) to hypoxanthine (Hx), which is then converted to uric acid by xanthine oxidase, with an increase in absorbance at 293 nm. Each well (200 µl total volume) contained 30 µl of sample, 50 mM potassium phosphate buffer (pH 7.5), 50 mM HEPES (pH 7.3), 0.01 units of xanthine oxidase, and substrate (2 mM Ado plus 100 µM deoxycoformycin, 100 µM MTA, or 2 mM Ino). The reaction was allowed to proceed at room temperature and the change in absorbance at 305 nm and 293 nm was measured over time.

Enzyme kinetics. $K_{m,\,app}$ and $V_{max,\,app}$ for nucleoside phosphorolysis and nucleoside synthesis were calculated using the nonlinear regression function of SigmaPlot 2004 (Systat Software, Inc.). The assays were carried out at varying

concentrations of one substrate and fixed concentrations of the second substrate. The substrate conversions were maintained below 10%. With Ado-PNP, Ado, Ino, Ade and ribose-1-phosphate concentrations were varied between 40 – 400 μM. Phosphate concentrations were varied between 250 – 5000 μM. With MTAP, the concentration of MTA was varied between 2 – 10 μM, and the concentration of Ado was varied between 120 – 600 μM. For both MTA and Ado, the phosphate concentration was kept at 50 mM.

NanoLC-tandem mass spectrometry. Mass spectrometry analysis was conducted at the Targeted Metabolomics and Proteomics Laboratory at the University of Alabama at Birmingham as described previously (25). Briefly, protein bands from SDS-PAGE gels were excised and destained. Following trypsin digestion, peptides were applied to a C18 reverse-phase cartridge, and the eluted peptides were analyzed on an Applied Biosystems-MDS-Sciex (Concorde, Ontario, Canada) 4000 Qtrap mass spectrometer. The tandem mass spectrometry data thus obtained was processed to provide protein identification using an in-house MASCOT search engine (Matrix Science) with the *M. smegmatis* NCBInr protein database. One missed cleavage site for trypsin was allowed in the analysis.

Results

Identification of Adenosine Cleavage Enzymes

Partial purification of Ado cleavage was achieved by applying a cell extract of *M. smegmatis* to the HiTrap Q anion exchange column. Two-ml fractions were collected as the proteins eluted with a linear gradient of 150 to 750 mM NaCl, and the fractions were tested for cleavage of Ado, Ino, and MTA by the xanthine oxidase method. Ino cleavage to Hx was seen with maximal activity in fraction 21 (Figure 2).

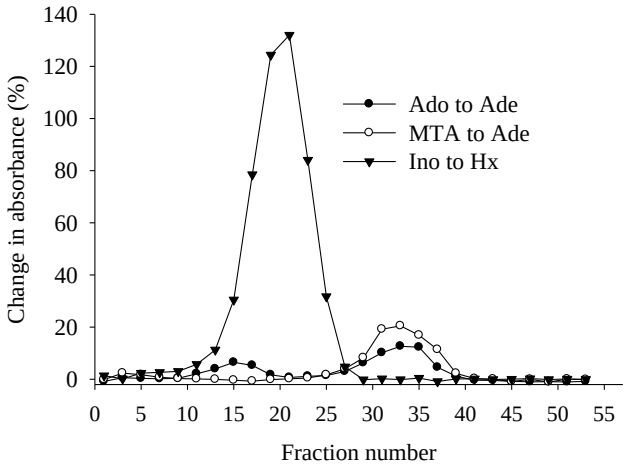

Figure 2. Elution of Ado, MTA, and Ino cleavage activities from *M. smegmatis* cell extract applied to the HiTrap Q anion exchange column. *M. smegmatis* cell extract was applied to the HiTrapQ anion exchange column and eluted with 150 – 750 mM NaCl gradient. Every other fraction was tested for Ade formation from Ado or MTA, and Hx formation from Ino by the xanthine oxidase assay. The formation of Ade and Hx is expressed as the percent change in initial absorbance at 305 nm (for Ado and MTA) and 293 nm (for Ino) after a 2 hour incubation with Ado, MTA, and Ino.

This fraction had negligible Ado cleavage, which indicated the presence of PNP, since mycobacterial PNP can cleave Ino but not Ado. Two peaks of Ado cleavage were detected, with maximal activity in fractions 15 and 33. MTA cleavage was also seen in fraction 33, suggesting that Ado cleavage in fraction 33 could be due to MTAP and/or MTAN. Fraction 15 had no MTA cleavage, and thus indicated the presence of a novel Ado cleavage activity that was different from PNP, MTAP, or MTAN.

Purification of Adenosine Cleavage Enzymes

Further purification of fraction 15 (Ado cleavage enzyme) was achieved by using a HiTrap™ Phenyl hydrophobicity interaction column and a Superdex 200 PG size exclusion column, successively. Superdex fractions were tested for Ado to Ino conversion, which would indicate the presence of Ado deaminase. Fractions that contained Ado cleavage activity, but not Ado deaminase activity, were pooled and used for further characterization experiments. Our purification scheme led to the removal of more than 99.9% of total protein and a 30-fold purification of the Ado cleavage activity (Table 1).

TABLE 1. Purification of Adenosine Phosphorylase from *M. smegmatis*

Step	**Total Protein** (mg)	**Enzyme activity** (nmol/mg-min)	**Purification** (fold)	**Total Activity** (nmol/min)	**Recovery** (%)
Cell extract	98	0.06	1	5.9	100
HiTrap Q	3.3	0.4	7	1.4	20
HiTrap Phenyl	0.034	1.9	32	0.056	1
Superdex 200 PG	ND[a]	– [b]	>32	0.021	0.4

[a]ND, not detected
[b] –, not determined

Although the resulting final fraction had Ado cleavage activity, the protein concentration of that sample could not be determined without using most of the sample, and a single band responsible for Ado cleavage activity could not be identified by Coomassie or silver staining the SDS-PAGE gel. However, since there was no evidence that the sample contained other purine enzymes that could affect substrate or product concentrations, that sample was suitable for further characterization experiments. PNP activity (corresponding to fractions 19-23) and MTA cleavage activity (corresponding to fractions 29-33) were also further purified by using the HiTrap Phenyl hydrophobicity interaction column followed by the Superdex 200 PG size exclusion column.

Characteristics of Adenosine Cleavage Activity

The cleavage of Ado seen in fraction 15 could be due to a hydrolase or a phosphorylase. To determine whether the Ado cleavage enzyme was a member of the hydrolase family, the purified enzyme was incubated in a reaction mix containing Ado, HEPES (pH 7.3) and water, but no phosphate. Ade formation was not detected under these conditions (Figure 3A), suggesting that another substrate or co-factor was needed for enzyme activity. When this reaction was repeated in the presence of 50 mM phosphate, Ade formation was seen, indicating that phosphate was required for activity. Additionally, Ado formation was observed when the enzyme was incubated with Ade and ribose-1-phosphate (Figure 3B). Taken together, these results indicated that the Ado cleavage activity in fraction 15 was due to a phosphorylase that reversibly catalyzed adenosine + phosphate to adenine + alpha-D-ribose-1-phosphate.

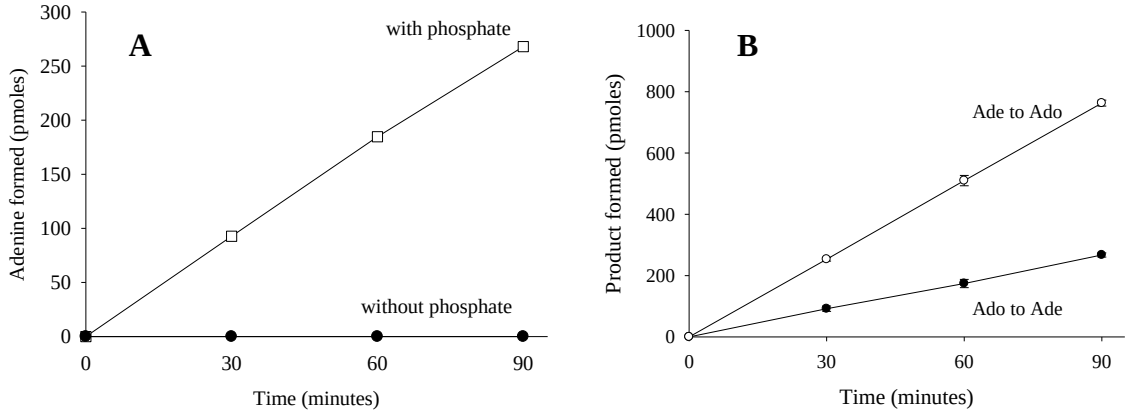

Figure 3. Ado cleavage requires phosphate and is reversible. In Panel A, fraction 15 was incubated with Ado in the presence or absence of 50 mM phosphate, and Ade formation was monitored by HPLC. In panel B, the enzyme was incubated with Ado and 50 mM phosphate and the formation of Ade was monitored by HPLC. The enzyme was also incubated with Ade and ribose-1-phosphate and the formation of Ado was monitored by HPLC. Each data point represents the mean ± standard deviation for 3 determinations. Some error bars are too small to be seen or hidden by the symbol.

To establish that the Ado cleavage activity was due to an enzyme other than PNP, MTAP, or MTAN, the activity of the Ado cleavage enzyme with the natural purine or pyrimidine nucleosides was evaluated. Ado cleavage enzyme accepted Ado, Ino, and guanosine as substrates, with Ado being the best substrate (Table 2). Other substrates included 2'-deoxyadenosine, 2-fluoroadenosine, and methyl-Ado with 58%, 137% and 31% of the Ado activity respectively. No activity was detected with xanthosine, pyrimidine nucleosides (cytidine, thymidine, and uridine), MTA, or S-adenosyl-L-homocysteine (SAH). Since MTAP can cleave MTA, and MTAN can cleave both MTA and SAH, our data suggested that the Ado cleavage enzyme was neither MTAP nor MTAN. Taken together, our data suggested that the Ado cleavage enzyme was specific

for purines and tolerated modifications at the 2'-position on the ribose moiety and the 2-position of the Ade moiety. Thus, our results indicated that the Ado cleavage enzyme is a member of the PNP family. Since a trimeric mycobacterial PNP that can cleave Ino and guanosine but not Ado has been described by others (5, 11, 19), we will refer to the Ado cleavage enzyme described in this work as Ado-PNP.

TABLE 2. Substrate specificity of *M. smegmatis* Ado-PNP

Nucleosides (100 µM)	**Enzyme Activity** [a] **pmoles min^{-1} ml^{-1}**
Purines	
Adenosine	19 ± 2
2'-Deoxyadenosine	11.28 ± 0.05
Inosine	8 ± 4
Guanosine	6 ± 1
Xanthosine	--
5'-Methylthioadenosine	--
S-Adenosyl-L-homocysteine	--
Adenosine Analogs	
2-Fluoroadenosine	26.3 ± 0.4
2-Methyladenosine	5.8 ± 0.5
Pyrimidines	
Cytidine	--
Thymidine	--
Uridine	--

[a] Mean ± standard deviation of at least 3 determinations

-- no activity detected

As shown in Table 2, both Ado and Ino were cleaved by Ado-PNP. Since *M. smegmatis* expressed high levels of PNP, the Ino cleavage seen in our Ado-PNP sample could be due to contaminating trimeric PNP. If Ado and Ino are both substrates for one enzyme, then Ado cleavage would be inhibited by the presence of Ino. Ado cleavage (100 µM) was inhibited by 45% by 1000 µM Ino (n=3), suggesting that both Ino and Ado are

substrates of Ado-PNP. Furthermore, 400 μM 9-benzyl-9-deazaguanine, a potent human PNP inhibitor (6, 22), inhibited Ino cleavage by *M. smegmatis* PNP by more than 75% (data not shown), but had no effect on either Ado or Ino cleavage by Ado-PNP. Taken together, these results indicated that Ado and Ino are both substrates of Ado-PNP.

The native molecular weight of the Ado-PNP was estimated by gel filtration chromatography on the Superdex 200 PG column. A calibration curve was produced based on the UV absorbance spectrum (generated from the Amersham-Pharmacia FPLC Controller LCC-501-Plus) of ferritin, conalbumin, carbonic anhydrase, and ribonuclease A molecular weight standards. Fractions eluting from the column were assayed for Ado cleavage using the HPLC assay. One peak of Ado cleavage activity was seen. Based on the elution volume of the fraction with maximum activity, the molecular mass of Ado-PNP was calculated to be 125 ± 7 kDa (n=6).

The stability of Ado-PNP at different temperatures was studied by incubating Ado-PNP at 4, 25, 37, or 60˚C for 3, 6, or 12 hours (data not shown). Ado-PNP activity was assayed at 37˚C after each of these incubations. Ado-PNP retained most of its activity at 4, 25, and 37˚C over 12 hours, but the enzyme was inactive after being incubated at 60˚C. To investigate the effect of pH on enzyme activity, Ado-PNP was incubated with Ado at different pH values which were obtained by adjusting a solution of citric acid and disodium phosphate buffer to pH values of 3-8 or by adjusting a solution of sodium diphosphate buffer to pH values of 7-11. The formation of Ade at each pH was measured by reverse-phase HPLC and the optimal pH of the reaction was between pH 6 and 7 (data not shown). The enzyme was inactive below pH 5. At pH 11, the enzyme had 44% of its activity at pH 7.

Kinetic properties of Ado-PNP

The apparent steady state kinetic constants of Ado-PNP were determined for Ado phosphorolysis and synthesis (Table 3). Substrate saturation curves were described by a hyperbolic function (data not shown), indicating that Ado-PNP followed Michaelis – Menten kinetics. Given that the protein concentration of Ado-PNP was not known, discrepancies in enzyme activity could exist between different batches of purified enzyme. Thus, to study the kinetic properties of Ado-PNP, the same batch of purified enzyme was used to calculate the $K_{m, app}$ and $V_{max, app}$ of Ado-PNP. The $K_{m, app}$ for Ado (98 ± 6 μM) was higher than that of Ade (43 ± 7 μM). As shown in Table 4, Ino had 43% of the Ado activity at 100 μM substrate. To determine whether the difference in activity was due to substrate binding or catalytic efficiency, the kinetic parameters for Ino cleavage were determined. The $K_{m, app}$ for Ino (220 ± 30 μM, n=3) was more than twice that of Ado. However, there was no difference in V_{max}/K_m between Ino and Ado. Thus the difference in Ado and Ino cleavage could be due to a difference in substrate binding.

TABLE 3. Apparent steady-state kinetic constants of *M. smegmatis* Ado-PNP

Substrate	**Co-substrate**	**K_m [a] (μM)**	**V_{max} [a] (pmoles min^{-1} ml^{-1})**
Nucleoside phosphorolysis			
adenosine	50 mM phosphate	98 ± 6	450 ± 10
phosphate	200 μM adenosine	700 ± 100	350 ± 20
Nucleoside synthesis			
adenine	200 μM ribose-1-phosphate	43 ± 7	1360 ± 80
ribose-1-phosphate	200 μM adenine	70 ± 10	1470 ± 90

[a] Mean ± standard deviation of at least 3 determinations

Characteristics of MTA cleavage enzyme

As shown in Figure 2, Ado could be cleaved by a second enzyme, which could also cleave MTA (fraction 33). An SDS-PAGE gel of fractions 29 to 37 had several protein bands, with one band that correlated with MTA cleavage activity (data not shown). This band from fraction 33 was excised from the gel. NanoLC-tandem mass spectrometry analysis suggested the presence of numerous proteins, two of which were MTAP and MTAN. Since sequence homology predicted that *M. smegmatis* expressed both MTAP and MTAN, it is possible that fraction 33 contained both MTAP and MTAN (the assay used could not differentiate between MTAP and MTAN activities). However, further purification of this fraction led to an enzyme that was only active in the presence of 50 mM phosphate (data not shown), thereby indicating the presence of a phosphorylase and not a nucleosidase.

M. smegmatis MTAP (purified as described above) was also tested for its ability to cleave purines and pyrimidines. MTAP was most active with MTA, and also cleaved Ado and 2-fluoroadenosine with 16% and 19% of the MTA activity, respectively (Table 4). No activity was detected with methyl-Ado, suggesting that MTAP was not tolerant of some modifications at the 2-position on the Ade moiety. Similar to human MTAP, cleavage of 2'-deoxyadenosine was not detected with *M. smegmatis* MTAP. SAH was not a substrate under the conditions tested, indicating that this enzyme was not MTAN. No activity was detected with Ino, guanosine, xanthosine, or the pyrimidine nucleosides. Based on the different substrate specificity for Ado and MTA, Ado-PNP and MTAP are different enzymes.

TABLE 4. Relative activity of *M. smegmatis* Ado-PNP, MTAP, and PNP

Nucleosides (100 µM)	**Relative Activity (%)**		
	Ado-PNP	**MTAP**	**PNP**
Purines			
Adenosine	100	16	--
2'-Deoxyadenosine	58	--	--
Inosine	43	--	100
Guanosine	32	--	68
Xanthosine	--	--	--
5'-Methylthioadenosine	--	100	--
S-Adenosyl-L-homocysteine	--	--	--
Adenosine Analogs			
2-Fluoroadenosine	137	19	--
2-Methyladenosine	31	--	--

-- no activity detected

Human MTAP was shown to cleave Ado with 12.5% of the activity seen with MTA (12). However, human MTAP had a K_m of 1.5 ± 0.2 µM with MTA and 760 ± 90 µM with Ado, which corresponds to a 500-fold higher K_m with Ado than with MTA (32). The V_{max} with Ado was 62% of the V_{max} with MTA, and therefore represented an 800-fold higher catalytic efficiency with MTA than with Ado (32). Hence, Ado is considered to be an inefficient substrate for human MTAP. Since Ado cleavage was observed with *M. smegmatis* MTAP, we investigated the kinetic properties of the mycobacterial enzyme with MTA or Ado as substrates in the presence of 50 mM phosphate. *M. smegmatis* MTAP had a $K_{m,\ app}$ of 1.6 ± 0.2 µM with MTA and 310 ± 40 µM with Ado, with a

similar V_{max} for both substrates thereby indicating that MTA was a more efficient substrate than Ado for *M. smegmatis* MTAP.

Using the same size exclusion column and its calibration curve discussed above, the native molecular mass of MTAP was calculated to be 64 ± 5 kDa. Since the MSMEG_0990 amino acid sequence predicted a protein of 27 kDa, our data suggested that MTAP expressed in *M. smegmatis* was a dimer. All previously described MTAP, including human MTAP, are known to be active as trimers (2, 26) with the exception of *Sulfolobus solfataricus* MTAP, which is a homohexamer with a molecular mass of 160 kDa (3).

Substrate specificity of PNP

To compare our Ado-PNP and MTAP results to *M. smegmatis* PNP, the substrate specificity of *M. smegmatis* PNP (purified as described above) was studied. Ino was the best substrate for PNP, which also accepted guanosine with 68% of the Ino activity (Table 4). No activity was detected with Ado, 2'-deoxyadenosine, methyl-Ado, 2-fluoroadenosine, MTA, xanthosine, SAH, or the pyrimidine nucleosides. This is consistent with previous reports that mycobacterial PNP is specific for 6-oxopurines (5, 11, 19). Further, the molecular mass of PNP was estimated to be 76 kDa, which is consistent with the description of trimeric *M. tuberculosis* PNP (5, 8). Taken together, our findings indicated that Ado-PNP, MTAP, and PNP are different enzymes.

