The metabolism of 2-methyladenosine in
Mycobacterium smegmatis

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INTRODUCTION

With one-third of the world population infected with Mycobacterium tuberculosis and 2-9 million deaths annually, M. tuberculosis is one of the world’s most important pathogens (Arachi, 1991; Butler, 2000). Furthermore, the World Health Organization has found M. tuberculosis that was resistant to first-line anti-tuberculosis drugs in all 35 countries and regions surveyed (Pablos-Mendez et al., 1998). Due to the emergence and distribution of multi-drug resistant M. tuberculosis, it is necessary to develop new drugs with novel mechanisms of action.

The Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) was established by the National Institute for Allergy and Infectious Diseases to stimulate research and design of new anti-tuberculosis drugs (Orme, 2001). Through this mechanism we have discovered numerous adenine (ade) and adenosine (ado) analogues with selective activity against M. tuberculosis. Although nucleoside analogues have not been considered as antibacterial agents, they have been used successfully in the treatment of cancer and viral infections for many years. It is likely that the molecular targets of these agents will inhibit enzymes that are not targets for the existing anti-mycobacterial agents and, thus, represent a new class of drugs with novel mechanisms of action against M. tuberculosis. Therefore, cross-resistance with existing anti-mycobacterial drugs is not expected with these agents.

In terms of potency and selectivity, one of the better agents in this class of compounds was 2-methyladenosine (methyl-ado) has demonstrated selective activity against Mycobacterium tuberculosis, which indicates that differences in the substrate preferences between mycobacterial and human purine metabolic enzymes can be exploited to develop novel drugs for the treatment of mycobacterial diseases. Therefore, in an effort to better understand the reasons for the antimycobacterial activity of methyl-ado, its metabolism has been characterized in Mycobacterium smegmatis. In a wild-type strain, methyl-ado was phosphorylated by adenosine kinase to methyl-AMP, which was further converted to methyl-ATP and incorporated into RNA. In contrast, a mutant strain of M. smegmatis was isolated that was resistant to methyl-ado, deficient in adenosine kinase activity and was not able to generate methyl-ado metabolites in cells treated with methyl-ado. These results indicated that phosphorylated metabolites of methyl-ado were responsible for the cytotoxic activity of this compound. Methyl-ado was not a substrate for either adenosine deaminase or purine-nucleoside phosphorylase from M. smegmatis. Treatment of M. smegmatis with methyl-ado resulted in the inhibition of ATP synthesis, which indicated that a metabolite of methyl-ado inhibited one of the enzymes involved in de novo purine synthesis. These studies demonstrated the importance of adenosine kinase in the activation of methyl-ado to toxic metabolites in M. smegmatis.

Keywords: purine metabolism, adenosine, adenosine kinase
sine (methyl-ado; MIC of 3 µg ml\(^{-1}\) for \(M.\) \textit{tuberculosis}\) H37Rv; \textit{Chen et al.}, 2000; \textit{Barrow et al.}, 2001). The salvage and metabolism of natural purines in mycobacteria (reviewed by \textit{Barclay} \\& \textit{Wheeler}, 1989) is similar to that in human cells. However, very little is known about the substrate preferences of the mycobacterial enzymes involved in ado metabolism. There are four known enzymes that could metabolize ado and its analogues: adenosine kinase (AK), adenosine deaminase (AD), purine-nucleoside phosphorylase (PNP) and nucleoside hydrolase. Although AK activity has been measured in \textit{Mycobacterium leprae}, \textit{Mycobacterium microti} and \textit{Mycobacterium avium} (\textit{Wheeler} 1987a, b), very little has been done to characterize the other mycobacterial enzymes that could metabolize ado analogues. Adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase activities have also been measured in \textit{M. leprae}, \textit{M. microti} and \textit{M. avium} (\textit{Wheeler} 1987a, b) and their genes (apt and hpt, respectively) have been identified in the \textit{M. tuberculosis} genome (\textit{Cole et al.}, 1998). These activities could also be involved in the metabolism of methyl-ado, if methyl-ado is found to be a substrate for the purine cleavage enzymes and/or AD. The PNP and AD genes have also been identified in the \textit{M. tuberculosis} genome (\textit{Cole et al.}, 1998). However, the AK gene in \textit{M. tuberculosis} has not yet been identified (\textit{Mizrahi et al.}, 2000).

