A CHARACTERIZATION OF TWO ENZYMES IN THE METHIONINE SALVAGE PATHWAY: METHYLTHIOADENOSINE / S-ADENOSYLHOMOCYSTEINE NUCLEOSIDASE AND METHYLTHIORIBOSE KINASE

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

The rise in drug resistance among the organisms responsible for numero infectious diseases of humans and livestock requires the continued search for new dru which interfere with metabolic events that are distinctly different from the host. Tl catabolism of the nucleosides 5'-deoxy-5'-methylthioadenosine (MTA) and S-adenosy homocysteine (SAH), byproducts of S-adenosylmethionine (SAM) dependent polyamir biosynthesis and methylation reactions, differs significantly between mammals and mar pathogenic bacteria and protozoans. As such, the enzymes responsible for the breakdow of MTA and SAH, and subsequent salvage to methionine, represent attractive targets f the development of novel chemotherapeutic agents.

To this end, the initial objective of my thesis research was to purify and characteriz the first two enzymes involved in MTA catabolism, MTA/SAH nucleosidase and 5 methylthioribose (MTR) kinase, from the pathogenic gram-negative bacillus, *Klebsiell pneumoniae*. Critical to the successful homogeneous purification of these two enzym was the development of a novel affinity chromatography resin which incorporated th substrate analogs 5'-(*para*-aminophenyl)thioadenosine (PAPTA) and 5-(*para* aminophenyl)thio-ribose (PAPTR). The enzymes displayed apparent monomeri molecular masses of 26.5 kDa (MTA/SAH nucleosidase) and ~46 kDa (MTR kinase Kinetic measurements performed on the purified enzymes revealed a $K_{m[MTA]}$ of 8.7μM for the nucleosidase and a $K_{m[MTR]}$ of 12.2 μM for the kinase. Automated amino termina sequence analysis of the two peptides allowed the identification of the first thirty-fiv residues of the nucleosidase, and nineteen of the first twenty residues of the kinase.

The amino acid sequence data obtained from the *Klebsiella* enzymes led directly int the second objective of the work, which entailed the cloning, sequencing and expressio of the nucleosidase and kinase genes. A search of the protein sequence databases reveale

a ~95% homology between the *Klebsiella* MTA/SAH nucleosidase peptide and t putative translation product of the *pfs* gene, an open reading frame reported approximately 4 minutes on the *E. coli* chromosome. Based on the *E. coli* sequenc oligonucleotide primers were designed, and a 967 base pair fragment of the *E. c* chromosome (containing the complete open reading frame) was amplified usi polymerase chain reaction (PCR). Nucleic acid sequence analysis of the cloned PC product revealed several alterations to the reported gene and resulted in the extension the open reading frame by an additional 13 codons. The *E. coli* gene sequence w subcloned and expressed as both a glutathione-S-transferase (GST) fusion protein and tryptophan inducible native full-length protein (232 amino acids). The identity of the *p* gene as encoding MTA/SAH nucleosidase was confirmed by kinetic measurements whi yielded a $K_{m[MTA]}$ of ~0.5 μM for both recombinant forms of the enzyme, in clo agreement with values reported in the literature for the native protein. Efforts to clone t MTR kinase gene by screening a *Klebsiella* genomic λgt11 library using degenera oligonucleotide probes based on NH_2-terminal sequence data, have to date be unsuccessful (Appendix A), and are the subject of ongoing investigation.

The remaining objective of this thesis was to employ the recombinant *E. c* nucleosidase as a model for drug design and highlight important features involved substrate recognition and catalysis. A series of nucleoside analogs were examined f their ability to inhibit MTA nucleosidase activity. The results provided evidence for t involvement of the purine C-6 amino and 5'- alkylthio group in substrate recognition, a the purine N-7 atom in catalysis. The findings are consistent with a proposed mechanis of catalysis which requires the protonation of N-7 in the transition state prior to nucleophilic attack by a water molecule on C-1 of the sugar. Preliminary studies on truncated version of the enzyme (rMTAN-8) suggest the involvement of the first eig

amino acids in substrate recognition, possibly in coordination of the thio group of tl nucleoside.

The results of investigations into the development of nucleosidase specif monoclonal antibodies, as well as studies on the differential inhibitory activity of MT analogs toward bacterial and cultured bone marrow cell growth are presented in addition appendices (B and C) following the main chapters of the thesis.

CHAPTER 1

INTRODUCTION

A. The Toll of Microbial Disease.

At the dawn of the Industrial Era, it is estimated that four out of five children bo never attained adulthood, but rather succumbed to a seemingly endless litany of epidem infectious diseases: small pox, yellow fever, scarlet fever, black plague, dysenter cholera, tuberculosis, Whooping cough, measles, tetanus, etc [1]. Other reports ha attributed half of all the cumulative deaths in mankind to malaria [2]. While the accurac of these reports may be difficult to assess, the underlying message is not. Infectio diseases have caused untold suffering, and remain a leading cause of annual huma mortality.