Discussion

Ado cleavage to Ade is common in bacteria and parasites. Most bacterial PNPs can cleave both 6-aminopurine and 6-oxopurine nucleosides, but *M. tuberculosis* PNP is known to only accept 6-oxopurines (5, 11, 19). In this work, we identified two enzyme activities in *M. smegmatis* that could cleave Ado and these activities were biochemically determined to be Ado-PNP and MTAP. Either enzyme could account for previous reports of Ado cleavage in *M. tuberculosis* or *M. smegmatis* cultures (9, 23). However, because MTAP did not cleave methyl-ado, only Ado-PNP could be responsible for the methyl-Ado cleavage observed in cell cultures. The $K_{m,\ app}$ for Ade and ribose-1-phosphate with *M. smegmatis* Ado-PNP was determined to be 43 and 70 µM, respectively, which is similar to the K_m for Ade and ribose-1-phosphate with *E. coli* PNP (16) and *B. cereus* AdoP (30). Our kinetic studies showed that Ado-PNP has a lower $K_{m,\ app}$ for Ade than Ado, suggesting that *in vitro* the formation of Ado from Ade is favored. However, as with the other phosphorylases the equilibrium *in vivo* is predicted to be in favor of Ado cleavage due to the rapid utilization of adenine and ribose-1-phosphate by other enzymes.

According to Bzowska et al., PNPs can be classified in three groups based on their substrate specificity and quaternary structure: low molecular mass homotrimers, high molecular mass homohexamers, and others (8). Similar to Bzowska's classification, Pugmire and Ealick have grouped PNPs based on their quaternary structure, and attribute trimeric PNPs to mammals and hexameric PNPs to bacteria, with a few exceptions (26). The authors also mention that some bacteria, such as *E. coli*, express both a trimeric PNP and a hexameric PNP. In *E. coli*, hexameric PNP (encoded by *deoD*) cleaves Ado, Ino, and guanosine (8, 16, 26), whereas the trimeric PNP could cleave all purine nucleosides

except for Ado and 2'-deoxyadenosine (10, 28). This trimeric PNP is often referred to as xanthosine phosphorylase. Similar to *E. coli*, *B. subtilis* and *B. cereus* also express two nucleoside phosphorylases namely inosine-guanosine phosphorylase and AdoP (15, 29, 30). Sgarrella et al., described an Ado specific phosphorylase (AdoP) in *B. cereus*, and have shown that AdoP could cleave Ado and 2'-deoxyadenosine (30). Ino could also be cleaved by this enzyme with 1.5% of the relative activity of Ado, and no activity was observed with guanosine, xanthosine, or the pyrimidine nucleosides. Utagawa et al., have shown that *Enterobacter aerogenes* has a PNPase activity responsible for the cleavage of Ino at 34% of the relative cleavage of Ado (33). Even though the Ado cleavage enzyme described in our study had a preference for Ado, the enzyme also cleaved Ino and guanosine and therefore the enzyme is not AdoP but rather a PNP (purine nucleoside: orthophosphate ribosyltransferase, E.C. 2.4.2.1). Accordingly, our work suggests that *M. smegmatis* possesses two PNPs: the trimeric PNP (MSMEG_1701) which is an inosine-guanosine phosphorylase and Ado-PNP, which is similar in substrate specificity to hexameric *E. coli* PNP.

Analysis of the amino acid sequence of Ado-PNP could determine the similarity of Ado-PNP to the trimeric PNP as well as provide the basis for a phylogenetic relationship between the two PNPs. The low expression of Ado-PNP in mycobacteria was a significant hurdle in this work, and unfortunately, we have not yet been able to identify the gene that encodes for this enzyme. The purification scheme used in this work yielded an Ado-PNP sample that could be biochemically analyzed, because it was free of other purine enzymes. However, the protein concentration of our purified preparation was very low, and we were not able to identify a single band in an SDS-PAGE gel stained

with either Coomassie or silver stain reagent. Therefore, more work is needed to identify the gene responsible for this activity. A number of genes that could cleave purine and pyrimidine nucleosides have been identified in the *M. tuberculosis* genome by sequence homology. Our Ado-PNP substrate specificity results indicated that Ado-PNP was not encoded by PNP, MTAP, or MTAN. Although the enzymes expressed by *Rv0535(MTAP)* and *Rv0091(MTAN)* are only tentatively identified, we have cloned and expressed these two genes and have shown that they do express MTAP and MTAN, respectively (unpublished results). Rv3393 has been identified as a probable nucleoside hydrolase (iunH) by sequence homology. We have also cloned and expressed this gene and have shown that it is able to hydrolytically cleave uridine, but not Ado (unpublished results). Finally, Rv2293c has been annotated as encoding for a conserved hypothetical protein that participates in the nucleoside metabolic process and could be membrane bound. The PHYRE server was used to generate the predicted secondary structure of Rv2293c, which was then compared against a library of known protein structures (17). While the sequence identity was low, the PHYRE server nonetheless predicted Rv2293c to be a member of either the purine and uridine phosphorylase or the hydrolase superfamily. Although we have attempted to clone and express this enzyme, we have not yet been successful.

The importance of expressing two PNPs in mycobacteria is not known. Mycobacteria could express Ado-PNP, even in low amounts, to supplement PNP, MTAP, and MTAN activities to maximize purine salvage and complement *de novo* purine synthesis. In the current experiments, PNP activity was much greater than Ado-PNP activity. In our previous studies we have noted variable expression of Ado cleavage in mycobacterial cell cultures (9, 23), which suggests that Ado-PNP expression may be

under the control of an unknown variable. Ado-PNP could be important in sensing and regulating Ado and Ade concentrations, which in turn could affect the expression of the other enzymes shown in Figure 1. Ado-PNP could be expressed in mycobacteria in response to exogenous Ado from macrophages or to salvage the ribose moiety as a carbon source.

Our work suggests that MTAP could play a role in the metabolism of Ado in *M. smegmatis*. Although MSMEG_0990 and Rv0535 have been annotated as a probable MTAP in *M. smegmatis* and *M. tuberculosis* respectively, the current work describes the first characterization of a bacterial MTAP. *Pseudomonas* were thought to possess MTAP (1), however, recent work in *P. aeruginosa* showed that the enzyme was actually a methylthioinosine phosphorylase instead of MTAP (14). Our preliminary characterization of *M. smegmatis* MTAP indicated that mycobacterial MTAP may be different from human MTAP. Unlike human MTAP which is a trimer, *M. smegmatis* MTAP may be a dimer. Similar to the human enzyme, our kinetic data indicated that MTA was a much better substrate than Ado. However, *M. smegmatis* MTAP had more than a 2-fold lower K_m with Ado compared to human MTAP, suggesting that Ado was a better substrate for *M. smegmatis* MTAP than for human MTAP. Therefore, it is possible that mycobacterial MTAP could selectively activate Ado analogs.

A sequence alignment showed more than 92% sequence identity (196 identical amino acid positions) between MTAP from *M. smegmatis* and *M. tuberculosis*, which suggests that the two mycobacterial MTAPs will likely share similar properties. In contrast, there was less than 36% sequence identity (93 identical amino acid positions) between *M. tuberculosis* and human MTAP, which predicts that mycobacterial and

human MTAP may differ in substrate specificity and other properties. We have cloned and expressed the *Rv0535* gene, which is predicted to express a probable MTAP in *M. tuberculosis* and have shown that it is indeed MTAP (unpublished results).

Purine metabolism is an attractive target for TB drug discovery since a purine-based drug would provide a novel mechanism of action and therefore MDR and XDR resistant *M. tuberculosis* strains would likely be sensitive to Ado analogs. Further, Barrow et al., have shown that methyl-Ado was toxic to latent mycobacteria (4), indicating that an Ado analog could work against both active and latent TB infections. Therefore, our discovery of two adenosine cleaving activities in mycobacteria could contribute to an anti-TB drug development effort. Since human cells do not express Ado-PNP, these enzyme activities in mycobacteria could be exploited to selectively activate a non-toxic Ado or MTA analog to a toxic product in mycobacterial cells. The differences in substrate specificity between human and parasitic MTAP have been investigated for anti-parasitic drug development (18) and these studies have shown 2'-deoxyadenosine, 3'-deoxyadenosine, and 2', 3'-dideoxyadenosine are poor substrates for human MTAP while *Trypanosome brucei brucei* MTAP can cleave these substrates (13). Similarly, a complete structure-activity relationship analysis with these two enzymes could reveal differences in substrate preferences that could be exploited for anti-tuberculosis drug development.

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IDENTIFICATION OF RV0535 AS 5'-METHYLTHIOADENOSINE PHOSPHORYLASE FROM *MYCOBACTERIUM TUBERCULOSIS*

by

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In press for Tuberculosis

Abstract

5’-methylthioadenosine (MTA) is a natural purine that is metabolized by methylthioadenosine phosphorylase (MTAP, E.C 2.4.2.28) in Eukarya and Archaea but generally not in bacteria. In this work, Rv0535, which has been annotated as a probable MTAP in *Mycobacterium tuberculosis,* was expressed in and purified from *Escherichia coli* BL21 (DE3). The purified protein displayed properties of a phosphorylase and MTA was the preferred substrate. Adenosine and S-adenosyl-L-homocysteine were poor substrates and no activity was detected with 5’-methylthioinosine, the other natural purines, or the natural pyrimidines. Kinetic analysis of *M. tuberculosis* MTAP showed that the K_m value for MTA was 9.1 µM. Rv0535 was estimated as a 30 kDa protein on a denaturing SDS-PAGE gel, which agreed with the molecular mass predicted by its gene sequence. Using gel filtration chromatography, the native molecular mass of the enzyme was determined to be 60 ± 4 kDa, and thus indicated that *M. tuberculosis* MTAP is a dimer. Differences in active site between mycobacterial and human MTAPs were identified by homology modeling based on the crystal of the human enzyme. A complete structure-activity relationship analysis could identify differences in substrate specificity between the two enzymes to aid in the development of purine-based, anti-tuberculosis drugs.

Introduction

One third of the world's population is infected with *Mycobacterium tuberculosis*, the causative pathogen of tuberculosis (TB). As the second leading infectious disease in the world, TB causes approximately 2 million deaths every year (6, 25). TB remains a global health emergency because of the rising number of infections with Multiple Drug Resistant (MDR) and Extensively Drug Resistant (XDR) strains of the mycobacterium, increasing HIV co-infections, the lack of an effective vaccine that protects both children and adults, and poor patient compliance with current drug regimens. To eradicate this deadly disease, there is an urgent need to develop a new class of drugs that has an alternate mechanism of action than current drugs to treat active, latent, and drug-resistant TB. Rationally designed nucleoside analogs could fulfill the above criteria because initial studies on nucleoside analogs have shown cytotoxicity against active and latent mycobacteria. While most nucleoside analogs are currently used to treat cancer and viral infections, they are not used against TB and therefore, resistance to nucleoside analogs is unlikely initially. Thus, to aid in the development of nucleoside analogs against TB, it is important to identify and understand differences between mycobacterial and human purine metabolic enzymes.

Methionine is an essential amino acid that acts as the initiator amino acid during protein synthesis. It can be converted to S-adenosylmethionine (SAM) which is important for transmethylation reactions (1, 14). Spermidine synthase uses decarboxylated SAM to produce polyamines (Figure 1A), and in the process releases 5'-methylthioadenosine (MTA), a natural purine that can inhibit spermine synthase, spermidine synthase, and mammalian S-adenosyl-L-homocysteine (SAH) hydrolase (15, 17, 9). In bacteria, MTA

levels are kept low by MTA /SAH nucleosidase (MTAN, E.C 3.2.2.16), which hydrolyzes the glycosidic bond of MTA to produce adenine (Ade) and 5'-methylthioribose. Ade participates in the purine salvage pathway to produce Ade nucleotides while 5'-methylthioribose is phosphorylated by a kinase to 5'-methylthioribose-1-phosphate, which can be used in the methionine salvage pathway. In Eukarya, including trypanosomes, and Archaea, MTA phosphorylase (MTAP, E.C 2.4.2.28) cleaves MTA in the presence of phosphate to produce Ade and 5'-methylthioribose-1-phosphate (Figure 1B) (1, 8). Although MTAP had been described in *Pseudomonas aeruginosa* (1), a recent study showed that the enzyme was 5'-methylthioinosine phosphorylase (MTIP) instead of MTAP (8). Thus, it is believed that MTAP is not present in bacteria.

However, we have previously shown that unlike other bacteria, *Mycobacterium smegmatis* expressed a phosphorylase that could cleave MTA, thereby marking the first report of a bacterial MTAP (3). According to Uniprot, *M. smegmatis* MTAP shares 196 amino acids (75% identity) with *Mycobacterium tuberculosis* Rv0535 (GenBank Accession no. **O06401)**, which has been annotated as a probable MTAP (23). In order to prove the identity as Rv0535 as encoding MTAP and to investigate its biochemical properties, Rv0535 was expressed in and purified from *Escherichia coli* BL21 (DE3) cells. Biochemical analysis of the recombinant gene product allowed the definitive assignment of Rv0535 as the MTAP gene in *M. tuberculosis*. Furthermore, our initial studies indicated differences between human and mycobacterial MTAPs that could be exploited to design a nucleoside analog that can be cleaved to a toxic product by the mycobacterial enzyme only, leading to selective toxicity against mycobacterial cells. A

greater understanding of the differences between the two enzymes could aid in a TB drug discovery effort.

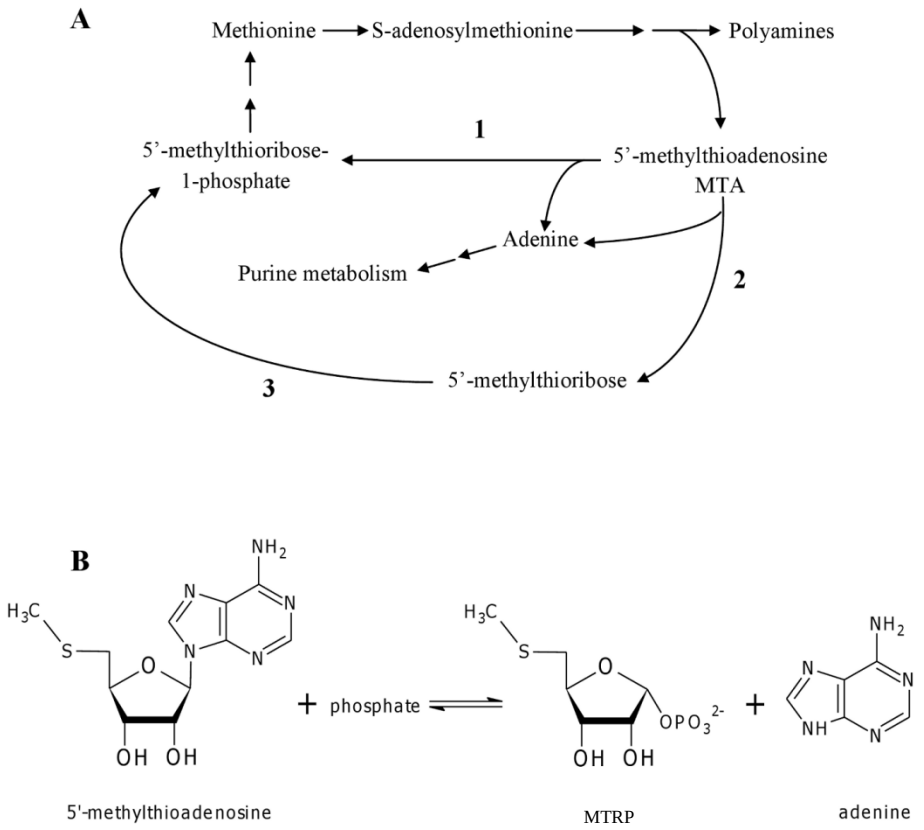

Figure 1. (A) Metabolism of MTA. Reaction 1 is usually catalyzed by MTAP from Eukarya and Archaea, whereas bacteria recycle MTA via Reaction 2 (by MTAN) and Reaction 3 (by methylthioribose kinase). (B) MTAP catalyzes the reversible phosphorolysis of MTA to Ade and ribose-1-phosphate.

Materials and Methods

Reagents. The natural nucleosides, nucleobases, and ribose-1-phosphate were purchased from Sigma Aldrich. 5'-Methylthioinosine and MT-DADMe-Immucillin A were gifts from Dr. Vern Schramm (Albert Einstein College of Medicine of Yeshiva University). Complete EDTA-free Protease inhibitor tablets were purchased from Roche. Sodium dodecyl sulfate – 12% polyacrylamide gel electrophoresis (SDS-12% PAGE) minigels, Tris/Glycine/SDS running buffer, Coomassie stain, Silver Stain Plus reagents, Precision Plus All Blue Protein™ standards, and Bradford dye reagent were purchased from Bio-Rad Laboratories. EZ-Run™ Pre-stained protein marker was purchased from Fisher Scientific.

Cloning of the *Rv0535* gene from *M. tuberculosis* H37Rv genomic DNA. The *Rv0535* gene was amplified by PCR from strain H37Rv genomic DNA, using the high fidelity DNA polymerase Dynazyme EXT (Finnzymes, Inc., MA), and NdeI primer (5'-AATTCATATGATGCACAACAATGGGCGCATG-3') and BamH1 primer (5'-ATTAGGATCCTCATGGCAGCTCGAACGGCAA-3'). The PCR product was inserted into the Nde1/BamH1 site of the pET28a (+) expression vector (Novagen/EMD Chemicals Inc., CA). The entire coding sequence in the recombinant vector was verified by automated DNA sequencing to confirm the identity, integrity, and absence of PCR-introduced mutations in the cloned fragment.

Protein expression and purification. The resulting recombinant vector was transformed into *E. coli* BL21 (DE3) (Novagen) competent cells. A single colony of transformed cells was selected from an Luria-Bertani (LB) agar plate containing kanamycin (50 µg/ml), and grown aerobically in 10 ml of LB media supplemented with

kanamycin (50 µg/ml) at 37 °C. The resulting culture was transferred into 1.2 L of the same media and grown at 24 °C until the A_{600} reached 0.6 – 0.8. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was grown aerobically at 37 °C for 5 hours. The cells (6g) were then harvested by centrifuging for 20 min at 12,227 × *g* at 4 °C, and the cell pellet was rinsed twice with Buffer A (20 mM Tris-HCl, 300 mM NaCl, and 20 mM imidazole, pH 7.5). The pellet was resuspended in 20 ml Buffer A containing one Complete EDTA-free protease inhibitor tablet. The cell suspension was lysed using a French pressure cell and then centrifuged for 1 hour at 100,000 × *g* at 4 °C. The clarified supernatant was collected and applied to a pre-equilibrated nickel affinity column (HisTrap HP, GE Healthcare). The column was washed with 5 column volumes of Buffer A, and the proteins were eluted using a linear imidazole gradient of Buffer A to Buffer B (20 mM Tris-HCl, 300 mM NaCl, and 500 mM imidazole, pH 7.5). Fractions with the most MTA cleavage were pooled and dialyzed overnight against two changes of 1 L of Buffer C (50 mM Tris HCl, 150 mM NaCl, 1 mM dithiothreitol, and 20% glycerol, pH 7.6) at 4 ˚C (Spectra/Por 4 Membrane Tubing, 12,000 to 14,000 Da MWCO, Fisher Scientific). Protein concentrations were obtained by the Bradford method (2) using bovine serum albumin as standard.

Activity Assays. Enzyme activity was followed by measuring the formation of product using reverse phase high performance liquid chromatography (HPLC) as described previously (3). The xanthine oxidase coupled spectrophotometric method of Savarese et al., (19) and Jensen and Nygaard (10) was modified to a 96-well plate format

and was used to rapidly detect MTA cleavage in fractions eluting from the purification column as described previously (3).