In the current studies, we utilized biochemical and genetic approaches to characterize the metabolism of methyl-ado in \textit{M. smegmatis} in order to better understand its mechanism of action. \textit{M. smegmatis} is a fast-growing mycobacterium that has been widely used as a model for studying the physiology and genetics of pathogenic mycobacteria. It is hoped that increased knowledge about purine metabolism in mycobacteria and the mechanism of action of this agent will aid in the development of new selective drugs for the treatment of \textit{M. tuberculosis} and other mycobacterial diseases.

**METHODS**

**Bacterial strains and culture methods.** \textit{M. smegmatis} strain mc\textsuperscript{155} was obtained from the American Type Culture Collection. \textit{M. smegmatis} strains were grown in Middlebrook 7H9 medium supplemented with ADC (Difco Laboratories) and 0.05% Tween 80. The number of cells was determined by counting colonies on plates of Middlebrook 7H10 agar with OADC (Difco Laboratories) enrichment. The MIC for methyl-ado against \textit{M. smegmatis} based on an agar dilution assay was 100 µg ml\(^{-1}\). Methyl-ado-resistant \textit{M. smegmatis} mutants were generated by transposon mutagenesis using the EZ::TN (\langle KAN-2\rangle) Tnp Transposome Kit (Epigenic Technologies) according to the manufacturer’s instructions. Kanamycin was added to a final concentration of 25 µg ml\(^{-1}\). The insertion of the transposon was confirmed by PCR using Tn-F1 (5’-AGTCTCCTTTCCGACCATTCAAG-3’) and Tn-F2 (5’-TCCGACTCGTCCAACTCATAC-3’) as primers that specifically amplify 429 bp of the kanamycin-resistance gene in the EZ::TN (\langle KAN-2\rangle) transposon. The transposition efficiency of the transposome was 124 X 10\(^{6}\) per µg DNA. One mutant strain (SR101) was selected for further characterization. Spontaneous methyl-ado, F-ado (2-fluoroadenosine) and F-ade (2-fluoroadenine) resistant \textit{M. smegmatis} mutants were also selected by picking colonies that grew on Middlebrook 7H10 agar containing 200 µg ml\(^{-1}\) methyl-ado, F-ado or F-ade. The mutants appeared with a frequency of approximately 1 in 10\(^{6}\) cells. The F-ado resistant mutant was also resistant to methyl-ado and \textit{vice versa}. However, the F-ado resistant mutant remained sensitive to methyl-ado and F-ado, and the methyl-ado/F-ado resistant mutants were still susceptible to F-ade. Two mutant strains (SR1201 and SR1301) that represent resistant mutants for F-ade and F-ado (\textit{respectively}) were isolated for further characterization. Methyl-ado, F-ado and F-ade were synthesized in our laboratories using procedures similar to those described by \textit{Hirota et al.} (1992) and \textit{Montgomery \\& Hewson} (1960).

**Enzyme assays.** \textit{M. smegmatis} cells were harvested in the late-exponential growth phase and resuspended in 0.1 M Tris/HCl buffer (pH 7.4) and 1 mM DTT. The cells were homogenized using a Mini-Beadbeater from BioSpec Products. Particulate matter was removed by centrifugation and the supernatant was dialyzed twice against the same buffer (500 times the volume of the extract). Protein concentration was determined by the Bradford (1976) assay using bovine serum albumin as the standard. [2,8-\(^{3}H\)]adenosine and [8-\(^{3}H\)]methyladenosine were obtained from Moravek Biochemicals. All of the enzyme assays were performed at least twice, and an appropriate amount of extract was used to obtain a linear increase in product formation with respect to time.