Even in modern times, infectious diseases have inflicted damage on the huma species of epic proportions. Outbreaks of bubonic plague in India caused more than 1 million deaths at the turn of the century [3]. A pandemic of influeza in 1918 caused a estimated 20 million deaths in less than one year, over half a million of which occured i the U.S. [4]. Diseases like malaria, which had nearly disappeared due to combined vectc control and public health campaigns, have returned in full force to tropical regions of th planet [5-7]. Today, malaria alone is responsible for approximately 1 million deaths pe year, predominantly in children under the age of five [8]. Poor sanitation and povert existing in most of the world continue to ensure that losses of human life due t respiratory and diarrheal diseases (viral, bacterial, and protozoal) approach 10 millio annually [9]. According to World Health Organization estimates, infectious disease accounted for over 50% of the yearly mortality in subsaharan Africa, and caused roughl 25% of the worldwide annual human mortality in 1990. For children under the age o

five, infectious diseases are the leading cause of death, accounting for approximately tw thirds of the annual mortality in this age group [10]. In the United States, the mortali rate for infectious disease increased by over 50% during the past decade, and is now tl third leading cause of death [11-13]. The recent emergence of the AIDS epidemic, Han virus, *E. coli* O157:H7, and nosocomial acquired *Enterococcus* infections, underscore tl need for vigilance in the detection of new scourges, and the recognition that organisn once regarded as relatively innocuous, may not remain so in the future [14, 15].

Despite the optimistic predictions of the 1960's and 70's when vaccinatic campaigns led to the erradication of small pox, it is unlikely that drugs or vaccines wi eliminate any of the prevalent human diseases in the foreseeable future. Worse, tl development of single and multiple drug resistant microorganisms has effectivel eliminated many antibiotics from our arsenal of therapies. As a result, there remains need for new drugs with novel modes of action for treatment of infectious diseases.

B. The Birth of the Modern Antibiotic Era.

Antibiotics have been in use by mankind since antiquity. For centuries prior to th arrival of Europeans in the New World, native South American tribes had been usin quinine containing extracts of Cinchona tree bark to combat fevers caused by malaria an other maladies [16]. In the Old World, ancient Egyptians and Romans used myrrl ground malachite, and verdegris to treat wounds and sepsis. Indeed, descriptions c many of these remedies and their toxic side effects appear in the writings of the earl physicians, Hypocrates and Celsus [17, 18].

The modern antibiotic era has its conception in work done by Louis Pasteur an Robert Koch. In 1876, Koch demonstrated that anthrax was due to an infectious bacillu Based on this observation, a critical understanding was reached that if a disease wa caused by a particular organism, then the therapy must entail the death of the offending

Table 1.1. Distribution of deaths from three groups of causes, by region, 1990.[c]

	Number of deaths (x1000) attributed to:			
Region[a]	I. Communicable, maternal and perinatal causes	II. Noncommun-icable causes	III. Injuries	Total
EME	439 (6.2)[b]	6 238 (87.6)	445 (6.2)	7 121
FSE	136 (3.6)	3 264 (86.8)	362 (9.6)	3 762
CHN	1 343 (15.1)	6 519 (73.4)	1 023 (11.5)	8 885
LAC	966 (32.3)	1 733 (57.9)	293 (9.8)	2 992
OAI	2 306 (41.8)	2 736 (49.6)	477 (6.6)	5 519
MEC	2 026 (46.2)	1 966 (44.8)	392 (8.9)	4 384
IND	4 060 (43.3)	4 700 (50.2)	611 (6.5)	9 371
SSA	5 415 (68.2)	1 898 (23.9)	624 (7.9)	7 937
WORLD	**16 690 (33.4)**	**29 055 (58.1)**	**4 227 (8.5)**	**49 971**

[a] EME, Established Market Economies; FSE, Former Socialist Economies; CHN, Chin LAC, Latin America and the Caribbean; OAI, Other Asia and Islands; MEC, Middle Eastern Crescent; IND, India; SSA, Sub-Saharan Africa.

[b] Figures in parentheses are percentages.

[c] Murray, C.J.L. and A.D. Lopez (1994) Global and regional cause-of-death patterns in 1990. *Bulletin of the World Health Organization*, **72** (3): 447-480.

Table 1.2. World estimated mortality (in thousands) of leading infectious diseases in 1990.[a]

	Number of deaths (in thousands)			
	Ages			
Cause of Death	0-4	5-14	15-70+	All Ages
Respiratory infections (combined viral, bacterial & parasitic)	2 732.0	245.0	1 337.4	4 314.4
Diarrhoeal diseases (combined acute, persistent & dysenteric)	2 478.0	210.5	184.2	2 872.7
Tuberculosis	71.7	151.4	1 792.4	2 015.5
Measles	862.7	143.2	0.5	1 006.4
Malaria	632.0	152.7	141.7	926.4
Tetanus	450.2	28.0	26.8	505.0
Pertussis	277.3	43.9	-[b]	321.2
HIV	58.0	9.8	223.0	290.8
Meningitis	125.0	68.1	48.7	241.8
Syphilis	77.2	-	116.2	193.4
Trypanosomiasis	4.2	19.3	31.6	55.1
Leishmaniasis	7.2	24.4	22.1	53.7
Onchocerciasis	-	-	29.8	29.8
Chagas' disease	-	-	23.1	23.1
Other infections (unnaccounted, combined)	336.1	168.4	377.1	881.6
Total deaths (Infectious diseases)	8 111.6	1 264.7	4 354.6	13 730.9
Total deaths (All causes)	12 654.6	2 265.8	35 050.7	49 971.1
Total population (in millions)	630.6	1 081.7	3 555.1	5 267.4

[a] Murray, C.J.L. and A.D. Lopez (1994) Global and regional cause-of-death patterns in 1990. *Bulletin of the World Health Organization*, **72** (3): 447-480.