Enzyme kinetics. Steady-state kinetic constants were determined by varying the concentration of one substrate at fixed saturating concentration of the co-substrate. The amount of product formed was measured by reverse-phase HPLC and the substrate conversions were maintained below 10%. The data was fitted using the nonlinear regression function of SigmaPlot 2004 (Systat Software, Inc).

NanoLC-tandem mass spectrometry. Mass spectrometry analysis was conducted at the Targeted Metabolomics and Proteomics Laboratory at the University of Alabama at Birmingham as described previously (16). Briefly, the protein band from an SDS-PAGE gel was excised and destained. Following trypsin digestion, peptides were applied to a C18 reverse-phase cartridge, and the eluted peptides were analyzed on an Applied Biosystems-MDS-Sciex (Concorde, Ontario, Canada) 4000 Qtrap mass spectrometer. The tandem mass spectrometry data thus obtained was processed to provide protein identification using an in-house MASCOT search engine (Matrix Science) with the *M. tuberculosis* NCBInr protein database. The search parameters accounted for the oxidation of methionine residues, and a fixed carbamidomethylation of cysteines. One missed cleavage site for trypsin was allowed.

Results

Expression and purification of recombinant Rv0535

The Rv0535 sequence was amplified by PCR and the product was ligated into the pET28a (+) expression vector. The recombinant plasmid was then introduced into *E. coli* BL21 (DE3) competent cells. After induction with IPTG, the cells were harvested by centrifugation and lysed using a French pressure cell. The cytoplasmic fraction of the cells was loaded onto a denaturing SDS-PAGE gel and protein bands were visualized with Coomassie stain. As shown in Figure 2, after induction with IPTG, a new protein band that corresponded with the predicted monomer size of Rv0535 (30.276kDa, with histidine tags) was detected. The new protein band was excised from the gel and analyzed by NanoLC-tandem mass spectrometry, which identified the protein as Rv0535 (with 68% sequence coverage).

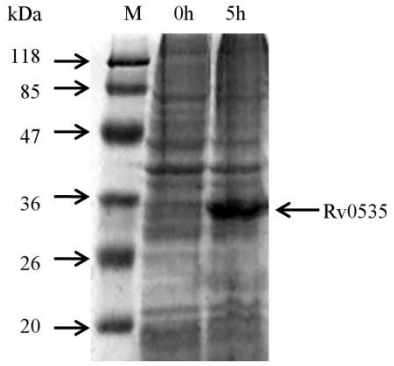

Figure 2. SDS-PAGE (12%, Coomassie stain) analysis of crude cell extracts from *E. coli* BL21 (DE3) cells before and after Rv0535 induction with IPTG. M, EZ-Run Pre-stained protein marker.

The pET28a (+) expression vector yielded recombinant Rv0535 that was fused to His-Tag at the N-terminus. Thus, a nickel affinity column (HisTrap HP) was used to purify the recombinant protein, after its expression had been induced with IPTG in *E. coli* BL21 (DE3) cells. The cytoplasmic fraction of the cells was applied to the purification column that was attached to an automated FPLC system. The column was washed with 5 column volumes of Buffer A, after which the concentration of imidazole was increased linearly, and one-ml fractions were collected. Fractions (including column flow-through and wash fractions) were tested for their ability to cleave MTA to Ade using the xanthine oxidase assay. Two peaks of MTA cleavage were seen. The first peak was in the flow-through and wash fractions and the second peak was in fractions 20-30 (Figure 3).

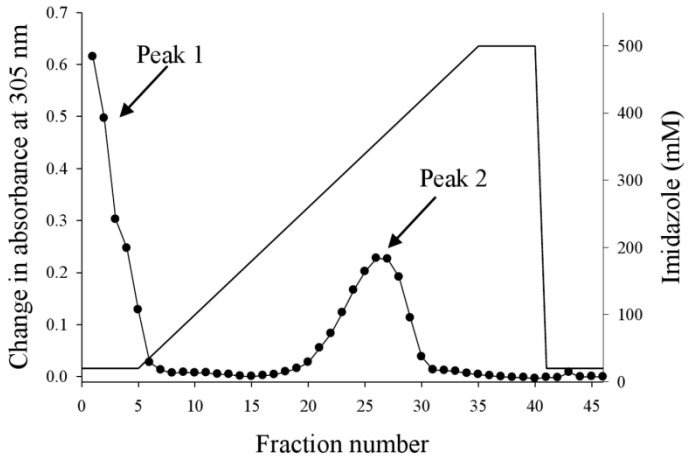

Figure 3. Elution profile of Rv0535 from the HisTrap HP column. Crude cell extract from *E. coli* BL21 (DE3) cells expressing Rv0535 was applied to the nickel affinity column and eluted with 20 – 500 mM imidazole gradient. Each fraction was tested for MTA cleavage, and the formation of Ade was measured by the change in absorbance at 305 nm after a 3-h incubation with MTA.

MTAP can only cleave MTA in the presence of phosphate, whereas MTAN can hydrolytically cleave MTA in the absence of phosphate. To determine whether peak 2 (fractions 20-30) contained MTAP, fractions 20-30 were pooled and dialyzed, after which MTA cleavage was assayed in the presence or absence of 50 mM phosphate. MTA to Ade conversion was measured by HPLC. As shown in Figure 4A, MTA cleavage was only seen in the presence of 50 mM phosphate. To further investigate whether fractions 20 -30 contained a phosphorylase, the forward and reverse reactions were assayed. Since methylthioribose-1-phosphate is not commercially available, this experiment was performed with 1 mM Ado plus 50 mM phosphate or 1 mM Ade plus 1 mM ribose-1-phosphate. Both Ado phosphorolysis and synthesis were observed (Figure 4B), indicating that the cleavage of Ado is reversible. Because inosine (Ino) cleavage was not observed in the purified sample (see substrate specificity below), *E. coli* PNP cannot account for the phosphorolytic cleavage or synthesis of Ado observed in our experiments. Similar to the formation of Ado from Ade and ribose-1-phosphate, we anticipate that this sample (fractions 20-30) can catalyze the formation of MTA from Ade and methylthioribose-1-phosphate. Taken together, these results indicated that the MTA cleavage activity in peak 2 was due to a phosphorylase.

Peak 1 contained the column flow through and wash fractions, suggesting the presence of a second MTA cleavage enzyme that did not bind to the HisTrap column. When the phosphate requirement of these fractions was analyzed, MTA cleavage was observed in the absence of phosphate, indicating the presence of a hydrolase (data not shown). According to sequence homology, the BL21 (DE3) strain of *E. coli* is expected

to express an MTAN, and therefore peak 1 is due to the elution of endogenous *E. coli* MTAN that did not bind to the HisTrap column.

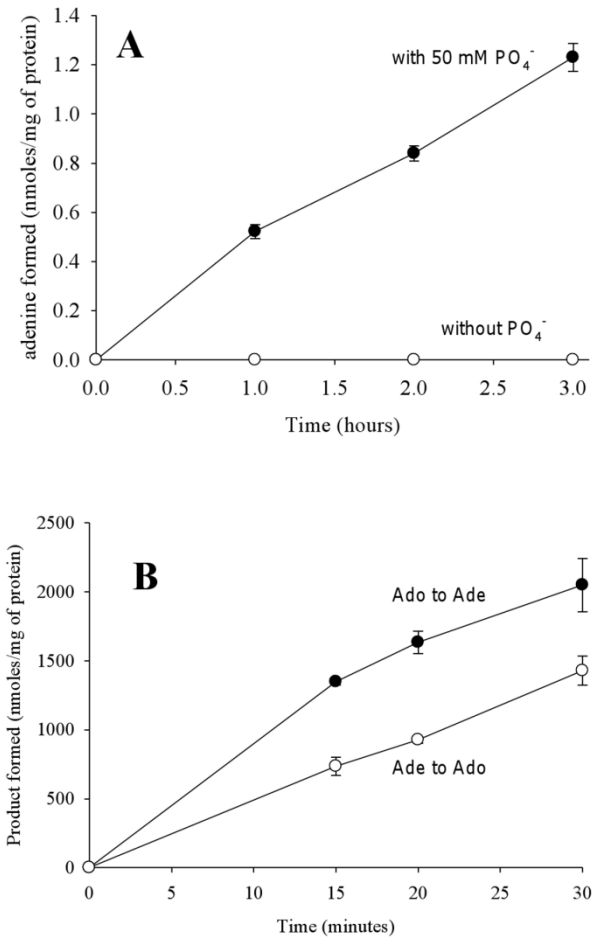

Figure 4. Ade formation requires phosphate and is reversible. (A) Purified Rv0535 was incubated with MTA in the presence or absence of 50 mM phosphate and Ade formation was monitored by HPLC. (B) The phosphorolysis and synthesis of Ado by Rv0535 was monitored by measuring the formation of Ade and Ado respectively by HPLC. Each data point represents the mean ± standard deviation for 3 determinations.

Purified Rv0535 (peak 2) was loaded and visualized on a silver stained, SDS-PAGE gel (Figure 5), which revealed one major band corresponding to the predicted monomer size of Rv0535. As shown in Table 1, our one-step purification scheme led to a 46-fold purification, with the removal of more than 99.9% of total protein. Table 1 also showed that only 5% of the total activity was recovered. While total activity represents MTA cleavage, the value in the crude cell extract does not discriminate between the contributions from endogenous *E. coli* MTAN or recombinant Rv0535. There was no difference in MTA cleavage in the crude cell extract in the presence or absence of phosphate (data not shown), suggesting that almost all of the activity detected in the crude was due to endogenous MTAN from the host cells. Since the purified Rv0535 fractions (Peak 2) could only cleave MTA in the presence of phosphate, it is likely that most of the Rv0535 was recovered during the purification step.

Table 1. Purification of *M. tuberculosis* Rv0535 from *E. coli* BL21 (DE3)

Purification Step	**Total protein (mg)**	**Total Activity (nmoles hr^{-1})**	**Specific Activity** * **(nmoles mg^{-1} h^{-1})**	**Purification (fold)**	**Yield (%)**
Crude cell extract	180	89,000	480	1	100
HisTrap HP	0.20	4,400	22,000	46	5

* Activity determined with 100 µM MTA plus 50 mM phosphate

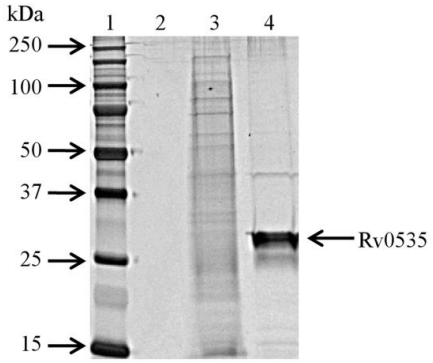

Figure 5. SDS-PAGE gel (12%; silver stain) of pooled Rv0535 fractions. Lane 1, molecular weight marker (Precision Plus All Blue Protein Standards); lane 2, empty; lane 3, crude cell extract (*E.coli* BL21 (DE3) expressing Rv0535); lane 4, pooled MTAP fractions from Peak 2 (Figure 3). Lanes 3 and 4 each contain 0.5 µg of protein.

Substrate specificity of Rv0535

In our previous study, we have shown that endogenous *M. smegmatis* MTAP could cleave MTA and Ado but not the other natural purines or pyrimidines (3). Although *M. smegmatis* and *M. tuberculosis* MTAP share 196 amino acids (74% identity), it is possible that they have different substrate specificity. Therefore, we studied the substrate preference of Rv0535 by evaluating its ability to cleave the natural purines, including SAH, and the pyrimidines. As shown in Table 2, MTA was the preferred substrate of Rv0535. Ado cleavage was also seen at 2% of the MTA cleavage activity. This is in contrast to *M. smegmatis* MTAP, where Ado cleavage was 16% of the MTA cleavage activity (3). SAH cleavage was very low (0.8% of MTA cleavage), further indicating that Rv0535 does not encode for the bacterial MTAN, which can cleave both

MTA and SAH. No activity was detected with the other purines (Ino, guanosine, or xanthosine) or the pyrimidines (cytidine, thymidine, or uridine). Similar to human and *M. smegmatis* MTAP, cleavage of 2'-deoxyadenosine was not observed with Rv0535 (7, 3).

MTA can be deaminated to 5'-methylthioinosine (MTI), a reaction that led to the misidentification of an MTIP as an MTAP in *P. aeruginosa* (8). To investigate whether the enzyme activity seen in our purified sample was due to an MTIP, the ability of Rv0535 to cleave MTI was assessed by HPLC. No activity was seen with MTI, indicating that Rv0535 was not MTIP. Further, *P. aeruginosa* MTIP can cleave Ino and Ado but not MTA (8), which is in contrast to our results above. Taken together, our substrate specificity results support our assessment of Rv0535 as an MTAP instead of an MTIP.

Table 2. Substrate specificity of Rv0535

Nucleosides (100 µM)	**Enzyme Activity (nmoles mg^{-1} h^{-1})***	**Relative activity (%)**
Purines		
5'-Methylthioadenosine	99,000 ± 3,000	100
Adenosine	2,100 ± 200	2.1
S-Adenosyl-L-homocysteine	800 ± 100	0.81
2'-Deoxyadenosine	--	--
Inosine	--	--
Guanosine	--	--
Xanthosine	--	--
5'-Methylthioinosine	--	--
Pyrimidines		
Cytidine	--	--
Thymidine	--	--
Uridine	--	--

-- less than 700 nmoles mg^{-1} h^{-1}
* values represent mean ± standard deviation for 3 determinations

Kinetic properties of Rv0535

The steady-state kinetic constants of Rv0535 were determined for MTA and phosphate (Table 2). The initial rate of reaction at different substrate concentrations was used to generate saturation curves, which were described by a hyperbolic function and thus displayed Michaelis-Menten kinetics (Figure 6). The K_m values for MTA and phosphate were 9 ± 3 µM (n=3) and 260 ± 80 µM (n=3), respectively. The K_m value for Ado cleavage was 1700 ± 200 µM (n=3), with 16% of the relative V_{max} with MTA, and more than a 6-fold decrease in K_{cat}, thereby indicating that Ado was a poor substrate for Rv0535.

Table 3. Steady-state kinetic parameters of Rv0535 in the direction of MTA phosphorolysis.

Substrate	**Co-substrate**	**K_m (µM)**	**K_{cat} (s^{-1})**
MTA	50 mM phosphate	9 ± 3	0.4 ± 0.2
Phosphate	200 µM MTA	260 ± 80	0.26 ± 0.02

Values represent mean ± standard deviation for 3 determinations

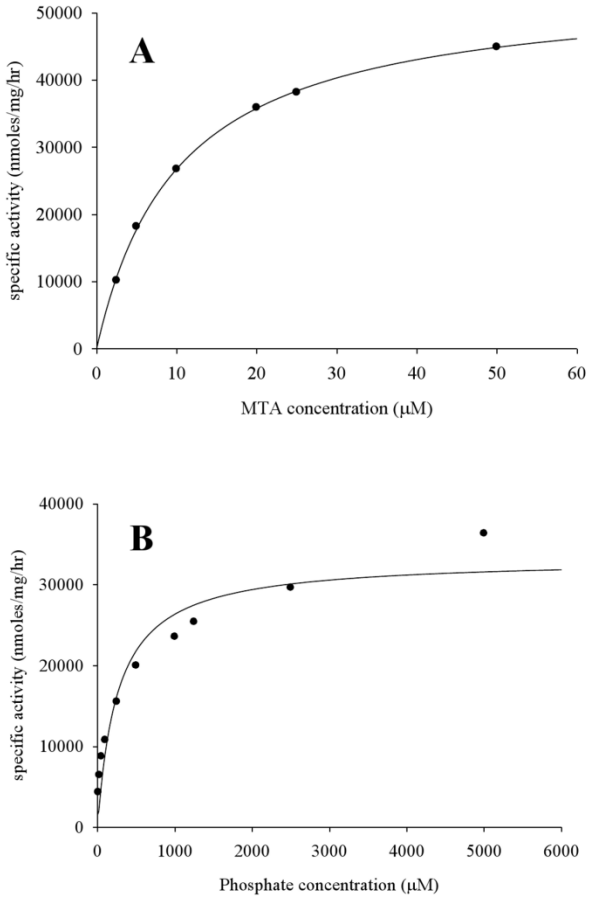

Figure 6. Determination of steady-state kinetic constants. Representative saturation curves for (A) MTA as the variable substrate in the presence of 50 mM phosphate, and (B) phosphate as the variable substrate in the presence of 200 μM MTA.

Inhibitors of Rv0535

Fabianowska-Majewska et al., have shown a 92 % decrease in MTA cleavage activity in the presence of 1 mM Ado (500 μM MTA) and therefore, even though Ado is a poor substrate for human MTAP, it could inhibit the human enzyme (7). To see whether Ado could inhibit Rv0535, Ade formation was monitored in the presence of 100 μM MTA and either 1 mM Ado or 3 mM Ado. No inhibition was seen at either concentration of Ado (data not shown). Thus, unlike the human enzyme, Rv0535 was not inhibited by Ado.

Transition state analogs for bacterial purine enzymes with inhibitory constants in the femtomolar and picomolar range have been synthesized (12, 21), some of which are being investigated as antimicrobials (18). MT-DADMe immucillin A is one of the best transition state analogs, and is a potent inhibitor of human MTAP. We investigated the effect of this transition state analog on MTA cleavage by Rv0535. The IC_{50} with MT-DADMe immucillin A was 25 ± 6 nM or 70 ± 6 nM nM in the presence of 10 μM or 100 μM MTA, respectively (data not shown), which indicated that MT-DADMe A immucillin was a potent inhibitor of Rv0535. Since transition state analogs have been described as slow-onset inhibitors, it is likely that the IC_{50} values obtained in our studies are greater than the true overall inhibition constant values of this inhibitor.

Other characteristics of Rv0535

The native molecular weight of Rv0535 was estimated by gel filtration column chromatography (Superdex™ 200 PG, GE Lifesciences). Briefly, the column was calibrated using the UV absorbance spectra of the molecular weight protein standards

(aldolase, ovalbumin, ribonuclease A, and aprotinin). Purified Rv0535 (200 µl) was applied to the column and eluted with an isocratic run of Buffer C. Fractions eluting from the filtration column were tested for MTA cleavage activity. The purified protein eluted as a single peak and based on its elution volume, the molecular weight of the native form of the enzyme was calculated as 60 ± 4 kDa (n=3) (Figure 7). Since the monomeric form of Rv0535 is approximately 30 kDa, our size exclusion data indicates that Rv0535 is a functional dimer. Although dimeric MTAPs are uncommon, our data agrees with the molecular weight of the *M. smegmatis* MTAP (64 ± 5 kDa, with a monomer of 27 kDa) (3).

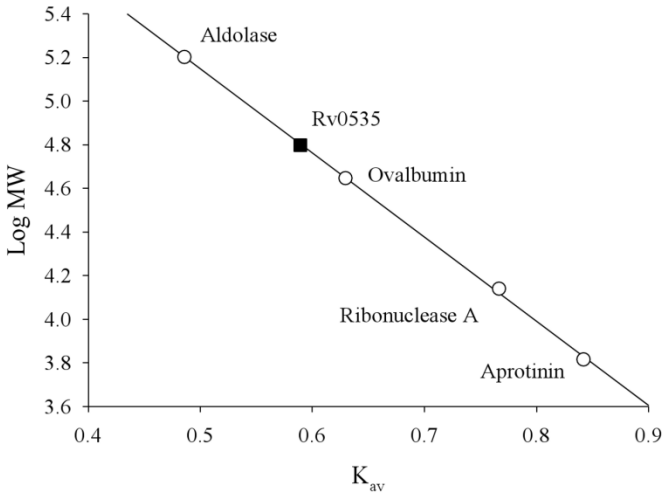

Figure 7. Estimation of the molecular weight of native Rv0535. Purified Rv0535 was applied to a Superdex 200 PG size exclusion column, and MTA cleavage activity was measured in fractions eluting from the column. The fraction with the most activity was used to calculate K_{av} according to the following equation: $K_{av}=(V_e-V_o)/(V_c-V_o)$, where V_o is the column void volume, V_e is the elution volume, and V_c is the geometric column volume. V_o was determined based on the V_e of Blue Dextran 2000. MW, molecular weight.