AK activity was determined as the amount of \(^{3}H\)AMP or \(^{3}H\)methyl-AMP formed from [\(^{3}H\)]adenosine or [\(^{3}H\)]methyladenosine at 37 °C, respectively. The reaction mixture (1 ml) consisted of 100 mM Tris/HCl (pH 7.4), 5 mM MgCl\(_{2}\), 20 µM deoxycoformycin (an inhibitor of AD activity), 2 mM ATP, 2 mM DTT, 20 µM [2,8-\(^{3}H\)]adenosine or [8-\(^{3}H\)]methyladenosine (1000 Ci mol\(^{-1}\)); 37 TBq mol\(^{-1}\)) and crude extract. The reactions were initiated by addition of enzyme and were incubated at 37 °C. Samples (50 µl) were taken at various time points and mixed with 10 µl 0.1 M EDTA to terminate the reaction. The mixtures were transferred to a 2.5 cm DE-81 disc, and the discs were batch washed three times in 1 mM ammonium acetate (pH 5.0), dried and placed in a scintillation vial for determination of radioactivity.

AD activity was assayed in mixtures that contained 0.1 M Tris/HCl (pH 7.4), 100 µM ado and 100 µg crude enzyme extract ml\(^{-1}\). The reaction was started by addition of the enzyme extract and was carried out at 37 °C. Samples (100 µl) were withdrawn at 15 min intervals and the reaction was stopped by heating at 100 °C for 5 min. The product of the reaction, inosine (ino), was separated from ado using reverse-phase HPLC (5 µm BDS Hypersil C-18 column, 150 × 4.6 mm; Keystone Scientific). The mobile phase was a 30 min linear gradient from 1% to 5% acetonitrile in 10 mM ammonium dihydrogen phosphate (pH 4.5) at a flow rate of 1 ml min\(^{-1}\). Ado and ino were detected by their absorbance at 260 nm as they eluted from the column.

PNP activity was assayed by monitoring the consumption of \(^{3}H\)methyladenosine (50 µCi, 3.4 MBq) added to reaction mixtures containing 400 nM Tris/HCl (pH 8.0), 30 mM phosphate, 100 µg crude enzyme extract ml\(^{-1}\), and 100 µM nucleoside (ino, ado or methyl-ado). The reaction was started by addition of the enzyme extract and was carried out at 25 °C. Aliquots were withdrawn at various times and the reaction was stopped by heating at 100 °C for 5 min. The products of the reaction [hypoxanthine, ade and 2-methyladenine (methyl-ade)] were separated from the substrates using reverse-phase HPLC as described above, except that the mobile phase was 5% acetonitrile in 50 mM
ammonium dihydrogen phosphate buffer (pH 4-5). Deoxycoformycin (10 μM) was included in the experiments with ado to inhibit AD activity. The substrates and products were detected as they eluted from the column by their absorbance at 260 nm.

APRT (adenine phosphoribosyltransferase) activity was assayed as described by McClarty & Fan (1993) with slight modifications. The reaction mixture consisted of 0.1 M Tris/HCl buffer (pH 7.4), 5 mM MgCl₂, 1 mM 5-phosphoribosyl 1-pyrophosphate, 10 μM [U⁻³⁵Cl⁻]adenine (100 Ci mol⁻¹) and 100 μg crude extract ml⁻¹. The reaction was initiated by the addition of the enzyme extract and then incubated at 37 °C. Samples (50 μl) were collected at 20 min intervals and spotted onto DE-81 discs, which were washed and counted for radioactivity as described for the AK assay.