[b] A dash (-) indicates less than 1000 deaths.

microbe. Close on the heels of Koch's work, Louis Pasteur in 1877 described the killi of anthrax bacilli by other bacteria, a phenomena later termed "antibiosis". This served a a foundation for later work in which cultures of various bacteria or fungi were screen for compounds that exhibited antibiotic activity toward pathogenic organisms [18]. I 1880, Laveran had discovered the malarial parasite, and shortly thereafter demonstrat that quinine could kill the intracellular organisms [19, 20].

The birth of the modern antibiotic era can be traced to work done by Emmeric Ehrlich, and Guttman at the turn of the century. Emmerich isolated and described t antibacterial properties of pyocyanase from *Pseudomonas aeruginosa* (*Bacill pyocaneus*) around the turn of the century [21]. In the next two decades, pyocyanas optochin (ethylhydrocupreine), and other compounds would be tested clinically again infectious diseases [18, 22]. While promising, these early drugs suffered from the sa drawbacks reported by the ancient physicians: agents containing lead or copper salts h dangerous toxicities in their therapeutic range, while biological products display problems with both unreliable potency and toxicity.

It was recognized early on that if drugs were developed with sufficient specifici for the microbe, then associated toxicity problems could perhaps be abrogated. Pa Ehrlich, working with synthetic dyes, coined the phrase "magic bullets" for agents whi specifically stained and killed microbes while leaving human cells untouched [23 Guttman and Ehrlich reported in 1891 that malaria could be treated with methylene bl [24]. Ehrlich's work led to the development of arsphenamine, an arsenical drug effecti against *Treponema pallidum*, the causative agent of syphilis. Shortly thereafter, Gerha Domagk discovered the sulfanilamides while screening thousands of dyes for antibacteri activity [18]. By the mid 1930's, clinical trials with the sulfanilamides against numero microbial diseases had begun. Penicillin, first described by Alexander Fleming in 19 [25], entered clinical trials around 1940.

Over the next forty years, thousands of drugs were discovered, either by screeni natural products isolated from various sources, or by randomly testing large numbers chemical compounds. Multiple generations of antibiotics were developed by chemical modifying known compounds to alter their pharmocologic profile or enhance th antibiotic activity. The general effectiveness in this empiric approach to drug discover coupled with the remarkable successes of World Health Organization-sponsored vacci programs, and pesticide campaigns to eliminate disease vectors, led to hea proclamations that the war against infectious diseases had been won [1, 18].

C. Drug Resistance, the Post-Partum Depression of the Antibiotic Era.

Unfortunately, the initial successes against infectious disease were relatively sho lived. Problems with drug resistance arose almost immediately with the birth of t antibiotic era. In 1910, Ehrlich reported resistance to arsenicals in trypanosomes [23 Drug resistance to optochin was noted during clinical trials against pneumococ pneumonia in 1917 [22]. In 1940, at approximately the same time that penicillin w entering into human use, the first observations of ß-lactamase activity in gram-negati *Escherichia coli* appeared [26]. Within 10 years, widespread penicillin resistance w encountered in nosocomial infections caused by the gram-positive organis *Staphylococcus aureus* [27]. Similarly, sulfonamide-resistant gram-negative organism surfaced within a decade of the introduction of this drug into clinical use [28]. By 196 fifteen years of widespread chloroquine use in malarious regions of the world led to th emergence of resistant *Plasmodium falciparum* strains in both Asia and South America [7, 29].

In 1960, the emergence of multiple drug resistance in strains of *Shigella dysentaria* was documented in Japan [30]. Within a decade, outbreaks of multi-drug resista *Staphylococcus aureus* and *Streptococcus pneumoniae* had been reported [18, 31]. B

1993, multiple drug resistance had been described in disease outbreaks in such far flu locations as Burundi (*Shigella dysenterae*-1992), Ecuador (*Vibrio cholerae*-1993), a India (*Salmonella typhi*-1990). The discovery of isoniazid and rifampin resista *Mycobacterium tuberculosis* in both Miami and New York serves as a serious loc example of this phenomenon [32].

In response to the rising tide of resistance, new drugs were developed a incorporated into antimicrobial therapeutic regimens. These drugs are either structu congeners of previous compounds, or completely different classes of agents with nov mechanisms of action. To date, greater than twenty classes of antimicrobial agents ha been developed for clinical use [33, 34]. Predictably, within a few years of us resistance to the newest chemotherapy and its dissemination has been documented [35 The ever increasing cost of drug development and marketing, now estimated at $100-3(million per drug, along with industry complacency has drastically reduced the number new antibiotics available for human use [36]. In 1993, only one new antibacteri compound was approved by the Food and Drug Administration [37]; in 1994, there we none [38]. This factor, coupled with the increased incidence of nosocomially acquire multiple drug resistance, including resistance to vancomycin, has led to dire predictions a "post-antimicrobial era", when none of the drugs available are effective in the treatme of disease [39].

D. Future Drug Development: Empiric and Rational Approaches.

If we are to stave off the onslaught of drug resistance, obviously the developme and manufacture of new chemotherapeutic agents is of paramount importanc Historically, an "empiric" approach to drug discovery has predominated. By this metho active compounds are discovered by random large scale screening of chemicals f antimicrobial activity. Following the identification of lead compounds, analogs a

synthesized and tested to find drugs with optimal activity. This general approach w successfully used to identify most of the therapeutic agents which are in use toda Empiric development of drugs will probably continue to be used in the future, despi distinct disadvantages: (1) it is costly, (2) it does not inherently consider the problem drug specificity, and (3) it has failed, particularly in the case of viral, fungal and parasit diseases to produce a large variety of medically tolerable compounds.