To investigate the effect of pH on enzyme activity, Rv0535 was incubated with MTA at different pH as described previously (3). The formation of Ade at each pH was measured by HPLC and the optimal pH of the reaction was between pH 7 and 7.5 (Figure 8A). The enzyme was inactive above pH 10 and below pH 6, indicating that the enzyme is inactive at its theoretical pI of 5.75.

The stability of Rv0535 at various temperatures was also studied. The purified protein was incubated at 4 °C, 25 °C, and 37 °C for specific amounts of time, after which MTA cleavage (at 37 °C) was assayed by HPLC. As shown in Figure 8B, there was a 12% or 40% decrease in MTA cleavage activity after the enzyme had been incubated at 4 °C for 30 min or 4 h respectively. In contrast, there was a 20% or 90% decrease in MTA cleavage activity after being incubated at 37 °C for 30 min or 4 h respectively. While there was no difference between the stability of the enzyme at 25 °C or 37 °C after a 30 min incubation at either temperatures, there was a considerable decrease in the rate of reaction when the assay was performed at 25 °C instead of 37 °C (data not shown). Therefore, enzyme assays were carried out at 37 °C. Additionally, there was no change in activity in aliquots stored at -20 °C (data not shown). Therefore, our data indicated that the recombinant protein was not stable at 4 °C, once the aliquot had been thawed. Because of the instability of the protein at 4 °C, necessary precautions were taken when handling the enzyme and to limit its loss of activity, the enzyme was incubated no longer than 15 minutes at 37 °C for the HPLC enzyme assay. Although the reason for the instability is not known, it could be due to the histidine tags that are on the N-terminus of the recombinant protein. It is possible that the removal of the polyhistidine tags by

peptidases, using a different tag, or purifying endogenous *M. tuberculosis* MTAP could resolve the stability issues.

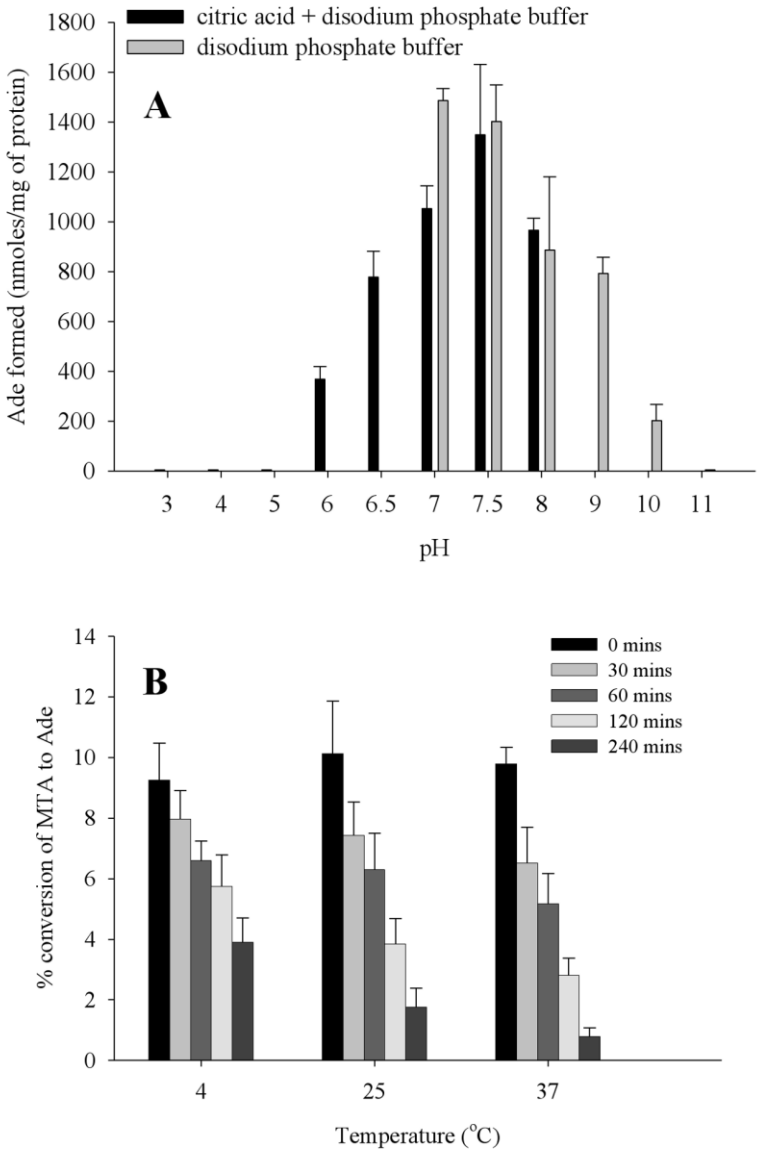

Figure 8. Effect of pH (A) and temperature (B) on Rv0535 activity. The amount of Ade formed was measured by HPLC, after the enzyme had been incubated at different pH (A) or at 4 °C, 25 °C, or 37 °C for different time periods (B). Error bars indicate mean ± standard deviation for 3 determinations**.**

To evaluate whether other bacteria express MTAP, the Basic Local Alignment Search Tool (BLAST) function of Uniprot (23) was used to compare the amino acid sequence of *M. tuberculosis* MTAP (Rv0535) to other sequence databases. The program produced 250 hits with sequence similarity ranging from 50% to 100%. More than 95% of these hits correspond to predicted or probable MTAPs, as opposed to MTAPs that have been purified and characterized. From the 250 hits, one sequence from each parent species, organism or strain that also had more than 55% similarity with Rv0535 was selected for a phylogenetic analysis. The resulting 7 sequences were all probable bacterial MTAPs. Therefore, while mycobacterial MTAPs were the first bacterial MTAPs to be identified, it is possible that other bacteria also express MTAP.

Given that there are a few differences in substrate specificity between *M. tuberculosis* and *M. smegmatis* MTAP, *M. smegmatis* MTAP (75% identity) was also included in the analysis. To represent MTAP from Archaea, MTAP sequences from *Sulfolobus Solfataricus* (43% identity) and *Pyrococcus furiosus* (46% identity) were added. MTAPs that have been well studied were also included, namely human MTAP (to represent mammalian MTAP, 38% identity), and *Trypanosoma brucei brucei* MTAP (to represent trypanosome MTAP, 40% identity). Since MTAP and MTAN can both cleave MTA, *M. tuberculosis* MTAN (15% identity) and *E. coli* MTAN (14% identity) were also included in the analysis. The selected sequences were aligned using MUltiple Sequence Comparison by Log- Expectation (MUSCLE) and a phylogenetic tree was created using the NCBI Genome Workbench (version 2.4.0 (13)).

As shown in Figure 9, the sequences that had been selected from the BLAST hits are closely grouped together. *M. tuberculosis* MTAP is in closer proximity to pathogenic

bacteria than to non-pathogenic bacteria, with the exception of *M. smegmatis*. Further, MTAPs that have been characterized were grouped separately from the predicted MTAPs. The Archaea and trypanosome MTAPs are closer to human MTAP as compared to bacterial MTAPs. While human and *M. tuberculosis* MTAP share 38% sequence identity, human and *M. tuberculosis* MTAN only share 18% identity. Similarly, *M. tuberculosis* MTAP and MTAN share 15% sequence identity. As suggested by the phylogenetic tree, despite having similar functions, MTAP sequences are different from MTAN sequences.

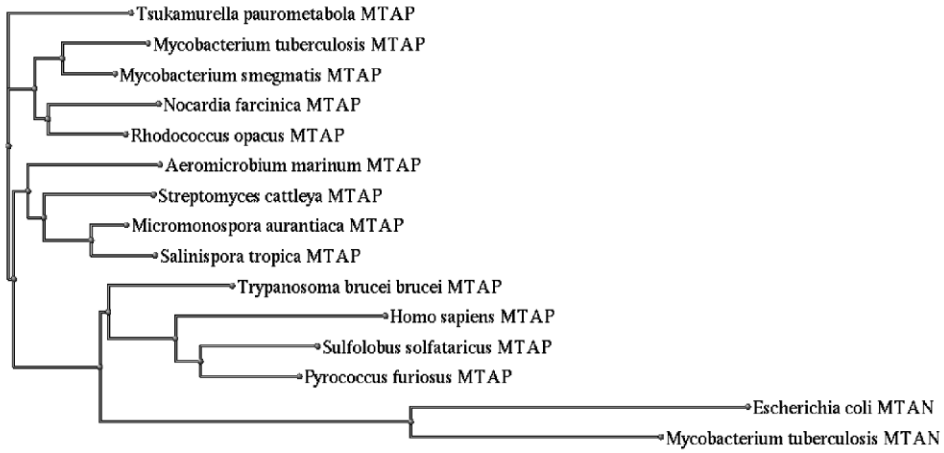

Figure 9. Phylogenetic analysis of MTA catabolizing enzymes from mycobacteria and other organisms. Amino acid sequences of MTAPs and MTANs from various sources were aligned with *M. tuberculosis* MTAP (Rv0535) and a phylogram was produced to represent the alignment.

Molecular model of Rv0535

The crystal structure of human MTAP has been determined and was used to generate a homology model of *M. tuberculosis* MTAP. This approach could help explain the substrate specificity of *M. tuberculosis* MTAP, as well as predict modifications to nucleoside analogs that would lead to their increased or decreased cleavage by the enzyme. To this end, an *M. tuberculosis* MTAP homology model was built using human MTAP (PDB no. 1CG6) as a template. The human MTAP sequence as present in the crystal structure was aligned with the *M. tuberculosis* MTAP sequence and short loop structures missing in the crystal structures were modeled with MODELER. The sequence identity in the modeled region was 40.5%, and residues that interact with the substrate were identified.

Similar to other MTAPs, we predict that each subunit of *M. tuberculosis* MTAP contains one active site. Our size exclusion data indicated that *M. tuberculosis* MTAP was a dimer, whereas human MTAP is known to be a trimer. Therefore, our modeling data is limited to comparisons of a single subunit from the *M. tuberculosis* MTAP structure to one subunit of the human MTAP structure. Residues that form interactions with the substrate were identified, and all but one residue, were components of one subunit. The *M. tuberculosis* model predicted that one amino acid residue (His126) from a neighboring subunit could contribute to weak steric interactions with the substrate. This could indicate that the active site of *M. tuberculosis* MTAP is not buried and is located at the subunit interface.

As shown in Figure 10, the active site of *M. tuberculosis* MTAP consists of about 17 amino acid residues. In general, the residues closer to the N-terminal domain are likely to participate in phosphate binding (Ser14, Arg55, His56, Cys88, Ala89, Met180, and Thr181) whereas the C-terminal domain residues could participate in nucleoside binding (Ser162, Thr203, Asp204, and Asp206). Ser14 could participate in both phosphate binding and interaction with the 3'-hydroxy group on the ribose moiety. Thus, it is possible that the active site *of M. tuberculosis* MTAP could be divided into a phosphate-binding site, a purine-binding site, and a ribose-binding site.

Of the 17 active site amino acids, 5 residues (Ser14, Cys88, Val178, Ala217, and Phe221) were different from human MTAP (Figure 10B and C). Ser14 and its human counterpart (Thr18) are both amino acids with polar, uncharged side chains and are both capable of forming analogous hydrogen bonding interactions with the substrate. However, the additional methyl group in Thr18 may have steric effects on the 5'-methylthio-group in MTA. Thr93 (from the human enzyme) forms more favorable interactions with the co-crystallized sulfate compared to Cys88. Val178 and its human counterpart (Ile194) are in close proximity to the purine ring, and although Ile194 has an additional methyl group and is therefore bulkier than Val178, steric/non-polar interactions are similar between the two species. The side-chain of Ala217 is in close proximity to the 5'-methylthio-group of the substrate and results in distinct non-polar interactions. The human counterpart of Ala217 (Val233) is significantly bulkier than Ala217 but Val233 forms more favorable non-polar interactions with the substrate. Thus, the substitution of Val233 with Ala217 in *M. tuberculosis* MTAP leads to a net loss in favorable non-polar interactions. Phe221 has a phenyl ring in close proximity to the 5'-

methylthio-group of MTA and adds considerable bulk to that region as compared to its human counterpart (Leu237). The non-polar interactions formed between the human MTAP residue Leu237 and the 5'-methylthio-group of MTA are more favorable than the analogous steric interaction involving Phe221, the corresponding residue in *M. tuberculosis* MTAP.

Given that the molecular model of *M. tuberculosis* MTAP identified amino acids that participate in substrate interactions, we compared the aligned sequences of other bacterial MTAPs (from *M. smegmatis*, *Nocardia farcinica*, *Rhodococcus opacus*, and *Tsukamurella paurometabola*) to determine whether these amino acids were conserved among bacterial MTAPs. Of the 17 residues involved in the active site of *M. tuberculosis* MTAP, 12 were conserved among these bacteria and human MTAP. Most active site amino acids were conserved among bacterial MTAPs: there were only two active site residues that were different between the various bacterial enzymes. *M. tuberculosis* Ser14, which we suggest participates in both phosphate and ribose binding pockets, is conserved in *M. smegmatis* and *R. opacus* MTAPs, whereas *N. farcinica* and *T. paurometabola* MTAPs have a Thr at that position, similar to human MTAP. *M. tuberculosis* Phe221 is conserved in *M. smegmatis*, *N. farcinica*, and *R. opacus* MTAPs, but not in *T. paurometabola* MTAP, which has a Leu at that position, similar to human MTAP. In general, MTAPs can fall in two categories, with amino acids resembling either *M. tuberculosis* or human MTAP. Given the differences that exist between bacterial MTAPs, it is possible that they have different substrate specificity.

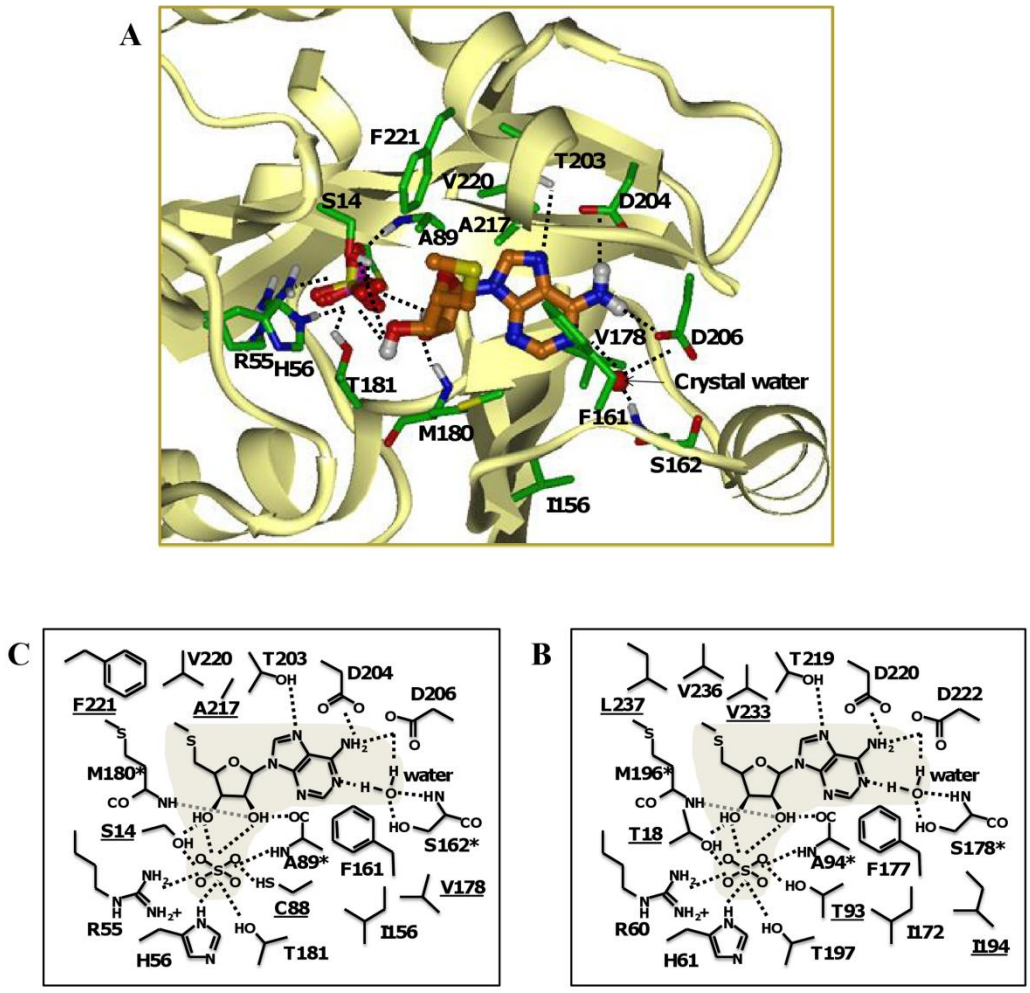

Figure 10. *M. tuberculosis* MTAP homology model built using human MTAP (PDB no. 1CG6) as a template. (A) Ribbon structure of a single subunit of *M. tuberculosis* MTAP with MTA and sulfate in the active site. Schematic representation of the interactions formed between MTA and the *M. tuberculosis* MTAP (B) and human MTAP (C). Amino acids differences between *M. tuberculosis* and the human sequence are underlined. Residues with backbone atoms involved in interactions with the substrate are marked (*). Polar interactions are shown as dashed lines.

Discussion

MTAP is primarily expressed in Eukarya, including trypanosomes, and Archaea, while MTAN is expressed in bacteria. In our previous work in *M. smegmatis*, we had noted the phosphorolytic cleavage of MTA and presented the first evidence of an MTAP activity in mycobacteria. Based on sequence homology, both MTAP and MTAN are predicted to exist in *M. smegmatis* and *M. tuberculosis*. Rv0535 was expressed in *E. coli* BL21 (DE3) cells and the purified protein was biochemically characterized. The natural purines and pyrimidines were tested as substrates, and MTA was cleaved with maximal activity, thereby indicating that MTA was the preferred substrate. MTI was also investigated since MTIP from *P. aeruginosa* had been incorrectly defined as MTAP. In our work, we showed that MTI was not a substrate for this enzyme, and therefore Rv0535 does not encode for MTIP. Thus, using a recombinant protein strategy combined with biochemical analysis, we confirm the correct annotation of Rv0535 as MTAP in *M. tuberculosis*.

In this work, we report that *M. tuberculosis* MTAP had a K_m of 9 µM with MTA, which is higher than the K_m of human MTAP (1.5 ± 0.2 µM) (24), but lower than that of *S. solfataricus* MTAP (24 µM) (4). Further, the K_{cat} of *M. tuberculosis* MTAP with MTA was 0.4 ± 0.2 s^{-1}, which is low in comparison to other bacterial purine metabolic enzymes, especially *E. coli* MTAN (MTA, 4.0 ± 0.1 s^{-1}) (22) and *M. tuberculosis* PNP (inosine, 5.4 ± 0.1 s^{-1})(5). However, *Streptococcus pneumoniae* MTAN has a comparable K_{cat} (MTA, 0.25 ± 0.04 s^{-1}; SAH, 0.37 ± 0.05 s^{-1}) (22). Variability in the specific activity of the recombinant enzyme was observed (in the range of 20,000 – 100,000 nmoles/mg/hr) and could be due to different purification efficiency and the instability of

the protein at 4 ^{o}C. It is possible that the specific activity and K_{cat} reported in this study are an underestimation of the actual values. On the other hand, a high turnover may not be required because of the presence of another enzyme (MTAN) that can also cleave MTA.