**Uptake of **[^H]**labelled compounds.** The rate of uptake of [2,8-[^H]]adenosine and [8-[^H]]methyl-adenosine was determined by the ‘oil-stop’ method described by Paterson *et al.* (1981) with slight modifications. [2,8-[^H]]adenosine or [8-[^H]]methyladenose (10 μM; 100 Ci mol⁻¹) were added to exponentially growing cells (~ 5 x 10⁶ cells ml⁻¹). Samples (0.2 ml) were collected at 30 s intervals for 5 min and transferred to microcentrifuge tubes containing 0.3 ml Nycosil M25 oil (Nye Lubricants). The medium and cells were immediately separated by a 30 s centrifugation at 16000 g, which terminated the uptake. The aqueous and oil layers were removed by aspiration and the pellet was washed twice with 0.1 M Tris/HCl buffer (pH 7.4). The tips of the microcentrifuge tubes, which contained the cell pellets, were then sliced into scintillation vials. The cell pellets were dissolved by the addition of 1 ml Soluene-350 (Packard) and incubated at 50 °C for 2 h. Scintillation cocktail (15 ml; Research Products International) was added to each sample and after an overnight incubation at room temperature, the samples were counted for radioactivity.

**Metabolism of ado and methyl-ado.** Late-exponential phase cultures (10⁹ cells ml⁻¹) of *M. smegmatis* mc²155 and SRI101 were incubated at 37 °C with 10 μM of either [2,8-[^H]]adenosine or [8-[^H]]methyladenosine (100 Ci mol⁻¹) for 1 h. Extraction and analysis of the acid-soluble nucleotide pool and the acid-insoluble nucleic acid pool was carried out as described by Parker *et al.* (1998). Separation and detection of nucleotides was performed using HPLC equipped with a Partisil-10 SAX column (10 μm, 250 x 6.4 mm; Keystone Scientific). Elution was accomplished with a 50 min linear gradient from 5 mM NH₄H₂PO₄ (pH 2.8) to 750 mM NH₄H₂PO₄ (pH 3.7) buffer at a flow rate of 2 ml min⁻¹. The acid-insoluble pool was resuspended in 0.5 M perchloric acid and applied onto a GF/A glass fibre filter. The filter was washed three times with 0.5 M perchloric acid followed by two washes with 95% ethanol, dried and counted for radioactivity. Radioactive metabolites in the growth medium were removed by a 30 s centrifugation at 16000 g, which terminated the uptake. The aqueous and oil layers were removed by aspiration and the pellet was washed twice with 0.1 M Tris/HCl buffer (pH 7.4). The tips of the microcentrifuge tubes, which contained the cell pellets, were then sliced into scintillation vials. The cell pellets were dissolved by the addition of 1 ml Soluene-350 (Packard) and incubated at 50 °C for 2 h. Scintillation cocktail (15 ml; Research Products International) was added to each sample and after an overnight incubation at room temperature, the samples were counted for radioactivity.

**Measurement of ATP.** *M. smegmatis* mc²155 cultures (2 ml) in late-exponential growth phase were incubated with 370 μM ado, 370 μM methyl-ado or 370 μM methyl-ado plus 740 μM hypoxanthine for 1 h at 37 °C. *M. smegmatis* SRI101 cultures (2 ml) in late-exponential growth phase were incubated with 370 μM methyl-ado for 1 h at 37 °C. The cells were harvested by centrifugation at 16000 g for 2 min at 4 °C. Extraction and analysis of the acid-soluble nucleotide pool using strong anion exchange HPLC (SAX-HPLC) was performed as described above. The ATP peak from the chromatography was detected by its absorbance at 260 nm and was quantified by comparing to a linear standard curve that was obtained from running pure ATP through the same HPLC conditions.