The "rational" approach to drug design has its foundation in the identification cellular processes that are essential for the replication and survival of the pathoge Potential inhibitors are assessed first for their ability to block a particular enzymatic gene function. Specificity is enhanced by developing drugs that interfere with process that occur only in the target organisms, not in the host. Alternatively, the selecte biochemical reaction may be present in both the disease agent and the host, but may diff significantly in its pharmacological properties, thus allowing selective interference with th microbial process [40].

Advances in the field of molecular biology have led to an additional refinement this process. The cloning of genes encoding targeted enzymes, and subsequer expression allow for large quantities of purified protein to be produced. X-ra crystallography and nuclear magnetic resonance spectroscopy studies of the purifie protein are used to construct physical models of enzyme-substrate and enzyme-inhibitc interactions [40, 41]. Computer analysis of large chemical databases are used to identif subsets of possible inhibitors which are predicted to bind tightly to the active (or othe allosteric) site of the enzyme [42]. This approach has been pursued in a number c instances, including the development inhibitors for HIV reverse transcriptase [43], HI protease [44, 45], bacterial cell wall biosynthesis [46], and bacterial thymidylate synthas [47], to list but a few. Future drug discovery will probably rely increasingly on thi method in combination with the more widely used empiric approach.

The studies presented in this thesis serve as the foundation for drug developme aimed at interfering with the salvage of methionine. This pathway is considered potential target for chemotherapeutic intervention since it fulfills many of the crite required for rational drug design. Several intermediates in the methionine recycli pathway must be maintained at low levels since they act as inhibitors of polyami biosynthesis and methylation reactions, processes essential for cellular proliferation a differentiation [48-52]. In addition, distinct differences occur between the enzym involved in the salvage of methionine in humans and a number of medically importa microbes, providing an opportunity to develop agents with selective activity toward t pathogen [53-56].

E. Methionine and S-Adenosylmethionine Metabolism: The Production of MTA a SAH.

Methionine serves as both an initiating amino acid and as a structural component protein biosynthesis [57]. In addition, a significant fraction of the cellular methionine converted to S-adenosylmethionine (SAM, AdoMet), a molecule first discovered b Cantoni in 1952 [58]. Formation of this sulfonium compound is accomplished by SA (AdoMet) synthetase (EC 2.5.1.6), which catalyzes the transfer of the adenosyl moiety ATP to methionine, with concomitant hydrolysis of the triphosphate [59].

SAM is an extremely versatile molecule involved in a wide variety of enzymat group transfer reactions. Methylation reactions involved in the biosynthesis of sma molecules like catecholamines and histamine, post-transcriptional modification messenger RNA, post-translational modification of proteins, and lipid biosynthesis, a but a brief list of SAM dependent events [60, 61]. Depending on the cell type examine it has been estimated that 67-90% of the cellular SAM pools are metabolized throug methylation reactions [61-64]. The demethylated product, S-adenosylhomocystei

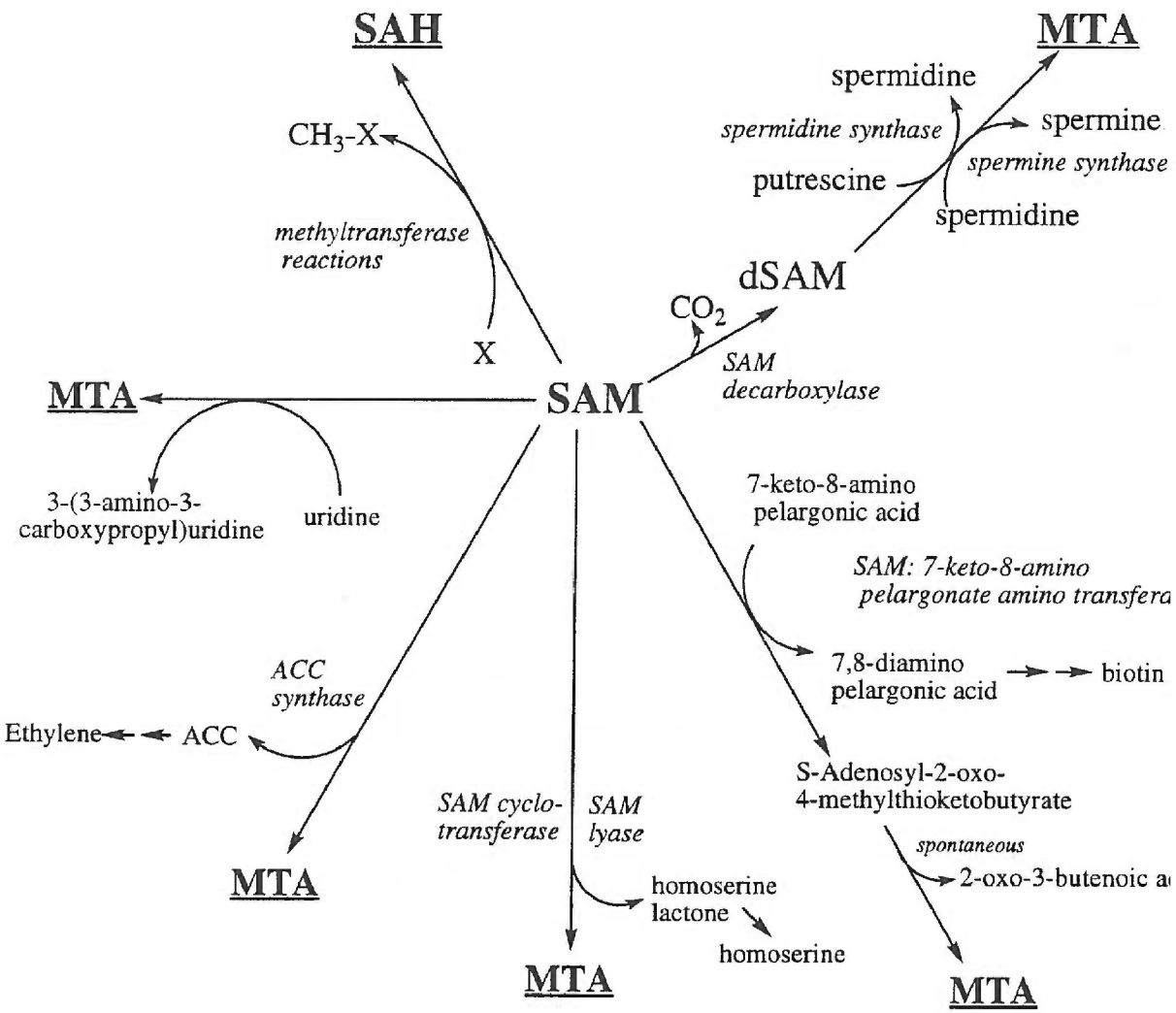
(SAH), acts as a potent feedback inhibitor of most methyl group transfer reactions [6 67]. The ratio of SAM:SAH existing in the cell is thought to play a role in governi cellular status with respect to proliferation and differentiation [68].