There are no known physiological explanations for the expression of both MTAP and MTAN in one organism. Until *M. tuberculosis* MTAN (Rv0091, Genbank Accession no. **CAA9827**) is expressed and its kinetic parameters are studied, the relationship between MTAP and MTAN will remain elusive. The concerted action of MTAN and methylthioribose kinase produces 5'-methylthioribose-1-phoshate, which can then be salvaged to methionine. To date, methylthioribose kinase has not been identified in the *M. tuberculosis* genome. Some bacteria such as *E. coli* do not possess methylthioribose kinase and therefore cannot recycle 5'-methylthioribose, which is excreted from the cell. If methionine salvage is important to the mycobacterium, it is possible that *M. tuberculosis* expresses both MTAP and MTAN to improve the efficiency of MTA cleavage, and MTAP produces 5'-methylthioribose-1-phosphate to rescue the methionine salvage pathway. It is also possible that because human cells express MTAP, mycobacterial MTAP is not expressed when the mycobacterium is found in its host. Further, it has been shown that methylthioribose kinase is expressed under starvation conditions in *Bacillus subtilis* (20). Thus, the expression of MTAP and MTAN under different growth conditions needs to be investigated.

The fact that human cells also express MTAP does not preclude *M. tuberculosis* target as a drug target. For example, parasites such as *Trypanosoma brucei brucei* also express MTAP but the substrate specificity of the parasitic MTAP differs from that of the

human form and these differences have been investigated for anti-parasitic drug development (11). The *M. tuberculosis* model indicated that the 5 different amino acid residues are in close proximity to or participate in the phosphate-binding site (Ser14 and Cys88), the purine-binding site (Val178), or the 5'-methylthioribose-binding site (Ser14, Ala217, and Phe221). Thus, it is possible that modifications to the Ade moiety or ribose moiety of the substrate could result in different substrate specificity between the mycobacterial and human MTAP. A complete structure-activity relationship study of Rv0535 using Ado analogs could help identify key differences between human and *M. tuberculosis* MTAP, which could be exploited in a drug discovery effort.

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STRUCTURE-ACTIVITY RELATIONSHIP OF 5'-METHYLTHIOADENOSINE PHOSPHORYLASE FROM *MYCOBACTERIUM TUBERCULOSIS*

by

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Format adapted for dissertation

Abstract

5’-Methylthioadenosine phosphorylase (MTAP, E.C 2.4.2.28) is a purine salvage enzyme that also participates in methionine salvage. MTAP has been described in Eukarya and Archaea, and recently the first bacterial MTAP was characterized in *Mycobacterium smegmatis*. Rv0535, which encodes for *M. tuberculosis* MTAP, has also been expressed and its biochemical properties have been studied. However, information about the active site of this mycobacterial enzyme is non-existent. Therefore, to supplement the initial studies of Rv0535, a structure activity relationship study involving 49 compounds was performed to identify which substrate modifications were tolerated. Two parent scaffolds were used, namely MTA and adenosine (Ado). Two nucleoside analogs, 2-fluoro-MTA and 2-fluoro-5’-deoxy-Ado, were better substrates than MTA. In general, the addition of an exocyclic fluorine atom at the 2-position of adenine (Ade) increased activity. Further, the endocyclic nitrogen atoms at the 3- and 7- positions of Ade were critical for activity. The proper orientation of the hydroxyl groups on the ribose moiety of Ado was also important, with the 2’- and 3’- hydroxyl in the *trans* orientation, and the 5’-hydroxyl group in the *cis* orientation. Moreover, the general removal of the 5’-hydroxyl group increased activity, as seen with 5’-deoxy-Ado and MTA. The information from this study could used to identify differences between human and mycobacterial MTAPs. These differences could then be exploited for the development of a nucleoside analog that can act as a subversive substrate and thus aid in a TB drug discovery effort.

Introduction

Tuberculosis (TB) is one of the leading infectious diseases in the world and has been declared a global health emergency by the World Health Organization. In 2010, it was estimated that 1.1 million lives were lost due to this disease (16) and that nearly one-third of the world's population was infected with latent TB (12). While untreated TB is almost always fatal, proper treatment consists of a combination of four drugs administered over 6 to 12 continuous months and has a success rate of 80-95% (13). Current TB drugs can be classified as first line or second line drugs, and target various cellular processes of *Mycobacterium tuberculosis*, which is the causative pathogen of TB. These processes include cell wall synthesis, RNA synthesis, folic acid and iron metabolism, and protein synthesis. However, there are no consistent guidelines for treating latent TB. Controlling and treating TB remains a challenge because of poor patient compliance with lengthy treatment, the emergence of drug-resistant TB strains, and increasing HIV-TB co-infections (9–12). Thus, there is a need to develop new TB drugs that can treat active, latent, and drug-resistant TB.

Purine metabolism is an essential process of all living cells and has been well studied in humans. Further, an enhanced understanding of purine metabolism has led to the development of anti-cancer and anti-parasitic nucleoside analogs. In 1994, the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) was established to screen for novel antituberculosis drugs. Two adenosine (Ado) analogs, 2-fluoro-adenosine (F-Ado) and 2-methyl-adenosine (methyl-Ado) were identified among the compounds that were toxic to mycobacteria. Further studies showed that methyl-Ado had a low MIC (4 µg/ml) and was cytotoxic to latent mycobacteria (1). In related studies

on the mechanism of action of methyl-Ado, it was shown that cell extracts from *M. tuberculosis* and *M. smegmatis* could cleave Ado to adenine (Ade) (6, 8). Since human cells cannot efficiently cleave Ado, this observation indicated a difference in purine metabolism between human and mycobacterial cells that could be exploited in the discovery of novel anti-TB drugs. It has since been shown that *M. smegmatis* expresses two enzymes that can cleave Ado, namely an adenosine purine nucleoside phosphorylase (Ado-PNP) and a 5'-methylthioadenosine phosphorylase (MTAP, E.C 2.4.2.28) (4). MTAP is a purine metabolic enzyme responsible for the cleavage of the natural purine, 5'-methylthioadenosine (MTA), which is generated as a by-product of polyamine synthesis. In the presence of phosphate, MTA is cleaved to produce Ade, which can participate in purine metabolism, and 5'-methylthioribose-1-phosphate, which is recycled to generate methionine (Figure 1). While MTAP was known to exist in Eukarya and Archaea, our recent work in *M. smegmatis* showed that some mycobacteria can also express MTAP (4).

MSMEG_0990 and Rv0535 have been annotated as MTAPs in *M. smegmatis* and *M. tuberculosis*, respectively. Rv0535 was expressed in *E. coli* BL21 (DE3) cells with an N-terminal histidine-tag, and purified using a nickel affinity column. The purified protein was characterized and two differences were observed between Rv0535 and human MTAP (3). First, while all functionally active MTAPs are known to be trimers (with the exception of *Sulfolobus solfataricus* which is a hexamer), *M. tuberculosis* MTAP is predicted to be a dimer. Moreover, the active site of one *M. tuberculosis* MTAP subunit was modeled based on the crystal structure of one human MTAP subunit ((PDB code 1CG6). The homology model of *M. tuberculosis* MTAP predicted that the active site of

the enzyme had three main binding pockets to accommodate the ribose sugar moiety, the purine base moiety, or the required phosphate. Of the 17 amino acid residues involved in the active site of *M. tuberculosis* MTAP, 5 were different from the human homolog. These residues were in close proximity to or participated in interactions with the three binding pockets. Therefore, it is possible that modifications to the sugar or purine moieties could result in different substrate specificity between the mycobacterial and human MTAPs.

Since MSMEG_0990 and Rv0535 were the first characterized bacterial MTAPs, little is known about their substrate specificity. It is possible that Rv0535 could be exploited in a drug discovery effort by activating subversive substrates to a toxic moiety in mycobacterial cells only. Thus, a better understanding of the active site of Rv0535 could be used for the rational design of novel subversive nucleoside analogs. To this end, we have evaluated 49 compounds, which represent modifications to the ribose and/or purine moiety of Ado or MTA.

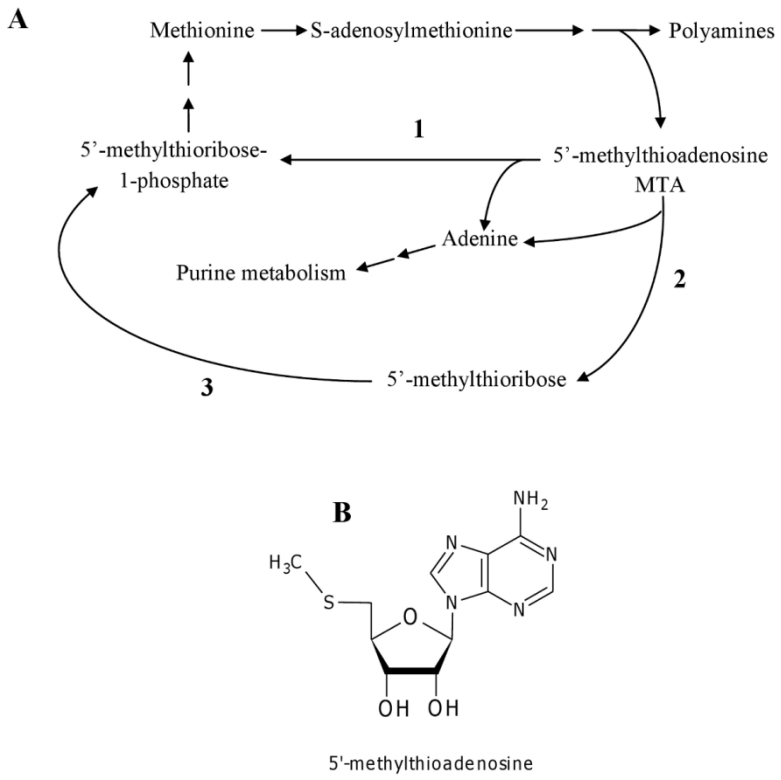

Figure 1. (A) Metabolism of MTA. Reaction 1 is usually catalyzed by MTAP from Eukarya and Archaea, whereas bacteria recycle MTA via Reaction 2 (by MTAN) and Reaction 3 (by methylthioribose kinase). Mycobacteria can metabolize MTA using MTAP. (B) Structure of 5'-methylthioadenosine

Materials and Methods

Chemicals. The nucleoside analogs used in this study were obtained from various sources. Ado **(1)**, 9-[β-D-ribofuranosyl]-purine **(15)**, 6-mercapto-purine riboside **(16)**, 8-bromo-Ado **(25)**, 8-aza-9-deaza-Ado **(26)**, 2'-deoxy-Ado **(27)**, 2'-O-methyl-Ado **(28)**, and 9-[β-D-arabinofuranosyl]-adenine **(29)** were purchased from Sigma-Aldrich (St. Louis, MO). 8-Azido-Ado **(23)** and β-L-Ado **(3)** were kindly provided by Dr. Mahmoud el Kouni (University of Alabama at Birmingham, Birmingham, AL). 2-Fluoro-3-deaza-Ado **(13)** was a gift from Dr. Alan C. Sartorelli (Yale University, New Haven, CT). All other compounds were provided by the chemical repository at Southern Research Institute (Birmingham, AL). Each compound was solubilized in water. Compounds that were not soluble in water were dissolved in DMSO. We evaluated the effect of DMSO on enzyme activity, and did not observe any considerable change in enzyme activity when DMSO was kept below 5%.

Cloning of the *Rv0535* gene from *M. tuberculosis* H37Rv genomic DNA. The *Rv0535* gene was amplified by PCR from strain H37Rv genomic DNA as described previously (3). The PCR product was inserted in the pET28a (+) expression vector (Novagen/EMD Chemicals Inc., CA). The resulting recombinant vector was transformed into *E. coli* BL21 (DE3) (Novagen) competent cells and selected using kanamycin (50 μg/ml). A 1.2 L culture was grown in Luria-Bertani media supplemented with kanamycin at 24 °C until the A_{600} reached 0.6 – 0.8. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was grown aerobically at 37 °C for 5 hours. The cells (6g) were then harvested by centrifugation and rinsed twice with Buffer A (20 mM Tris-HCl, 300 mM NaCl, and 20

mM imidazole, pH 7.5). The pellet was resuspended in 20 ml Buffer A containing one Complete EDTA-free protease inhibitor tablet. The cell suspension was lysed using a French pressure cell and then centrifuged for 1 hour at 100,000 × *g* at 4 °C. The clarified supernatant was collected and purified with nickel affinity column (HisTrap HP, GE Healthcare) using a linear imidazole gradient as described previously (3). Fractions with the most MTA cleavage were pooled and dialyzed overnight against two changes of 1 L of Buffer C (50 mM Tris HCl, 150 mM NaCl, 1 mM dithiothreitol, and 20% glycerol, pH 7.6) at 4 ˚C (Spectra/Por 4 Membrane Tubing, 12,000 to 14,000 Dalton MWCO, Fisher Scientific). Protein concentrations were obtained by the Bradford method (2) using bovine serum albumin as standard.

Substrate Assay. Enzyme activity was followed by measuring the formation of product using reverse-phase high performance liquid chromatography (HPLC) as described previously (4). Briefly, the purified enzyme was incubated with each compound at 37 ˚C in the presence of 50 mM potassium phosphate buffer (pH 7.5) and 50 mM HEPES (pH 7.3). For compounds compared to Ado, the substrate concentration was 250 µM while for compounds compared to MTA, the substrate concentration was 100 µM. After a specific amount of the time, the reaction was stopped by boiling at 100 ˚C for 5 minutes after which the samples were analyzed by reverse-phase HPLC. The substrates and products were detected as they eluted from the column by their absorbance at 260 nm. The substrate conversions were maintained below 10%. Due to the reported instability of the recombinant enzyme, the activity assays were conducted within 15 minutes at 37 °C. Some nucleobases were not available for use as standard. Therefore, all evaluations were

conducted in the presence or absence of enzyme, and the appearance of a new peak only in the presence of the enzyme was monitored and considered as a positive result.

Results and Discussion

M. tuberculosis MTAP (Rv0535) was identified and characterized recently (3). As one of the first bacterial MTAPs discovered, little is known about the active site of the enzyme. Thus, this structure-activity relationship (SAR) study was performed to identify substrates of the enzyme to help understand its active site. In our previous work, we evaluated the cleavage of the natural purines and pyrimidines. MTA was the preferred substrate, but Ado and S-adenosyl-L homocysteine cleavage was also detected at 2% and 0.8% of the MTA activity respectively (3). The cleavage of other natural purines and pyrimidines was not detected. Further, we noted that the K_m with Ado was 1700 μM whereas the K_m with MTA was 9 μM, indicating that Ado was a poor substrate for the enzyme (3).

Although Ado was a poor substrate for *M. tuberculosis* MTAP, Ado analogs were included in this SAR for several reasons. First, an SAR study of bacterial MTAPs has not been conducted, and therefore there is no predictor of substrate specificity. Because we have several Ado analogs available, we were able to evaluate a broad range of structures for substrate activity. It is hoped that the results obtained with the Ado analogs could be used to predict the substrate specificity of MTA analogs, since the number of MTA analogs is limited. Of note, because Ado cleavage was low at 100 μM (2% of MTA cleavage), a higher concentration of Ado and its analogs were used to increase cleavage rate. Assessment of Ado analogs was done by comparing these compounds to Ado (Table 1).

Thus, the compounds selected for this SAR study were based on two parent scaffolds, namely MTA (Table 2) and Ado. The cleavage of each analog was compared to their parent scaffold. In addition, Ado analogs that had similar or greater activity compared to Ado cleavage were compared to MTA at 100 μM (Table 3). Figure 2A shows the numbering convention used for naming the compounds.

To aid in our understanding of the SAR results, we used a homology model of *M. tuberculosis* MTAP (Figure 3). The active site of a single subunit of Rv0535 was modeled using human MTAP (PDB no. 1CG6) as a template. The human enzyme was co-crystallized with MTA and a sulfate molecule, which mimics the required phosphate. The homology model of Rv0535 revealed that 5 amino acid residues in the active site of the enzyme were different from the active site of the human enzyme. As shown in Figure 3, the active site of Rv0535 consists of three binding pockets that can accommodate the 5'-methylthioribose moiety, the Ade base, and the phosphate co-substrate. The ribbon diagram (Figure 3A) provides useful information for the spatial arrangement of amino acids and the substrate. Figure 3B depicts the interactions of the substrate with the amino acids present in the active site of the enzyme. The homology model is used in this work to explain the SAR results.

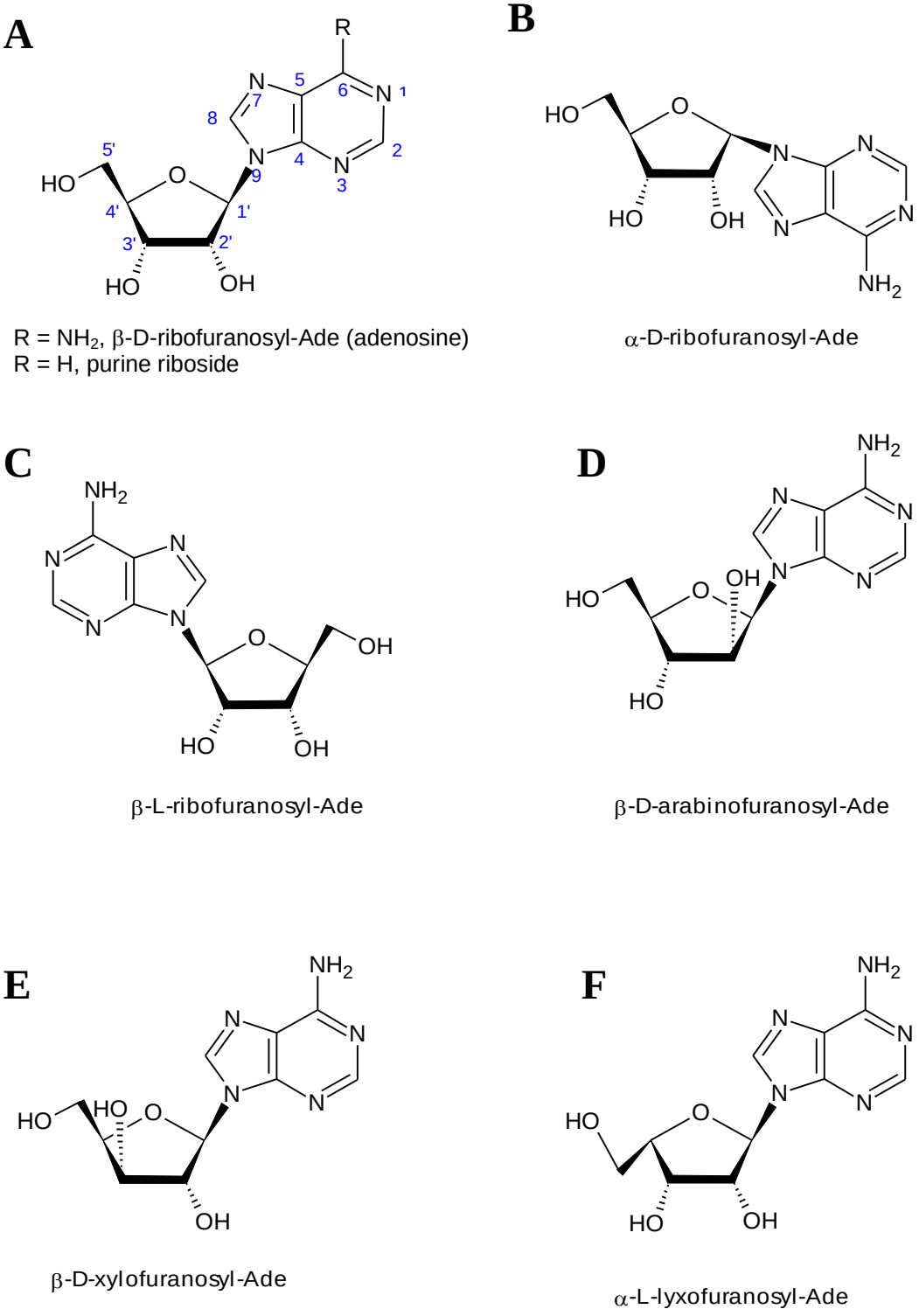

Figure 2. (A) Structure and numbering convention for Ado and purine riboside; (B-F) Stereoisomers and enantiomers of Ado used in this structure-activity relationship.