**RESULTS**

**Enzyme activities**

Mycobacteria are known to have three purine salvage enzymes (AK, AD and PNP) that could utilize ado and its analogues as substrates. Ade and its analogues are primarily metabolized by APRT. Therefore, extracts from wild-type *M. smegmatis* mc²155 and the resistant mutants (SRI101, SRI201 and SRI301) were prepared from late-exponential phase cultures and assayed for these activities (Table 1). AK activity in the methyl-ado/F-ado resistant *M. smegmatis* strains (SRI101 and SRI301) was substantially reduced compared to the activity in *M. smegmatis* mc²155, implicating this enzyme in the activation of methyl-ado and F-ado to toxic metabolites. Methyl-ado was a relatively poor substrate for this enzyme, since the specific activity from *M. smegmatis* mc²155 extracts with methyl-ado was 200-fold less than that when ado was used as a substrate. Methyl-ado was not a substrate for either AD or PNP that were present in the cell-free extracts, and ado was not a substrate for PNP. The inability to detect ado cleavage in *M. smegmatis* is consistent with the results of Wheeler (1987b), who did not detect any adenosine phosphorylase activity in *M. leprae*, *M. microti* or *M. avium* extracts. The activities of APRT, AD and PNP in the AK deficient cells were similar to the activity of these enzymes in mc²155, which indicated that these enzymes were not involved in the metabolism of methyl-ado.

In the F-ado resistant strain (SRI201) the APRT activity was not detectable, which indicated the importance of this enzyme to F-ado activation to toxic metabolites. AK and AD activities were similar in the SRI201 extract and the wild-type extracts.

**Uptake of [³H]adenosine and [⁸H]methyladenosine**

The uptake of ado and methyl-ado by *M. smegmatis* strains mc²155 and SRI101 was measured to determine the effect of the loss of AK activity on uptake. Although the rate of uptake of methyl-ado (0.18±0.02 and 0.20±0.04 pmol min⁻¹ per 10⁸ cells, respectively; mean ± sd, n = 3) was approximately 10% of that of ado (2.4±0.15 and 1.6±0.10 pmol min⁻¹ per 10⁸ cells, respectively; mean ± sd, n = 3) in *M. smegmatis* mc²155 and SRI101, the rate of uptake of these two compounds in the methyl-ado resistant mutant (SRI101) was similar to that in *M. smegmatis* mc²155. Therefore, the methyl-ado resistant phenotype of SRI101 is not a result of the deficiency in methyl-ado uptake. Moreover, the loss of AK in SRI101 has a limited effect on the uptake of ado.

**Metabolism of [³H]methyladenosine in *M. smegmatis* mc²155 and SRI101**

Our previous results indicated that AK was essential for the activity of methyl-ado against *M. smegmatis* mc²155. Characterization of the metabolism of methyl-ado and
metabolism of methyl-ado in M. smegmatis. Cultures of M. smegmatis mc²155 (●) and SRI101 (○) were incubated with 10 µM [³H]methyladenosine (100 Ci mol⁻¹) for 1 h. The cells were collected by centrifugation and the intracellular acid-soluble metabolites were obtained and analysed by SAX-HPLC as described in Methods. The radioactive acid-soluble metabolites of methyl-ado were detected by counting 1 min fractions that eluted from the column. The experiment was repeated with similar results.

![Graph](image)

**Fig. 1.** Metabolism of methyl-ado in M. smegmatis. Cultures of M. smegmatis mc²155 (●) and SRI101 (○) were incubated with 10 µM [³H]methyladenosine (100 Ci mol⁻¹) for 1 h. The cells were collected by centrifugation and the intracellular acid-soluble metabolites were obtained and analysed by SAX-HPLC as described in Methods. The radioactive acid-soluble metabolites of methyl-ado were detected by counting 1 min fractions that eluted from the column. The experiment was repeated with similar results.