Most of the remaining SAM (not involved in methylation) is decarboxylated l SAM decarboxylase (EC 4.1.1.50), a step which commits the molecule to polyami biosynthesis. The enzyme spermidine synthase (EC 2.5.1.16) catalyzes the transfer of t propylamine group from decarboxylated SAM (dSAM) to putrescine (1,4-diaminobutan to yield spermidine [69] In higher eukaryotes, the enzyme spermine synthase (E 2.5.1.17) catalyzes the addition of a second propylamine group from dSAM to spermidi to form spermine. The thioether, 5'-deoxy-5'methylthioadenosine (MTA) is produc stoichiometrically with each polyamine molecule [69], and acts as a potent feedba inhibitor of the propylamine transferases [48, 70, 71].

MTA results from a number of other minor, but physiologically significant, SA dependent reactions that are worth considering. The 3-amino-3-carboxypropyl (AC group of SAM is removed by a variety of hydrolases/lyases acting upon the nucleosid The action of coliphage T_3 SAM hydrolase, an early gene product, yields homoserine a MTA. The reaction is believed to play a key role in phage replication by impairing ho directed DNA methylation and spermidine biosynthesis [72, 73]. A related enzyme, SA cyclotransferase (EC 2.5.1.4), yields homoserine lactone and MTA [74, 75]. T homoserine lactone subsequently converts nonenzymatically to homoserine, which recycled back to methionine in some prokaryotic systems through a trans-sulfuration ste with cysteine [68].

The ACP group of SAM is also involved in the generation of unusual nucleosid purines, and amino acids. Rare tRNA's have been isolated from bacterial [76] a mammal cells [77] which contain ACP modified uridine residues. In the slime mol *Dictydostelium*, the ACP group is used to form discadenine, an unusual purine which

Figure 1.1. The biosynthesis of methylthioadenosine (MTA).


SAH
MTA
spermidine
spermine
spermidine synthase
spermine synthase
putrescine
spermidine
CH3-X
methyltransferase reactions
dSAM
CO2
X
SAM decarboxylase
MTA
SAM
3-(3-amino-3-carboxypropyl)uridine
uridine
7-keto-8-amino pelargonic acid
SAM: 7-keto-8-amino pelargonate amino transfera
7,8-diamino pelargonic acid
biotin
ACC synthase
Ethylene
ACC
S-Adenosyl-2-oxo-4-methylthioketobutyrate
SAM cyclo-transferase
SAM lyase
spontaneous
2-oxo-3-butenoic a
homoserine lactone
homoserine
MTA
MTA
MTA

functions as a regulatory molecule in sporulation [78]. The ACP group is also used synthesize diphthamide, an amino acid found only in elongation factor 2 (EF-2), component of eukaryotic translational machinery [79].