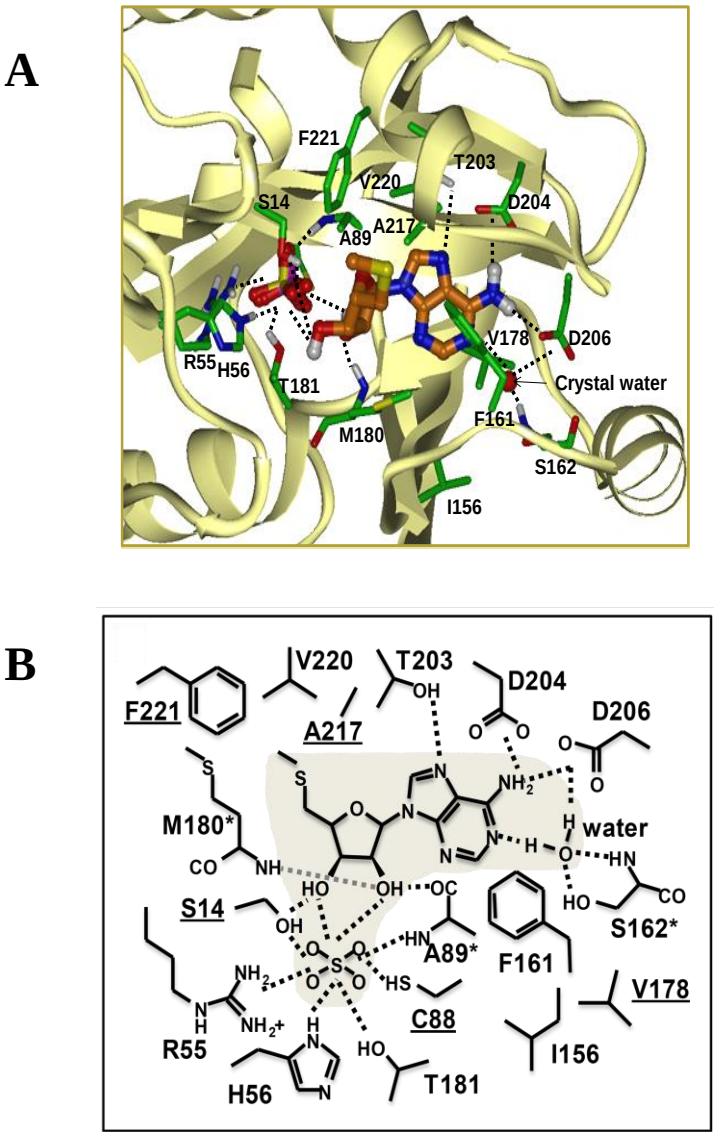

Figure 3. *M. tuberculosis* MTAP homology model built using human MTAP (PDB no. 1CG6) as a template. (A) Ribbon structure of a single subunit of *M. tuberculosis* MTAP with MTA and sulfate in the active site. (B) Schematic representation of the interactions formed between MTA and the *M. tuberculosis* MTAP. Amino acids different between *M. tuberculosis* and human MTAPs are underlined. Residues with backbone atoms involved in interactions with the substrate are marked (*). Polar interactions are shown as dashed lines.

Adenosine scaffold – modifications to the adenine moiety

Twenty-three compounds representing single or multiple modifications to the purine base were evaluated.

1-position

The homology model predicts the interaction of the amino group at the 1-position with Ser162 through water-mediated hydrogen bonding. The addition of a methyl group at that position decreased but did not abolish activity as N^1-methyl-adenosine had 15% of the Ado cleavage. Given the prediction of the model and the decreased activity with N^1-methyl-adenosine, it is likely that the 1-position of the Ade moiety is important and that other modifications might not be tolerated.

2-position

Seven compounds representing modifications to the 2-position of the Ade moiety were evaluated. Small molecule additions at that position were generally recognized by the enzyme. F-Ado was cleaved with 170% of Ado cleavage but the addition of a larger halogen (bromine) at the 2-position resulted in a decrease in activity compared to Ado (29% of the Ado cleavage activity). Methyl-Ado could be cleaved with 23% of the activity with Ado, whereas the addition of the larger ethyl group at the 2-position resulted in slightly less activity (15% of Ado cleavage activity). 2-Azido-Ado was a poor substrate since only 8% of Ado cleavage was detected. However, the replacement of the 2-carbon with a nitrogen (2-aza-Ado) led to increased cleavage (193%) compared to Ado. Thus, small modifications at the 2-position can enhance activity compared to Ado but larger modifications may not be well tolerated.

3-position

Two compounds with modifications at the 3-position of the Ade base were evaluated. The first was 3-deaza-Ado, in which the nitrogen atom at the 3-position is replaced with a carbon atom. This modification abolished activity. Since a fluorine atom at the 2-position could enhance activity compared to Ado, we evaluated 2-fluoro-3-deaza-Ado. Again, no cleavage was detected, suggesting that the nitrogen atom is essential for activity, and the loss of this atom cannot be compensated by a fluorine atom at the 2-position.

The homology model suggests that the nitrogen atom does not participate in hydrogen bonding in the active site. Therefore, it is possible that the loss of that atom results in a destabilization of interactions at other positions, such as N^1.

6-position

Six compounds representing modifications at the 6-position of the Ade moiety were evaluated. None of the compounds were better substrates than Ado. Further, no activity was detected with the removal of the amino group at the 6-position (purine riboside, Figure 2A). Addition of a methyl, a sulfur (in the mercapto compound), or both a methyl and a sulfur (methylmercapto) group(s) to purine riboside were better than purine riboside, but with no improvement in activity compared to Ado. As suggested by the homology model, the amino group at the 6-position participates in hydrogen bonding with Asp204 and Asp206. Given our results, it is possible that a methyl, mercapto, or methylmercapto group at this position can partly replace the function of the amino group.

The cleavage of 6-methylpurine-2'-deoxyriboside (MeP-dR) at 23% of the Ado cleavage activity was detected. This was unexpected since in our previous work, we had not detected the cleavage of 2'-deoxy-Ado (at 100 μM) (3), which indicated that the 2'-hydroxyl group to the ribose moiety was necessary for cleavage. However, at 250 μM, cleavage of 2'-deoxy-Ado was detected (see below). Therefore, the cleavage of a 2'-deoxy compound could be due to the higher concentration of analog used in this part of our SAR experiment.

7-position

When the nitrogen atom at the 7-position of the Ade moiety was replaced with a carbon atom in the form of 7-deaza-Ado, no cleavage was detected, suggesting that this nitrogen atom is crucial for activity. This is further supported by the homology model, in which the nitrogen atom can form hydrogen bonds with Thr203.

8-position

Six compounds representing modifications at the 8-position of the Ade moiety were evaluated. Activity was detected with three compounds. Similar to modifications at the 2-position, small additions at the 8-position were tolerated, with the smallest atom (fluorine) having the highest cleavage in this category. 8-Chloro-Ado and 8-bromo-Ado could also be cleaved but at 83% and 26% of the Ado cleavage activity, respectively. Thus, our results indicated that as the size of the halogen atom increases, the activity decreases. The addition of nitrogen atoms at that position abolished activity as the cleavage of either 8-aza-Ado or 8-azido-Ado was not detected. We also evaluated the cleavage of 8-aza-9-deaza-Ado. This compound, also known as formycin A, has been

described as a competitive inhibitor of bacterial PNP but not of human PNP (5). Since 8-aza-Ado was not a substrate, and it is known that glycosidic bond of 9-deaza-Ado is very stable, we did not expect formycin A to be a substrate for MTAP. Indeed, the cleavage of this 8-aza-Ado analog was not detected. Further studies are needed to evaluate whether this compound can inhibit Rv0535.

The homology model predicts the presence of Ala217 in close proximity to the 8-position of the Ade moiety. The human counterpart to Ala217 is the larger Val233, which may suggest that the mycobacterial enzyme may have more space to accommodate modifications at the 8-position.

Adenosine scaffold – modifications to the ribose moiety

Ribofuranosyl conformation

Two isomers of Ado were evaluated. MTAP could cleave β-D-Ado (Ado, Figure 2A), but not α-D-Ado (Figure 2B) or β-L-Ado (Figure 2C), indicating that Rv0535 preferred the β-D-ribofuranosyl conformation of Ado.

2'-position

Our previous work suggested that the 2'-hydroxyl was important for cleavage activity (3). For this SAR study, we evaluated 3 compounds representing modifications at the 2'-position of the ribose moiety at a higher concentration than previous studies (250 μM). We noted the cleavage of 2'-deoxy-Ado at 16% of the Ado cleavage activity. This is comparable to the activity seen with MeP-dR, which also lacks the 2'-hydroxyl group. However, no activity was detected with 2'-*O*-methyl-Ado, which is the addition of

a methyl group to the 2'-hydroxyl group. The cleavage of 9-[β-D-arabinofuranosyl]-adenine (araA) was not detected. Since in araA, the 2'-hydroxyl is in the *cis* position (Figure 2D), our results indicated that the proper orientation of the 2'-hydroxyl group was important for activity. Further, since no activity was detected with 2-fluoro-araA, the addition of a fluorine atom at the 2-position of the Ade base could not compensate for the *cis* orientation of the 2'-hydroxyl group.

The homology model predicts that the 2'-hydroxyl group interacts with Ala89 and with the phosphate binding pocket. Further, as shown in Figure 3A, the *trans* orientation of the 2'-hydroxyl group brings the hydroxyl closer to the phosphate binding pocket. It is also possible that this orientation is less constrained for interaction with Met180.

3'-position

We also investigated the effect of modifications to the 3'-position. We did not detect the cleavage of 3'-deoxy-Ado. Further, no activity was detected with 9-[β-D-xylofuranosyl]-adenine, in which the 3'-hydroxyl group is in the *cis* position (Figure 2E). Therefore, similar to araA, the proper orientation of the 3'-hydroxyl group is important for activity. This is also supported by the homology model, which predicts the interaction of the 3'-hydroxyl group with the phosphate binding pocket and Ser14.

2'- and 3'-positions

We investigated the cleavage of 2', 3'-dideoxyadenosine (ddA), which is used as a reverse transcriptase inhibitor for the treatment of HIV. Further, Ghoda et al., have shown that ddA was a substrate for *Trypanosoma brucei brucei* MTAP but not for human MTAP (7), suggesting that ddA analogs could be used as a subversive substrate for the

treatment of parasitic infections. Consistent with our results with 2'-deoxy-Ado and 3'-deoxy-Ado, ddA was not a good substrate for Rv0535, with 0.4% of Ado cleavage. Due to its poor cleavage by *M. tuberculosis* MTAP, ddA analogs would not be as good as a subversive substrate for the mycobacterial enzyme as it is for the trypanosomal enzyme.

5'-position

We investigated 2 compounds with modifications at the 5'-position of the ribose moiety of Ado and 2 additional compounds with modifications at the 5'-position of the ribose moiety with an exocyclic fluorine atom at the 2-position of the Ade base. Compared to Ado cleavage, we observed a 13-fold and 20-fold increase in activity with 5'-deoxy-Ado and 2-fluoro-5'-deoxy-Ado, respectively. MTA, which has a methylthio-group instead of a hydroxyl group at the 5'-position of Ado, is the preferred substrate of Rv0535 (3). Thus, the removal or substitution of the 5'-hydroxyl group enhanced cleavage with respect to Ado.

9-[α-L-Lyxofuranosyl]-adenine (Figure 2F) and 9-[α-L-lyxofuranosyl]-2-fluoro-adenine had 24% and 40% of the Ado cleavage activity, respectively, indicating that the proper orientation of the 5'-hydroxyl group was important. Therefore, it is likely that Rv0535 has a preference for the *cis* orientation of the 5'-hydroxyl group. Our results indicated that similar to F-Ado, the addition of a fluorine atom at the 2-position of the Ade moiety could enhance activity.

Table 1. *M. tuberculosis* MTAP activity with Ado analogs

	Compounds (250 μM)	Relative activity [a]	number of determinations
		% of Ado	
1	9-[β-D-Ribofuranosyl]-adenine (adenosine)	100	
2	α-D-Adenosine	--	2
3	β-L-Adenosine	--	2
	Modification on Adenine moiety		
	N^1-position		
4	N^1-Methyl-adenosine	15	1
	2-position		
5	2-Aza-adenosine	193	2
6	2-Fluoro-adenosine	170	2
7	2-Chloro-adenosine	50	2
8	2-Bromo-adenosine	29	2
9	2-Methyl-adenosine	23	1
10	2-Ethyl-adenosine	15	1
11	2-Azido-adenosine	8	1
	3-position		
12	3-Deaza-adenosine	--	2
13	2-Fluoro-3-deaza-adenosine	--	2
	6-position		
14	N^6-methyl-adenosine	37	2
15	9-[β-D-Ribofuranosyl]-purine (purine riboside)	--	2
16	6-Mercapto-purine riboside	24	1
17	6-Methylmercapto-purine riboside	30	1
18	6-Methyl-purine riboside	28	1
19	6-Methylpurine-2'-deoxyriboside	23	1
	7-position		
20	7-Deaza-adenosine (tubercidin)	--	2
	8-position		
21	8-Fluoro-adenosine	93	1
22	8-Aza-adenosine	--	2
23	8-Azido-adenosine	--	2
24	8-Chloro-adenosine	83	2
25	8-Bromo-adenosine	26	2
26	8-Aza-9-deaza-adenosine (formycin A)	--	2

Modification on Ribose moiety

	2'-Position		
27	2'-Deoxy-adenosine	16	1
28	2'-*O*-Methyl-adenosine	--	2
29	9-[β-D-Arabinofuranosyl]-adenine (araA)	--	2
30	9-[β-D-Arabinofuranosyl]-2-fluoro-adenine	--	2
	3'-Position		
31	3'-Deoxy-adenosine (cordycepin)	--	2
32	9-[β-D-Xylofuranosyl]-adenine	--	2
	2' and 3'-positions		
33	2',3'-Dideoxyadenosine	0.4	1
	5'-Position		
34	5'-Deoxy-adenosine	1,300	1
35	2-Fluoro-5'-deoxyadenosine	1,950	1
36	9-[α-L-Lyxofuranosyl]-adenine	24	2
37	9-[α-L-Lyxofuranosyl]-2-fluoro-adenine	40	2

[a]Activity expressed as percent of adenosine cleavage (3,200 nmoles/mg/hr)

-- Not detected

MTA scaffold

Ten compounds that represent modifications to the MTA scaffold were evaluated at 100 μM and compared to MTA cleavage. As predicted by the evaluation of Ado analogs, the two compounds that had a fluorine atom at the 2-position of the Ade moiety had greater activity compared to MTA. Both 2-fluoro-MTA and 2-fluoro-5'-ethylthioadenosine were cleaved at 140% of the MTA cleavage activity. Given that 2-fluoro-MTA had 140% of the MTA activity and that F-Ado had 170% of the Ado activity, it can be inferred that 2-fluoro-5'-ethylthioadenosine has at least 100% of the 5'-ethylthioadenosine activity. Therefore, it is likely that MTA and 5'-ethylthioadenosine (not available for testing) are comparable substrates for Rv0535.

Similar to 2-chloro-Ado, which had 50% of the Ado cleavage activity, 2-chloro-MTA had 45% of the MTA cleavage activity. Other modifications at the 2-position of Ade were not tolerated, since activity was not seen with neither 2-amino-MTA nor 2-azido-MTA. Since we did not evaluate 5'-ethylthioadenosine, it is not clear whether the 2-fluoro analog compensated for the bulkier ethyl group. Since there was no difference between 2-fluoro-MTA and 2-fluoro-5'-ethylthioadenosine, it is possible that the ethyl group did not have a considerable effect on the active site. On the other hand, 5'-isobutylthioadenosine had 50% of the MTA cleavage activity, indicating that the larger isobutyl group at the 5'-position was not as well tolerated in the active site as the methyl or ethyl group. 8-Methyl-MTA had decreased activity compared to MTA (24% of MTA cleavage activity), and 8-hydroxy-MTA was not a substrate, suggesting that modifications at the 8-position of Ade were not be well tolerated. No activity was detected with 8-aza-5'-chloro-adenosine. Since no activity was seen with 8-aza-Ado, our

result suggested that the 5'-chloro modification could not compensate for the modification at the 8-position of the Ade moiety. Similar to araA, no activity was seen with MTA in which the 2'-hydroxyl group in the *cis* position (araMTA).

Two other compounds, which are Ado analogs, were compared to the MTA scaffold, namely 9-(β-D-5-methyl-(*allo*)-ribofuranosyl)-2-fluoro-adenine and 9-(β-D-5-methyl-(*talo*)-ribofuranosyl)-2-fluoro-adenine, which are stereoisomers of each other. Both compounds have an additional hydroxyl group and a methyl group at the 5'-position as opposed to the methylthio-group in MTA. In the *allo* compound, the 5'-methyl group is oriented to the back of the plane of the ribose sugar in the direction of the 4'-oxygen, while in the *talo* compound, the 5'-methyl group points towards the 2'-hydroxyl group. The *allo* and *talo* compounds could be cleaved with 1.4% and 7% of the MTA cleavage activity, respectively. As discussed earlier, it is possible that the decreased activity with these two compounds is due to the 5'-hydroxyl group. These two compounds contain a 2-fluoro-adenine moiety. Based on previous observations that 2-fluoro-Ade modified compounds have greater activity than an Ade moiety, it is expected that 9-(β-D-5-methyl-(*allo*)-ribofuranosyl)-adenine will have less active substrate than 9-(β-D-5-methyl-(*allo*)-ribofuranosyl). Therefore, it is likely that the *allo* compound will be a poor substrate for Rv0535. Similarly, 9-(β-D-5-methyl-(*talo*)-ribofuranosyl)-adenine is predicted to also be a poor substrate for Rv0535, although it will still be better than the *allo* compound. The cleavage of 9-(β-D-5-methyl-(*allo*)-ribofuranosyl)-adenine and 9-(β-D-5-methyl-(*talo*)-ribofuranosyl)-adenine needs to be investigated.