Table 1. Specific activities of AK, AD, APRT and PNP in M. smegmatis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>AK Ado (µmol/min/mg protein)</th>
<th>Methyl-ado (µmol/min/mg protein)</th>
<th>AD Ado (µmol/min/mg protein)</th>
<th>Methyl-ado (µmol/min/mg protein)</th>
<th>APRT Ado (µmol/min/mg protein)</th>
<th>Ino (µmol/min/mg protein)</th>
<th>PNP Ado (µmol/min/mg protein)</th>
<th>Methyl-ado (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc²155</td>
<td>3400 ± 140</td>
<td>20 ± 10</td>
<td>33000 ± 1600</td>
<td>&lt; 1</td>
<td>34 ± 4</td>
<td>2.6 ± 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>SRI101</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>13000 ± 970</td>
<td>&lt; 1</td>
<td>27 ± 0.8</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>SRI201</td>
<td>2000 ± 110</td>
<td>–</td>
<td>28000 ± 2400</td>
<td>–</td>
<td>&lt; 1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SRI301</td>
<td>110 ± 10</td>
<td>–</td>
<td>22000 ± 1500</td>
<td>–</td>
<td>27 ± 4</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Activity units were defined as nmol min⁻¹ (mg protein)⁻¹. Enzyme extracts were obtained from the cells and enzyme activities were assayed as described in Methods. Values are means of at least two experiments ± SD. –, Not determined.

The radioactivity in the acid-insoluble portions in the M. smegmatis mc²155 and SRI101 extracts that were incubated with [³H]methyladenosine and [³H]adenosine was measured to determine the incorporation of these compounds into nucleic acids after the 1 h incubation period. M. smegmatis mc²155 was able to incorporate methyl-ado into nucleic acids (8 ± 0.5 pmol per 10⁸ cells; mean ± SD, n = 3), whereas no incorporation of methyl-ado into nucleic acids was detected in M. smegmatis SRI101. The amount of methyl-ado incorporated into the nucleic acids of M. smegmatis mc²155 was only 4% of that of ado incorporated (217 ± 8 pmol per 10⁸ cells; mean ± SD, n = 3), which is consistent with previous results that indicated that methyl-ado was a poor substrate for AK. Alkali treatment of acid-insoluble portions from M. smegmatis mc²155 incubated with [³H]methyladenosine or [³H]adenosine reduced the radioactivity to background level (data not shown), which indicated that the incorporation of methyl-ado into nucleic acids by the bacterium was mainly into RNA, not DNA.

The wild-type strain of M. smegmatis mc²155 incorporated only 2 times more ado into the acid-insoluble fraction than did M. smegmatis SRI101 (117 ± 8 pmol per 10⁸ cells, mean ± SD, n = 3), which was deficient in AK activity. Because AK activity was decreased more than 3000-fold in SRI101, our results suggest that during the first hour of incubation about half of the ado that was incorporated into nucleic acids of wild-type M. smegmatis was due to the generation of hypoxanthine and its subsequent metabolism rather than by direct phosphorylation by AK.

Reverse-phase HPLC separation of M. smegmatis mc²155 medium incubated with [³H]adenosine for 1 h showed that more than 94% of ado was converted to ino...
and hypoxanthine (Fig. 2). In the same period of time, less than 1% of the ado was associated with the mycobacteria, which indicated that most of the ado was deaminated by M. smegmatis rather than being phosphorylated by AK. In contrast, there was no detectable difference in the amount of methyl-ado in the medium before or after incubation with M. smegmatis mc²155 for 1 h (data not shown), which confirms the results (shown in Table 1) that indicated methyl-ado was a very poor substrate for AD. However, methyl-ade was detected in the medium after longer incubations (data not shown). The enzyme (or enzymes) involved in the generation of methyl-ade are not currently known. However, ado and methyl-ado were not substrates for PNP from M. smegmatis. However, methyl-ade was a very poor substrate for AD. However, methyl-ade was detected in the medium after longer incubations (data not shown). The enzyme (or enzymes) involved in the generation of methyl-ade are not currently known. However, ado and methyl-ado were not substrates for PNP from M. smegmatis.