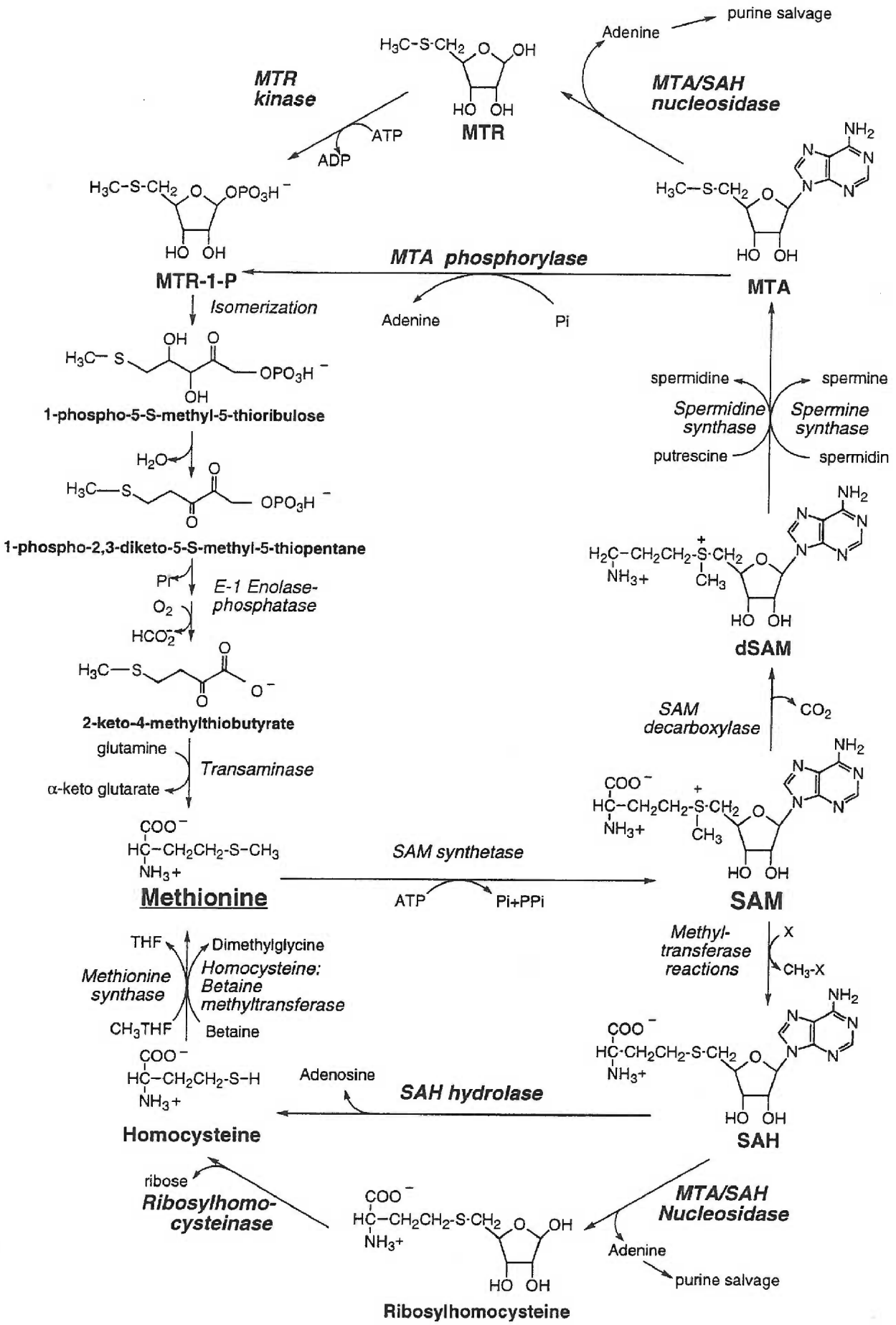
In the tissues of higher plants, the ACP group of SAM is converted to aminocyclopropane-1-carboxylic acid (ACC) by the action of ACC synthase (S-adenosy L-methionine methylthioadenose-lyase, EC 4.4.1.14) [80, 81]. The reaction represen the rate limiting step in the formation of ethylene, a plant hormone involved in growt senescence, and fruit ripening. A recent report by Good et al. has suggested that fr ripening in tomatoes could be controlled by transgenic expression of the coliphage T SAM hydrolase [82] . Presumably, the resulting depletion of SAM pools decreases th amount available for the synthesis of ACC. These findings are of interest since the provide insight into the potential agricultural importance of manipulation of SA metabolism and methionine recycling.

Lastly, in biotin biosynthesis, SAM acts as an amino group donor in th transamination of 7-keto-8-amino pelargonic acid. The product, 7,8-diaminopelargon acid, is the precursor to desthiobiotin [83]. The coproduct of the reaction, S-adenosyl-2 oxo-4-methylthiobutyrate, decomposes spontaneously to MTA and 2-oxo-3-butenoic aci

F. Methionine Recycling from MTA and SAH.

In each of the metabolic reactions of SAM considered in the previous paragraph MTA or SAH was produced. These thioethers represent energetically "expensive compounds that are recycled in a series of reactions that link methionine and purin salvage [61]. In addition, MTA and SAH act as feedback inhibitors of numerou reactions, thus requiring rapid catabolism to prevent their antiproliferative effects. I 1952, Shapiro showed that methionine auxotrophs of *Aerobacter aerogenes* (*Enterobact aerogenes*) could grow when cultures were supplemented with MTA [84]. This initi

Figure 1.2. Methionine recycling pathways from MTA and SAH.


MTR
MTR kinase
ATP
ADP
MTR-1-P
MTA/SAH nucleosidase
Adenine
purine salvage
MTA
MTA phosphorylase
Adenine
Pi
Isomerization
1-phospho-5-S-methyl-5-thioribulose
1-phospho-2,3-diketo-5-S-methyl-5-thiopentane
E-1 Enolase-phosphatase
2-keto-4-methylthiobutyrate
glutamine
α-keto glutarate
Transaminase
Methionine
SAM synthetase
ATP
Pi+PPi
SAM
spermidine
spermine
Spermidine synthase
Spermine synthase
putrescine
spermidin
dSAM
SAM decarboxylase
CO2
Methyl-transferase reactions
X
CH3-X
THF
Dimethylglycine
Methionine synthase
Homocysteine: Betaine methyltransferase
CH3THF
Betaine
Homocysteine
Adenosine
SAH hydrolase
SAH
ribose
Ribosylhomo-cysteinase
MTA/SAH Nucleosidase
Adenine
purine salvage
Ribosylhomocysteine

demonstration of a methionine recycling pathway from MTA was later repeated in *Candi utilis* [85, 86], *Ochromonas malhamensis* [85-88], apple tissue [89], and mammalian ce [90-92].