Table 2. *M. tuberculosis* MTAP activity with MTA analogs

	Compounds (100 μM)	**Relative activity** [a]
		% of MTA
38	5'-Methylthioadenosine (MTA)	100
37	2-Fluoro-5'-methylthioadenosine	140 ± 50
39	2-Chloro-5'-methylthioadenosine	45 ± 9
40	2-Amino-5'-methylthioadenosine	--
41	2-Azido-5'-methylthioadenosine	--
42	2-Fluoro-5'-ethylthioadenosine	140 ± 30
43	5'-Isobutylthioadenosine	50 ± 10
44	8-Methyl-5'-methylthioadenosine	24 ± 8
45	8-Hydroxy-5'-methylthioadenosine	--
46	8-Aza-5'-chloroadenosine	--
47	5'-Methylthio-9-[β-D-Arabinofuranosyl]-adenine	--
48	9-(β-D-5-Methyl-(allo)-ribofuranosyl)-2-fluoro-adenine	1.4 ± 0.1
49	9-(β-D-5-Methyl-(talo)-ribofuranosyl)-2-fluoro-adenine	7 ± 1

[a]Activity expressed as percent of MTA cleavage (18,000 nmoles/mg/hr)

Values represent mean ± standard deviation for 3 determinations

-- Not detected (n=2)

Ado analogs compared to MTA

From Table 1 of Ado analogs, four compounds with the highest activity were selected and compared to the MTA scaffold (at 100 μM). Only 1 compound, 2-fluoro-5'-deoxy-Ado was a better substrate than MTA, at 130% of the MTA cleavage. 5'-Deoxy-Ado had 50% of the MTA activity, while 2-fluoro-Ado and 2-aza-Ado had 4.9% and 3% of the MTA cleavage activity. Interestingly, our results indicated that 2-fluoro-5'-deoxy-Ado and 2-fluoro-MTA were comparable substrates and both had greater activity than MTA. Therefore, our results suggested in the presence of a fluorine atom at the 2-position of Ade, the active site of the enzyme did not discriminate between a methylthio- and a methyl at the 5'-position of Ade.

Table 3. Comparison of the most active Ado analogs to MTA

Compounds (100 μM)	**Relative activity** [a]
	% of MTA
5'-Methylthioadenosine (MTA)	100
2-Fluoro-adenosine	4.9 ± 0.7
2-Aza-adenosine	3 ± 1
5'-Deoxy-adenosine	50 ± 30
2-Fluoro-5'-deoxy-adenosine	130 ± 30

[a]Activity expressed as percent of MTA cleavage (18,000 nmoles/mg/hr)

Values represent mean ± standard deviation for 3 determinations

Comparison with human MTAP

Toorchen and Miller have shown that MTAP purified from human liver can cleave Ado with 0.1% of the MTA cleavage activity (11), which is at least 10-fold lower than the Ado cleavage by Rv0535. However, similar to Rv0535, the cleavage of 2'-deoxy-Ado or 2', 3'-dideoxyadenoinse was not seen with human MTAP (11). The cleavage of 5'-deoxy-Ado and 2-fluoro-MTA by human MTAP was similar to Rv0535 (11).

HL-60 human promyelocytic leukemia cells were shown to cleave 2-fluoro-5'-deoxy-Ado at 44% of the activity with MTA (10), which is 3-fold lower than the cleavage of this compound by Rv0535. Cytotoxic assays performed in HL-60 cells showed an IC_{50} of 0.05 µM with 2-fluoro-5'-deoxy-Ado (10). The IC_{50} was at least 1000-fold higher in an MTAP-deficient cell line, indicating that the cytotoxicity observed with this compound is due to MTAP (10). Although Rv0535 can cleave this compound better than the human enzyme, the compound's low IC_{50} in human cells implies that 2-fluoro-5'-deoxy-Ado is not a suitable compound as a subversive substrate in mycobacteria.

Kung et al., used recombinant human MTAP that had been purified to homogeneity to perform an SAR using MTA analogs with various modifications on the ribose moiety. We estimated that Rv0535 recognized 5'-ethylthioadenosine as substrate and was comparable to MTA. However, human MTAP cleaved 5'-ethylthioadenosine with 50% of the MTA cleavage activity (8) suggesting a difference between the human and mycobacterial enzymes. To confirm this difference, the cleavage of 5'-ethylthioadenosine by Rv0535 needs to be investigated. Similar to Rv0535, araMTA and

2’-deoxy-MTA were poor substrates for human MTAP, indicating that both enzymes prefer a 2’-hydroxyl group in the *trans* position for activity.

A few analogs have been evaluated with partially purified human MTAP in the Parker lab and the preliminary results are summarized in Table 4. Of the 14 analogs tested, MTA was the best substrate, followed by 2-fluoro-5’-deoxy-Ado, which had 71% of the MTA cleavage activity. In contrast, 2-fluoro-5’-deoxy-Ado was a better substrate than MTA for *M. tuberculosis* MTAP, indicating that the active site of Rv0535 can better accommodate this compound compared to human MTAP. This could be accounted for by the presence of an Ala217 in Rv0535, whereas in the human homolog this amino acid is a valine, which has an additional methyl group compared to alanine.

As with Rv0535, the addition of a fluorine at the 2 position of Ade in the lyxofuranosyl compounds led to an increase in activity with human MTAP compared to the non-fluorinated compounds. However, these compounds were still poor substrates. The cleavage of MeP-dR by human MTAP was not seen, whereas this compound can be cleaved by Rv0535. However, these assays were performed at different concentrations (100 µM with human MTAP). For an accurate comparison of the cleavage of this compound by human MTAP, the assay needs to be repeated at 250 µM.

The cleavage of 9-(β-D-5-methyl-(*allo*)-ribofuranosyl)-2-fluoro-adenine and 9-(β-D-5-methyl-(*talo*)-ribofuranosyl)-2-fluoro-adenine by human MTAP was detected. Since the *talo* oriented compound had 23% of the MTA cleavage activity (7% of MTA with Rv0535) and the *allo* oriented compound had 4% of the MTA cleavage activity (1.4% of

MTA with Rv0535), our results indicated that both Rv0535 and the human enzyme preferred the *talo* orientation to the *allo* orientation.

Table 4. Human MTAP activity with nucleoside analogs

Compounds	**Activity** [a] nmol mg^{-1} hr^{-1}	**Relative activity** % of MTA
	Parker lab	
5'-Methylthioadenosine (MTA)	149,000 (5)	100
2-Fluoro-5'-deoxyadenosine	106,000 (3)	71
2-Fluoro-2'-deoxyadenosine	543 (2)	0.4
9-[β-D-Arabinofuranosyl]-2-fluoro-adenine	-- (2)	0
9-[α-L-Lyxofuranosyl]-adenine	200 (2)	0.1
9-[α-L-Lyxofuranosyl]-2-fluoro-adenine	7,400 (3)	5
6-Methylpurine-2'-deoxyribose	-- (2)	0
9-(5-Deoxy-β-D-ribofuranosyl)-6-methylpurine	1,040 (2)	0.7
9-(β-D-5-Phenylthio-ribofuranosyl)-6-methylpurine	81 (4)	<0.1
9-(β-D-5-Methyl-(allo)-ribofuranosyl)-6-methylpurine	-- (2)	0
9-(β-D-5-Methyl-(talo)-ribofuranosyl)-6-methylpurine	4 (2)	<0.1
9-(β-D-5-Methyl-(allo)-ribofuranosyl)-2-fluoro-adenine	6,000 (2)	4
9-(β-D-5-Methyl-(talo)-ribofuranosyl)-2-fluoro-adenine	34,300 (2)	23
9-[α-L-Lyxofuranosyl]-6-methylpurine	-- (2)	0
	Toorchen and Miller study (11)	
2'-deoxy-adenosine		No activity
2',3'-dideoxyadenosine		No activity
5'-deoxy-adenosine		48
Adenosine		0.1
	Savarese et al., study (10)	
2-Fluoro-5'-deoxyadenosine		44
	Kung et al., study (8)[b]	
5'-Methylthioadenosine (MTA)		100
5'-Ethylthioadenosine		50
5'-Methylthio-9-[β-D-arabinofuranosyl]-adenine		0.003
5'-Methylthio-2'-deoxy-adenosine		0.03

[a] number of determinations indicated parentheses

[b] calculated from the ratio of K_{cat}/K_m

-- Not detected

Summary

The results presented in this work indicated that some modifications were better tolerated as substrates than others. The nitrogen atom at the 3 and 7 positions were required for activity. The amino group at the 6 position was also important. Replacement of this amino group by a hydrogen atom abolished activity, while other groups such as methyl, mercapto, or methylmercapto, resulted in decreased activity. The effect of exocyclic substitutions at the 2-position or the 8-position of the Ade moiety was dependent on size, since the smaller atoms could lead to enhanced activity. This was true for analogs compared to either the Ado or the MTA scaffold. Further, the addition of a fluorine atom at the 2-position of the Ade moiety enhanced activity as compared to the parent analog. Moreover, our results indicated that the β-D-ribofuranosyl conformation of Ado was preferred. The *trans* conformation of the 2'- or 3'- hydroxyl groups and the *cis* orientation of the 5'-hydroxyl group were necessary for activity. Finally, the removal of the 5'-hydroxyl group of Ado led to an increase in activity, especially when combined with the 2-fluoro substitution. Since some of the compounds presented in this work have not been evaluated with human MTAP, an extensive SAR study of human MTAP could help identify differences between human and mycobacterial MTAPs. These differences could be exploited for the development of subversive substrates that can only be activated to a toxic compound by *M. tuberculosis* MTAP.

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SUMMARY AND CONCLUSIONS

Adenosine cleavage in *M. smegmatis*

Ado cleavage enzymes are known to exist in nature and include bacterial PNPs, AdoP, Ado nucleosidase, and MTAP. In humans, Ado can only be cleaved by MTAP. Because human MTAP has a high K_m and a low K_{cat} for Ado, the cleavage of Ado by this enzyme is considered largely inefficient (23, 53). Neither human nor mycobacterial PNP cleave Ado (15, 22, 49). Chen et al., and Parker et al., have observed the cleavage of Ado in *M. smegmatis* and *M. tuberculosis* crude cell extracts (16, 34), thereby suggesting a difference in the metabolism of Ado between human and mycobacterial cells. At that time, there was no evidence at the protein level of an AdoP, Ado nucleosidase, or MTAP in *M. tuberculosis*. Therefore, the goal of this project was to identify Ado cleavage enzyme(s) in mycobacteria. The identification and characterization of these enzymes could expose differences in Ado metabolism between mycobacterial and human cells that could be exploited for the development of new anti-TB drugs.

M. smegmatis is a non-pathogenic and fast growing mycobacterium. In preliminary studies, *M. smegmatis* was also shown to have an Ado cleavage activity, similar to *M. tuberculosis*. Therefore, *M. smegmatis* was used to study the endogenous mycobacterial Ado cleavage enzymes. *M. smegmatis* cells were collected and lysed, after which their cytoplasmic content was loaded on successive purification columns. The elution profile from the first purification column indicated the presence of two enzymes that could cleave Ado. Further purification and characterization of these enzymes showed that one enzyme was Ado-PNP and the other was MTAP.

M. smegmatis Ado-PNP could cleave Ado, 2'-deoxy-Ado, Ino and guanosine. It could also cleave F-Ado and methyl-Ado, two Ado analogs. Thus, the substrate specificity of this enzyme indicated that Ado-PNP was different from the trimeric form of *M. smegmatis* PNP, which does not accept Ado as a substrate. The low expression of Ado-PNP in mycobacteria was a significant hurdle in this work. While the purified Ado-PNP sample was free of other purine enzymes, a single protein band corresponding to this enzyme could not be identified on an SDS-PAGE gel stained with either Coomassie or Silver Stain reagent. Therefore, the gene that encodes for Ado-PNP was not identified.

The second enzyme (MTAP) was also characterized. The preferred substrate for this enzyme was MTA, although the cleavage of Ado and F-Ado was also detected at 16% and 19% of the MTA cleavage activity, respectively. The enzyme was only active in the presence of phosphate, indicating that the enzyme was phosphorylase. Mass spectrometry analysis of a protein band from an SDS-PAGE gel indicated that the enzyme was MTAP (MSMEG_0990). This was unexpected since all studied MTAPs belong to either the Eukarya or Archaea family. Since *M. smegmatis* is closely related to *M. tuberculosis*, it is likely that Ado-PNP and MTAP identified in *M. smegmatis* are responsible for the Ado cleavage observed in *M. tuberculosis*.

Rv0535, *M. tuberculosis* MTAP

The isolation and characterization experiments of the endogenous activity presented in Chapter 1 indicated that *M. smegmatis* MTAP is expressed. We have also noted the phosphate-dependent cleavage of MTA in crude *M. tuberculosis* cell extract,

which is an activity that can only be attributed to MTAP. Thus, it is expected that *M. tuberculosis* also expresses endogenous MTAP, the product of the *Rv0535* gene.

Rv0535 was expressed in *E.coli* BL21 (DE3) cells and the protein was purified using a nickel affinity column. The purified protein was free of contaminating purine metabolic enzymes, and was biochemically characterized as *M. tuberculosis* MTAP. While MTA was the preferred substrate, MTAP could also cleave Ado and SAH with 2% and 0.8% of the MTA cleavage activity, respectively. Cleavage of the other natural purines, the pyrimidines, or 5'-methylthioinosine was not detected. The molecular mass of the native enzyme was determined, which indicated that *M. tuberculosis* MTAP was a dimer. This is interesting since all other MTAPs that have been previously characterized are trimers or hexamers (2, 39).

The work presented here shows conclusive evidence of an MTAP in both *M. smegmatis* and *M. tuberculosis*. To evaluate whether other bacteria express MTAP, the Basic Local Alignment Search Tool (BLAST) function of Uniprot (52) was used to compare the amino acid sequence of *M. tuberculosis* MTAP (Rv0535) to other sequence databases. This strategy identified other probable bacterial MTAPs based on sequence homology and suggested that while mycobacterial MTAPs were the first bacterial MTAPs to be identified, it is possible that other bacteria also express MTAP. Further, despite having similar functions, MTAP sequences are different from MTAN sequences.

A sequence alignment showed more than 92% sequence identity (196 identical amino acid positions) between MTAP from *M. smegmatis* and *M. tuberculosis*, suggesting that the two mycobacterial MTAPs could share similar properties. In contrast,

there was less than 36% sequence identity (93 identical amino acid positions) between *M. tuberculosis* and human MTAP, which could indicate that mycobacterial and human MTAP may differ in substrate specificity. A homology model of *M. tuberculosis* MTAP was built using the crystal structure of human MTAP as a template. The *M. tuberculosis* model suggested that the active site of *M. tuberculosis* MTAP contained a purine base binding pocket, a ribose binding pocket, and a phosphate binding pocket. Further, the model identified 5 active site amino acid residues that were different between human and *M. tuberculosis* MTAP. These amino acids were in close proximity to or participated in the phosphate-binding site (Ser14 and Cys88), the purine-binding site (Val178), or the 5'-methylthioribose-binding site (Ser 14, Ala217, and Phe221). Thus, it is possible that modifications to the Ade moiety or ribose moiety of the substrate could result in different substrate specificity between the mycobacterial and human MTAP.

Substrate-activity relationship study of Rv0535

A structure-activity relationship study of Rv0535 using MTA and Ado analogs was performed to better understand the topography of the active site of Rv0535. To this end, 49 nucleoside analogs with modifications to the purine base or the ribose moiety were investigated as substrates of Rv0535. Two parent scaffolds were used, namely MTA and Ado. Two nucleoside analogs, 2-fluoro-MTA and 2-fluoro-5'-deoxy-Ado, were better substrates than MTA. In general, the effect of exocyclic substitutions at the 2-position or the 8-position of the Ade moiety was dependent on size, since smaller atoms, such as fluorine, could lead to enhanced activity. Further, the endocyclic nitrogen atoms at the 3- and 7- positions of Ade were critical for activity. The amino group at the 6 position was also important. Replacement of this amino group by a hydrogen atom

abolished activity, while other groups such as methyl, mercapto, or methylmercapto resulted in decreased activity. Our results suggested that the β-D-ribofuranosyl conformation of Ado was preferred. The proper orientation of the hydroxyl groups on the ribose moiety of Ado was also important, with the 2'- and 3'- hydroxyl in the *trans* orientation, and the 5'-hydroxyl group in the *cis* orientation. Finally, the removal of the 5'-hydroxyl group of Ado led to an increase in activity, especially when combined with an exocyclic 2-fluoro addition. Since some of the compounds presented in this work have not been evaluated with human MTAP, an extensive SAR study of human MTAP could help identify differences between human and mycobacterial MTAPs.

Methionine pathway in *M. tuberculosis*

A more extensive view of the methionine pathway is shown in Figure 1 and the enzymes involved in this pathway are listed in Table 1. Methionine is converted to S-adenosyl-L-methionine (SAM, also known as AdoMet) by SAM synthase (also known as methionine adenosyltransferase) in a reaction that uses 1 ATP molecule. The *M. tuberculosis* and *M. smegmatis* homologs of this enzyme have been cloned and characterized (9). SAM can be decarboxylated (reaction 2), but while this activity has been reported in *M. bovis* (37), the *M. tuberculosis* homolog has not been shown to exist either by sequence homology or at the protein level. The importance of decarboxylated SAM for polyamine synthesis and the unlikelihood that a mycobacterium would have conserved enzymes downstream of decarboxylated SAM such as spermidine synthase and MTAP, argue that an SAM decarboxylase also exists in *M. tuberculosis*, but has yet to be discovered.

SAM can also be demethylated by methyltransferases (reaction 7) to produce S-adenosyl-L-homocysteine (SAH). Several SAM-dependent methyltransferases are known to exist in *M. tuberculosis* and have been implicated in the biosynthesis of mycolic acids (60, 61, 10). An SAH hydrolase cleaves SAH (reaction 8) to produce Ado and homocysteine, which can be converted to methionine by methionine synthase (reaction 12). Homocysteine can also be converted in a two step process to cysteine, another sulfur containing amino acid (reaction 9). Thus, the methionine pathway is linked to the formation of cysteine, which can be used as a source of sulfur in mycobacteria. However, because methionine was shown to be the preferred sulfur source for pathogenic mycobacteria (56), it is possible that altering the methionine pathway could affect sulfur metabolism.

In the synthesis of polyamines, the aminopropylic group of decarboxylated SAM is donated to spermidine by spermine synthase (to produce spermine) or to putrescine by spermidine synthase (to produce spermidine). In the process, MTA is generated (Reaction 3). A spermidine synthase has been annotated in *M. tuberculosis*, and given that this activity has been observed in *Mycobacteria* spp. (36, 38), the endogenous protein could exist in *M. tuberculosis*. MTA can be metabolized by either reaction 4 (MTAP) or 5 (MTAN). This reaction produces Ade, which can be used in purine metabolism. The phosphate-dependent cleavage of MTA in cell-free extracts of *M. tuberculosis* was observed, indicating the endogenous expression of MTAP. Recombinant *M. tuberculosis* MTAP (Rv0535) was expressed, purified and characterized. Thus, our results argue for the presence of a methionine salvage pathway in *M. tuberculosis*.

Prior to this work, MTAN was thought to be the enzyme responsible for the metabolism of MTA in mycobacteria. However, while MTAN has been annotated based on sequence homology, its existence has not been shown at the protein level. Most characterized MTANs are described as having a dual function because they can hydrolytically cleave MTA and SAH. As opposed to SAH hydrolase which cleaves SAH to produce homocysteine and Ado (reaction 8), MTAN cleaves SAH to produce Ade and S-ribosylhomocysteine.

There is no evidence for the existence of a 5'-methylthioribose kinase (enzyme 6 in Figure 1) in *M. tuberculosis*. In other bacteria that metabolize MTA via MTAN but lack this kinase, the resulting methylthioribose is thought to be excreted from the cell (32). In bacteria that possess the kinase, such as *B. subtilis*, it was shown that carbon, sulfur, or nitrogen deprivation can enhance the synthesis of this kinase (47). Therefore, the presence of methylthioribose kinase in *M. tuberculosis* needs to be investigated under different growth conditions.