**Effect of methyl-ado on the ATP pool of M. smegmatis mc²155 and SR101**

Certain purine analogues have been found to inhibit de novo purine synthesis in Escherichia coli, Salmonella typhimurium and Bacillus subtilis (Weng et al., 1995; Zalkin & Nygaard, 1996). The inhibition of de novo purine synthesis can result in a decline in the ATP pool unless exogenous purines are added to the growth medium. Therefore, it is possible that the metabolites of methyl-ado could inhibit enzymes associated with the formation of ATP. ATP pools of M. smegmatis mc²155 treated with ado, methyl-ado or methyl-ado plus hypoxanthine as well as M. smegmatis SR101 treated with methyl-ado for 1 h were measured using SAX-HPLC. Treatment with 370 µM methyl-ado decreased the ATP pool by 86% in M. smegmatis mc²155 (from 380 ± 50 to 50 ± 6 pmol ATP per 10⁹ cells, mean ± sd, n = 3). In contrast, the ATP pool in methyl-ado resistant M. smegmatis SR101 incubated with the same concentration of methyl-ado was comparable to that of mc²155 without the treatment of methyl-ado (340 ± 20 pmol ATP per 10⁹ cells, mean ± sd, n = 3). The ATP pool of M. smegmatis mc²155 incubated with methyl-ado plus 740 µM hypoxanthine (230 ± 10 pmol ATP per 10⁹ cells, mean ± sd, n = 3) was 4.6-fold higher than that of the strain incubated with methyl-ado alone, though it was only 64% of that of M. smegmatis mc²155 incubated with ado. The addition of 740 µM hypoxanthine to the medium containing 360 µM methyl-ado was also able to relieve the inhibitory effect of methyl-ado on M. smegmatis mc²155 growth (data not shown). In these experiments no methyl-ATP was detected in the cells treated with methyl-ado because of the insensitivity of the methodology used. However, it is clear from these studies that methyl-ATP formed in these cells did not replace a significant portion of the ATP in these cells. Therefore, the cytotoxic activity of methyl-ado metabolites occurred at relatively low concentrations with respect to the ATP levels in M. smegmatis.

**DISCUSSION**

Our results indicated that the anti-M. smegmatis activity of methyl-ado was due to its direct phosphorylation by AK to cytotoxic metabolites. Neither the cleavage of methyl-ado to methyl-ade nor its deamination to 2-methyl-inosine were involved in its cytotoxicity to M. smegmatis. Methyl-ATP and the incorporation of methyl-ado into RNA were detected in cells treated with methyl-ado. ATP is involved with many reactions in bacterial cells, and it is possible that inhibition of one or more of these enzymes by methyl-ATP is responsible for the anti-mycobacterial activity of methyl-ado. The incorporation of methyl-ado into RNA could disrupt the function of this molecule, which could also contribute to its activity against mycobacteria.

ATP levels were depressed in cells treated with methyl-ado. The fact that the depression of ATP levels by methyl-ado was dependent on AK activity indicated that the inhibition of ATP synthesis was not due to methyl-ado, but was due to one of its phosphorylated metabolites. The prevention of the cytotoxicity of methyl-ado by hypoxanthine and the ability of hypoxanthine to replenish ATP levels suggested that inhibition of de novo purine synthesis was responsible for the activity of methyl-ado against M. smegmatis. Methyl-ado has shown activity against M. tuberculosis in mouse models (data not shown), which indicates that the hypoxanthine in mouse plasma is not sufficient to affect the activity of methyl-ado in whole animals. Because ATP is a substrate of many of the enzymes involved with de novo purine synthesis, it is possible that the inhibition of one of these enzymes by methyl-ATP is responsible for the inhibition of de novo purine synthesis. Treatment of E. coli with
ado results in the feedback inhibition of the de novo pathway and repression of the synthesis of the de novo enzymes (Zalkin & Nygaard, 1996). Therefore, it is also possible that one or more of the phosphorylated metabolites of methyl-ado are acting as a negative feedback inhibitor of de novo purine biosynthesis.