In mammalian cells, the initial catabolism of MTA and SAH occurs by separ enzymes, MTA phosphorylase (EC 2.4.2.28) [93] and SAH hydrolase (EC 3.3.1.1) [9 The products of the hydrolysis of SAH are adenosine and homocysteine. Adenosine dispersed into the purine pools of the cell, predominantly through the actions of adenosi deaminase (EC 3.5.4.4) and nucleoside kinase (EC 2.7.1.15). Homocysteine converted to methionine, by either a vitamin B_{12} dependent methionine syntha (methyltetrahydrofolate:homocysteine methyltransferase, EC 2.1.1.13), or a vitamin B independent enzyme (betaine:homocysteine methyl-transferase, EC 2.1.1.5) [68, 95].

The initial step in the salvage of methionine from MTA was first demonstrated b Pegg and Williams-Ashman, who showed that MTA catabolism occured via the action of specific phosphate dependent nucleosidase (MTA phosphorylase, EC 2.4.2.28), whic yielded adenine and 5-methylthioribose-1-phosphate (MTR-1-P) [93]. This is the on adenine producing reaction within mammalian cells [96]. Adenine subsequently enters th cellular purine pools by the action of adenine phosphoribosyltransferase (APRTase; E 2.4.2.7). MTA phosphorylase has since been purified and characterized from a variety mammalian sources (rat, mouse, bovine, human, etc.) and tissue types (lymphocyte erythrocytes, liver, lung, testes, placenta, brain, etc.) (Table 1.5 and references therein The phosphorylase has been reported to function as a homodimer [97] or homotrimer th is unaffected by SAH, but competitively inhibited by adenine [98, 99]. The human MT phosphorylase gene has recently been cloned [100, 101] and expressed [102], allowir confirmation of its monomeric molecular weight (≈31kDa), and trimeric quaterna structure. MTA phosphorylase activity has also been reported in the archaebacteria [10

104], a number of eubacteria [105], fungi [106, 107], algae [108], and protozoans [10
113].

In contrast to mammalian cells, the initial catabolism of MTA occurs by a phospha independent nucleosidase in a number of other bacteria (especially in the fami *Enterobacteriaceae*)[105, 114], several protozoans [54, 55, 115, 116], and plants [11 120]. The bacterial enzyme, first described in *E. coli* [114], has a broader substra specificity than the mammalian phosphorylase, and irreversibly hydrolyzes both SAH ar MTA to yield adenine and the corresponding thiosugar, ribosylhomocysteine methylthioribose (MTR). Bacterial MTA/SAH nucleosidase (EC 3.2.2.9), has since bee purified and characterized from *E. coli* [121, 122] and *Klebsiella pneumoniae* [123]. Th bacterial enzymes function as 26-31kDa monomers that are insensitive to inhibition by th products, adenine and methylthioribose. In what appears to be an interesting hybrid mammalian and bacterial properties, the plant *Lupinus luteus* (Yellow lupine) phosphat independent nucleosidase acts as a 62kDa homodimer with a strict specificity for MT and is readily inhibited by adenine [118]. Similar enzyme activity profiles appear in oth plants as well, suggesting that separate MTA and SAH catabolizing enzymes are the nor for this kingdom [106,117].

The second step in the alternate (microbial and plant) pathway involves the AT dependent phosphorylation of MTR to yield MTR-1-P. The reaction is catalyzed by MT kinase (EC 2.7.1.100), an enzyme first described by Ferro and coworkers in lysates *Enterobacter aerogenes* [125]. MTR kinase has since been reported in a number bacterial [54, 116, 126], protozoal [54, 55, 116], and plant [119, 120, 128] species. Th salvage of ribosylhomocysteine, produced by the cleavage of SAH, remains poor characterized, but reportedly involves cleavage to ribose and homocysteine [143].

The remaining steps in the recycling of MTR-1-P to methionine appear to be th same for all cell types, although they have not been extensively studied. MTR-1-P is fir

Table 1.3. Enzyme activities in prokaryotes.

	Enzyme Activity			
Organism	MTA/SAH Nucleosidase	MTR Kinase	MTA Phosphorylase	Ref.
Archaebacteria				
Caldariella acidophila	–	nt	√	[103]
Sulfolobus solfataricus	–	nt	√	[124]
Eubacteria				
Citrobacter freundii	√	nt	–	[105]
Citrobacter intermedius	√	nt	–	[105]
Enterobacter aerogenes	√	√	–	[54, 106, 11· 116, 125]
Enterobacter cloacae	√	√	–	[54]
Escherichia coli (B, K-12)	√	nt[a]	–	[105, 114]
Erwinia carotovora	√	nt	–	[105]
Klebsiella pneumoniae	√	√	–	[105, 123, 126]
Proteus mirabilis	√	nt	–	[105]
Proteus vulgaris	√	nt	–	[106]
Salmonella typhimurium	√	nt	–	[114]
Serratia marcescens	√	nt	–	[105]
Acinetobacter calcoaceticus	–	nt	√	[105]
Agrobacterium tumefaciens	–	nt	√	[105]
Alcaligenes faecalis	–	nt	√	[105]
Arthrobacter globiformis	–	nt	√	[105]
Bacillus cereus	√	nt	–	[106]
Corynebacterium fascians	–	nt	√	[105]
Mycobacterium avium	–	nt	√	[105]
Nocardia asteroides	–	nt	√	[105]
Protaminobacter ruber	–	nt	√	[105]
Pseudomonas aeruginosa	–	nt	√	[105]
Pseudomonas maltophila	–	nt	√	[105]
Pseudomonas putida	–	nt	√	[105]
Rhodopseudomonas spheroides	–	nt	√	[105]
Staphylococcus aureus	√	nt	–	[106]
Streptomyces hygroscopicus	–	nt	√	[105]