While our results suggest the existence of a functioning methionine salvage pathway in *M. tuberculosis*, it is to be noted that MTAP is the only enzyme that has been isolated downstream of SAM decarboxylase since spermidine synthase and MTAN are both inferred from sequence homology. In *B. subtilis*, seven enzymes responsible for the conversion of 5'-methylthioribose-1-phosphate to methionine have been identified (45, 46). However, sequence homology did not reveal the presence of these enzymes in *M. tuberculosis*. More in-depth studies are needed to identify the missing enzymes in the methionine pathway of mycobacteria.

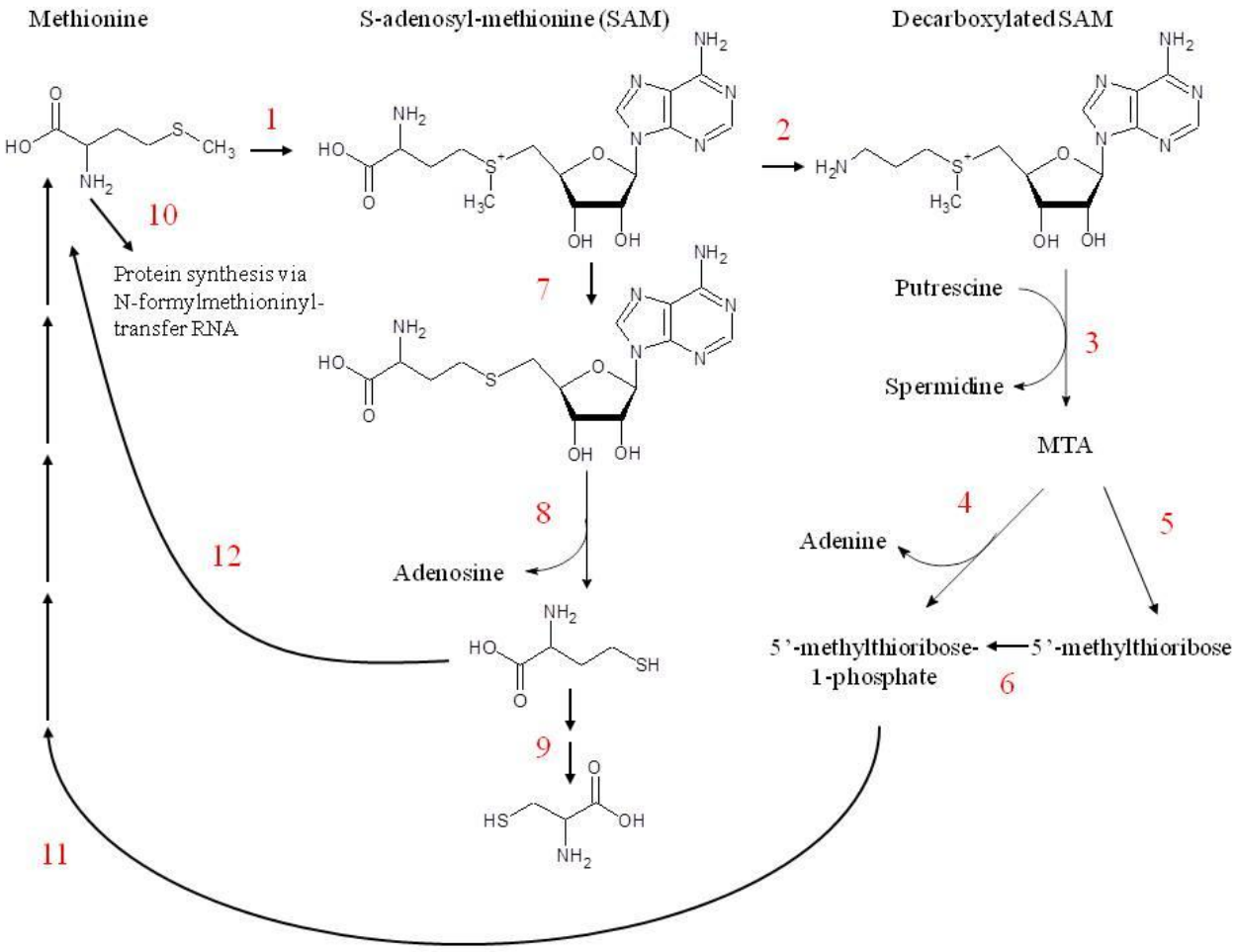

Figure 1. The Methionine pathway

Enzymes: 1. Methionine adenosyltransferase; 2. SAM decarboxylase; 3. Spermidine synthase; 4. MTAP; 5. MTAN; 6. 5'-methylthioribose kinase; 7. Methyltransferases; 8. SAH hydrolase; 9. Many; 10. Many; 11. Many; 12. Methionine synthase

Table 1. Enzymes involved in the metabolism of methionine in *M. tuberculosis*

Reaction	*Gene*	E.C.	Name	Status [a]
1	*metK (Rv1392)*	2.5.1.6	Methionine adenosyltransferase or SAM synthetase	Evidence at protein level (9)
2		4.1.1.50	SAM decarboxylase	Not annotated
3	*speE (Rv2601)*	2.5.1.16	Spermidine synthase	Inferred from homology
4	*mtap (Rv0535)*	2.2.4.28	MTA phosphorylase	Evidence at protein level (13, 14)
5	*mtn (Rv0091)*	3.2.2.16	MTA/SAH hydrolase	Inferred from homology
6		2.7.1.100	S-methyl-5-thioribose kinase	Not annotated
7	Many	2.1.1.37	Methyltransferases	
8	*sahH (Rv3248c)*	3.3.1.1	SAH hydrolase	Evidence at protein level (41)
9	Many including *cysM2 (Rv1077)*	4.2.1.22	Cystathionine β-synthase	
10	Many including *fmt (Rv1406)*	2.1.2.9	Methionyl-tRNA formyltransferase	Inferred from homology
11	Many			Not annotated
12	*metE (Rv1133c)*	2.1.1.14	Methionine synthase	Inferred from homology

[a] References indicated in between parentheses

Implication of culture conditions on purine gene expression

While *M. tuberculosis* has a functional *de novo* purine pathway (29, 31), *de novo* purine synthesis is expensive. During *de novo* synthesis, inosine monophosphate (IMP) is synthesized from 5-phospho-α-D-ribosyl-1-diphosphate (PRPP) in 10 reactions that require 4 ATPs. IMP is converted to adenosine monophosphate (AMP) and guanosine monophosphate (GMP), which can then be phosphorylated further for DNA and RNA synthesis. On the other hand, the purine salvage pathway uses available nucleobases to produce one mole of the respective monophosphate from one mole of PRPP and one ATP. Thus, *M. tuberculosis* could use the less expensive purine salvage pathway for rapid energy generation, when ATP is not readily available, or to salvage exogenous nucleobases from the host.

In purine metabolic studies, the intracellular life of the mycobacterium has to be considered. In the host, the mycobacterium experiences a nutrient-poor and oxidizing environment. Moreover, the mycobacterium can alter its metabolism once in the host, and can persist for several years and decades. Therefore in studying purine metabolism, it is important to consider the contribution of exogenous nutrients from the host cells in purine salvage, and the synthesis of required molecules through the *de novo* pathway. In the first chapter, we identified Ado-PNP in *M. smegmatis*, an activity that had also been observed in cell-free extracts of *M. tuberculosis*. We also noted the variable expression of this endogenous activity, which suggests that the expression of this enzyme is dependent on an unknown variable. Since *M. leprae* can salvage Ado, Ade, and hypoxanthine from media (55), the unknown variable could be the availability of purines in the media. Since our cultures were grown under aerobic conditions in Middlebrook media supplemented

with OADC (oleic acid, albumin, dextrose, and catalase), the effect of exogenous nucleosides and nucleobases on purine enzyme expression was not assessed. Similarly, the endogenous expression of MTAP needs to be verified under nutrient starvation, in dormancy models, and in infected macrophages.

Implications for drug discovery

The motivating premise behind this project is the discovery of a drug target to treat TB. Differences in purine metabolism between humans and viruses have been exploited to develop clinically relevant nucleoside analogs and it is possible that a similar approach could be used for the rational design of anti-TB drugs. The fact that *M. tuberculosis* and humans both express an MTAP does not mean that MTAP is not a drug target. For example, the herpes simplex virus thymidine kinase accepts acyclovir as a better substrate than human nucleoside kinases. The resulting phosphorylated acyclovir can act as a DNA chain terminator and selectively inhibit viral DNA polymerase. Similarly, anti-HIV nucleoside analogs selectively inhibit HIV reverse transcriptase even though human cells express numerous nucleoside kinases and DNA polymerases. This strategy has also been explored in parasites, such as *T. brucei brucei* and *T. brucei rhodesiense*. Infections with these trypanosomes can be cured by using the MTA analog, 5'-hydroxyethythioadenosine, which is a poor substrate for human MTAP (4). This analog is selectively cleaved by trypanosomal MTAP to produce hydroxyethylthioribose-1-phosphate, which can affect the methionine pathway including polyamine synthesis (3). The SAR of Rv0535 could be used to identify compounds that can be metabolized by Rv0535 but not human MTAP. Therefore even poor substrates of Rv0535, if selectively metabolized by the mycobacterial enzyme, could be explored as potential medicinal

compounds. This also holds true for Ado-PNP with the added advantage that human cells do not express an Ado-PNP. However, the first step to using Ado-PNP as a potential drug target would require the identification of its gene, a process that has been unsuccessful.

Proposed therapeutic approaches in regards to MTAP

The cleavage of MTA by MTAP yields Ade and 5'-methylthioribose-1-phosphate. A subversive approach would utilize MTAP to cleave a non-toxic MTA analog to produce a toxic moiety that can poison mycobacterial cells. This toxicity could reside in the Ade moiety as in the case of the highly toxic 2-fluoro-adenine, or in the sugar moiety, as in the case of 5'-hydroxyethythioadenosine. Moreover, a poisoned ribose moiety could be phosphorylated to a triphosphate form to interfere with DNA and RNA synthesis or affect the generation of methionine. The selection of the toxic moiety will be largely affected by substrate differences between human and mycobacterial MTAP.

The specific inhibition of *M. tuberculosis* MTAP could be considered as a drug development strategy. This would lead to an increase in MTA, the effect of which is unclear, since MTA could be shuttled out of the cell. However, it is possible that an increase in intracellular concentration of MTAP could lead to feedback inhibition of spermidine synthase, a process that has been shown to exist in mammalian systems (33, 40). Further, inhibition of MTAP could lead to the disruption of the methionine salvage pathway, if it shown to exist in *M. tuberculosis*.

The components of the methionine pathway are important for several reasons. First, MTAP is an important contributor of Ade, which participates in purine metabolism.

Second, the sugar moiety of MTA has been shown to participate in the synthesis of lipoarabinomannan (54), which is an important component of the mycobacterial cell wall. Third, MTAP could play a role in maintaining MTA levels low to prevent negative feedback inhibition of polyamine synthesis. Further, methionine is the preferred source of sulfur for mycobacteria and its salvage could be important under nutrient-deprivation. Methionine can be used as a precursor for the synthesis of cysteine. Moreover, methionine is also a precursor for SAM, which is important in the synthesis of mycolic acids. Thus, disrupting the methionine pathway could have implications in drug discovery.

Future directions

Ado-PNP

We have shown the activity of Ado-PNP in *M. smegmatis* but have not been able to identify the gene responsible for this activity. Identification of this gene could lead to the expression of the recombinant protein, which could be used to perform an SAR study with Ado analogs. The SAR study would be valuable for our understanding of this enzyme and its potential as a drug target. While scaling up the *M. smegmatis* and *M. tuberculosis* cultures could result in the purification to homogeneity of Ado-PNP, this approach would require a considerable amount of starting culture, since we have been so far been unsuccessful with 5-liter cultures. Therefore, genetic techniques must be used. As mentioned in Chapter 1, sequence homology predicts that *Rv3393* encodes for a probable nucleoside hydrolase. While Ducati et al., have suggested that this enzyme is an Ado hydrolase (21), preliminary observations in the Parker lab suggests that it can

hydrolytically cleave uridine, but not Ado. Therefore, a full characterization of this enzyme is needed.

Another candidate gene is *Rv2293c*, which has been annotated as a conserved hypothetical protein that participates in the nucleoside metabolic process. This gene was amplified and cloned in a pvv16 vector, which carries a hygromycin resistance marker. The resulting recombinant vector was transformed in *M. smegmatis*. Unfortunately, this attempt was unsuccessful. A similar strategy could be employed, using the pET28 vector, and transformed into *E. coli* BL21 (DE3) cells. Using *E. coli* as the expression system is advantageous because unlike *M. smegmatis*, *E. coli* does not express Ado-PNP, and would provide a clean background for the expression of Ado-PNP. The recombinant enzyme could then be purified and characterized.

To investigate the cause of the variability in expression of Ado-PNP, *M. smegmatis* and *M. tuberculosis* cultures could be grown in media supplemented with various nutrients, including Ado and Ade. Deoxycoformycin could also be used to inhibit mycobacterial Ado deaminase in mycobacteria. In theory, this would prevent the conversion of Ado to Ino and in order to metabolize Ado, expression of Ado-PNP should increase. Moreover, radiolabeled nucleotides could be used in media to assess the exogenous contribution of purine nucleosides and nucleobases to mycobacterial purine metabolism. These experiments could also be performed in infected macrophages. In addition, experiments could be set up to reflect hypoxic conditions, nutrient starvation, and oxidative stress, and the resulting cultures could be tested for the expression of Ado-PNP.

Previous unpublished work of the Parker lab has suggested the presence of an Ado-cleavage activity in media in which mycobacterial cells had been grown. Since our studies of Ado-PNP focused on the cytoplasmic fraction of cell extracts, this topic needs to be revisited. The presence of Ado cleavage activity in media could be due to cell lysis or enzyme secretion. If the enzyme is secreted, it would imply a function for salvaging purines from host cells.

M. tuberculosis MTAP

As discussed in Chapter 2, recombinant MTAP was unstable. It is possible that the polyhistidine tag was responsible for this instability. The use of a different expression system or the removal of the histidine tags using peptidases could resolve this instability issue. A stable purified sample is important to produce consistent and accurate activity assays. While the results presented in Chapter 2 are solid, the variability in activity could affect future of kinetic work.

In Chapter 2, we presented early inhibition results with the potent transition state analog, MT-DADMe immucillin A. Complete kinetic and inhibition studies could be performed. The results could then be compared to the results obtained with human MTAP, since this compound has been evaluated in human cell lines and mice (7). This compound as well as other transition state analogs that are potent inhibitors of Rv0535 could be used to investigate the effect of MTAP inhibition on mycobacterial cell growth. In addition, since MTAN is also predicted to exist in mycobacteria, it is possible that the inhibition of MTAP could lead to the expression of MTAN.

Rv0091 has been expressed in the Parker lab and preliminary results indicated that it encodes for MTAN. The relationship of MTAP and MTAN is interesting. The biochemical characterization of recombinant MTAN could identify the properties of this enzyme. This information could be used to purify MTAP from MTAN in a crude cell extract of *M. tuberculosis* and would clarify whether MTAP and MTAN are expressed at the same time. Nutrient starvation and supplementation experiments as well as macrophage studies could aid in our understanding of the expression of these two enzymes in *M. tuberculosis*.

In Chapter 3, the initial results for an SAR study are presented. Only 2 compounds had enhanced activity compared to MTA cleavage. Compounds that were not substrates, such as 8-aza-9-deaza-Ado, could be evaluated as inhibitors of Rv0535. Additional MTA analogs also need to be evaluated to obtain a better topology of the active site of the enzyme at the 5'-methylthio region of the substrate. Further, most of the Ado analogs used in Chapter 3 have not been evaluated against human MTAP. To aid in identifying differences in substrate specificity between human and *M. tuberculosis* MTAP, the SAR study presented in Chapter 3 could be reproduced using human MTAP.

Provided that MTAN exists at the protein level in *M. tuberculosis*, an SAR study could be conducted on the recombinant enzyme and compared to the SAR study of MTAP, thereby providing more information on the methionine pathway enzymes as drug targets. MTAN is one of several enzymes in the methionine pathway whose existence at the protein level needs to be shown. The results presented here suggest the existence of a methionine salvage pathway. However, the enzymes responsible for the final conversion of methylthioribose-1-phosphate to methionine have not been shown to exist in *M.*

tuberculosis. The identification and characterization of the enzymes shown in Figure 1 would lead to a better understanding of the methionine pathway, which in turn could provide some interesting insight to sulfur metabolism.

M. tuberculosis MTAN is also of interest for a different reason. Bacterial quorum sensing is the phenomenon by which bacterial cells communicate, and this is mediated by at least two autoinducer molecules, AI-1 and AI-2. Quorum sensing has been involved in biofilm formation, antibiotic resistance, and virulence. In some bacteria, AI-2 synthesis is also sensitive to carbohydrate depletion, osmolarity, and pH (50). In bacteria such as *E.coli*, *Salmonella typhimurium*, and *Vibrio cholera*, the *in vitro* synthesis of AI-2 from SAH only required two enzymes, namely Pfs and LuxS (43, 51). Pfs is another name for MTAN and functions in two ways. First, it can cleave SAH to produce S-ribosylhomocysteine, which can then be cleaved by LuxS to make AI-2 molecules(s). Second, MTAN is thought to keep MTA and SAH levels low since these molecules are inhibitors of SAM-requiring reactions (8, 19). While autoinducers or quorum sensing have not been reported in *M. tuberculosis*, biofilm formation has been seen in *M. avium* upon exposure to autoinducer molecules (24). It would be interesting to see the effect, if any, of autoinducer molecules on *M. tuberculosis*.

The structure of one AI-2 molecule is a 5 carbon backbone with 2 hydroxyl groups and 2 ketone groups (4,5-dihydroxy-2,3-pentadione) and is derived from the ribose moiety of SAH. Since the fate of the ribose moiety after MTA cleavage by MTAP or MTAN is not known in *M. tuberculosis*, some exploratory experiments using a radiolabeled MTA analog could be used to track the metabolism of 5'-methylthioribose-

1-phosphate (produced from MTAP) or 5'-methylthioribose-1-phosphate (produced from MTAN).

Conclusions

Purine metabolism is an attractive target for TB drug discovery since a purine-based drug would provide a novel mechanism of action and therefore MDR and XDR *M. tuberculosis* strains would likely be sensitive to Ado analogs. Further, Barrow et al., have shown that methyl-Ado was toxic to latent mycobacteria, indicating that an Ado analog could work against both active and latent TB infections. Therefore, our discovery of two Ado cleavage activities in mycobacteria could contribute to an anti-TB drug development effort. The first enzyme, Ado-PNP, is not expressed in human cells. Therefore, this mycobacterial enzyme activity could be exploited to selectively activate a non-toxic Ado analog to a toxic product in mycobacterial cells only. Future studies are needed to identify the gene that encodes for Ado-PNP and to perform a complete SAR on this enzyme. The second enzyme, MTAP, has a human homolog. However, mycobacterial MTAP could still be used as a drug target. In Chapter 3, we presented the initial results of an SAR study, which could be used to identify differences in substrates for human and mycobacterial MTAPs. These differences could then be exploited for the rational development of a nucleoside analog that can act as a subversive substrate, being activated to a toxic compound by the mycobacterial enzyme only. Additionally, while MTAP is a component of the methionine pathway, other enzymes in that pathway need to be investigated.

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