Although our results showed that AK is involved in the activation of methyl-ado, the basis of selectivity of this agent has not yet been determined. It is likely that differential activation of methyl-ado by mycobacterial and human cells contributes to the selective activity of methyl-ado, because methyl-ado is not phosphorylated by rabbit liver AK (Miller et al., 1979). However, it is also possible that selective inhibition of the molecular targets by the methyl-ado metabolites could also contribute to the mechanism of selectivity.

Methyl-ado and F-ade resistant mutants isolated in this work exhibited very low AK and APRT activities, respectively, which demonstrated that these compounds must be metabolically activated in M. smegmatis. In E. coli, S. typhimurium, B. subtilis and two halophilic archaea, Halobacterium halobium and Haloferax volcanii, F-ade resistant mutants also have a reduced APRT activity relative to wild-type (Saxild & Nygaard, 1987; Stuer-Lauridsen & Nygaard, 1998; Zalkin & Nygaard, 1996). However, contrary to our results in M. smegmatis, these F-ade resistant mutants were cross-resistant to F-ado. In these organisms, which do not have AK activity, F-ado is cleaved to F-ade by PNP, which is then activated by APRT to toxic metabolites. Although some methyl-ado was detected in the medium of M. smegmatis cultures, our results indicated that the metabolic pathway responsible for the generation of this metabolite is not involved in the activity of ado analogues in mycobacteria.

Aristeromycin, a carbocyclic analogue of ado isolated from Streptomyces citricolor cultures, was not inhibitory against pathogenic bacteria except for mycobacteria (Suhadolnik, 1970). Similarly, methyl-ado inhibited the growth of M. tuberculosis strain H37Ra and three clinical isolates of M. avium complex at MICs of 8–16 µg ml⁻¹, but not E. coli, Staphylococcus aureus and Enterococcus faecalis at concentrations of 64 µg ml⁻¹ (unpublished data). Searches conducted on databases including Medline, SWISS-PROT and GenBank revealed that only two bacteria, Chlamydia psittaci and Acholeplasma laidlawii, have AK activity (McClarty & Fan, 1993; Tryon & Pollack, 1984). Furthermore, there is no AK assigned to any bacterial genome sequence that is available in GenBank. Therefore, ado analogues requiring the activation by AK would be specific to mycobacterial species. Such specificity is important for anti-mycobacterial drugs, since patients may be required to take the drug for a long period of time and killing of enteric bacteria could cause problems tolerating the therapy.

Methyl-ado was found to be a substrate for AK but not for AD. This may point out an important direction for future drug development of ado analogues as anti-mycobacterial agents, because AD activity is the dominant enzyme activity for ado metabolism in M. smegmatis and could serve as the first enzyme in detoxification of ado analogues. Moreover, the fact that methyl-ado is a poor substrate of AK increases the significance of the inability of methyl-ado to serve as a substrate for AD. Thus, future drug development of ado analogues as antimycobacterial agents will require optimization with regard to both enzymes.

These studies and those of others (Barclay & Wheeler, 1989; Wheeler, 1987a, b) indicate that mycobacteria are very different from most other bacteria in their metabolism of ado: mycobacteria can directly phosphorylate ado and the mycobacterial PNP does not accept ado as a substrate. The presence of AK in mycobacteria allows for the development of novel agents that could be selectively activated by this enzyme to cytotoxic metabolites. Although human cells also express AK activity, our studies with methyl-ado suggest that differences in the substrate preferences between the mycobacterial and human enzymes can be exploited to develop novel drugs for the treatment of mycobacterial diseases. The purification and characterization of AK from M. tuberculosis is currently in progress to precisely determine how this enzyme differs from its human homologue.

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