(√)= enzyme activity present; (–) = enzyme activity absent; (nt) = not tested.

[a] MTR kinase activity has not been detected in *E. coli*, Michael K. Riscoe, unpublished observation.

Table 1.4. Enzyme activities in lower eukaryotes.

	Enzyme Activity			
Organism	MTA/SAH Nucleosidase	MTR Kinase	MTA Phosphorylase	Ref.
Protozoa				
Entamoeba histolytica	√(MTA)[a]	√	–	[55]
Entamoeba invadens	√(MTA)	√	–	[54]
Giardia lamblia	√(MTA)	√	–	[54, 116]
Ochromonas malhamensis	√(MTA)	√	–	[88, 116]
Plasmodium falciparum	√(MTA)	√	√[b]	[116]
Phytomonas davidii	–	–	√	[54]
Leishmania donovani	–	nt	√	[110, 112]
Trypanosoma brucei	–	nt	√	[112, 113]
Trypanosoma cruzi	–	nt	√	[111, 112]
Euglena gracilis	–	nt	√	[109]
Algae				
Acetabularia mediterranea	√	nt	–	[108]
Fungi				
Aspergillus nidulans	–	nt	√	[107]
Candida albicans[c]	–(SAH)[d]	nt	nt	[106]
Candida utilis[c]	–(SAH)	nt	nt	[106]
Saccharomyces cerevisiae	–(SAH)	–	√	[106, 127]

(√)= enzyme activity present; (–) = enzyme activity absent; (nt) = not tested.

[a] √(MTA) indicates that MTA nucleosidase activity was present. SAH nucleosidase activity was not tested.

[b] Trace amounts of phosphorylase activity here probably represent human erythrocyte enzyme.

[c] These *Candida* species lacked SAH nucleosidase and ribosylhomocysteinase activity, however, SAH hydrolase activity was demonstrated. Also, in these species, MTA is converted to methionine, whereas MTR is not. The inference is that the MTA nucleosidase/MTR kinase enzymes are probably replaced by a MTA phosphorylase similar to the findings for *Aspergillus* and *Saccharomyces*.

[d] –(SAH) indicates SAH nucleosidase activity was absent. MTA nucleosidase activity was not tested.

[e] √(MTA)/–(SAH) indicates that the nucleosidase recognized only MTA, SAH was not a substrate.

Table 1.5. Enzyme activities in higher eukaryotes.

	Enzyme Activity			
Organism	MTA/SAH Nucleosidase	MTR Kinase	MTA Phosphorylase	Ref.
Plants				
Pyrus malus (Apple)	nt	√	nt	[119]
Persea americana (Avocado)	√(MTA)[a]	√	–	[116, 119
Hordetum distichon (Barley)	–(SAH)[b]	√	nt	[106, 128
Zea mays (Corn)	–(SAH)	√	nt	[106]
Phaseolus vulgaris (Green bean)	–(SAH)	nt	nt	[106]
Pyrus communis (Pear)	nt	√	nt	[119]
Spinaces oleraces (Spinach)	–(SAH)	nt	nt	[106]
Fragaria (Strawberry)	nt	√	nt	[119]
Lycopersicon esculentum (Tomato)	√(MTA)	√	–	[119, 120 129]
Lupinus luteus (Yellow lupine)	√(MTA)/–(SAH)[c]	√	–	[118, 128
Vinca rosea (Periwinkle)	√(MTA)/–(SAH)	nt	–	[117]
Glycine max (Soybean)	nt	√	nt	[128]
Helianthus anuus (Sunflower)	nt	√	nt	[128]
Cucummis sativus (Cucumber)	nt	√	nt	[128]
Invertebrates				
Drosophila melanoganser	–	nt	√	[130, 131]
Vertebrates				
Mus (Mouse)	–	–	√	[116, 132-134]
Rattus (Rat)	–	nt	√	[93, 134-137]
Sus (Pig)	–	nt	√	[134]
Ovis (Sheep)	–	nt	√	[134]
Bos (Cow)	–	nt	√	[98, 134, 138-140]
Homo sapien (Human)	–	–	√	[97, 99, 116, 124, 141, 142]

(√)= enzyme activity present; (–) = enzyme activity absent; (nt) = not tested.

[a] √(MTA) indicates that MTA nucleosidase activity was present. SAH nucleosidase activity was not tested.

[b] –(SAH) indicates SAH nucleosidase activity was absent. MTA nucleosidase activity was not tested.

[c] √(MTA)/–(SAH) indicates that the nucleosidase recognized only MTA, SAH was not a substrate.

isomerized to 5-methylthioribulose-1-phosphate [144, 145], followed by dehydration 1-phospho-2,3-diketo-5-S-methylpentane [146]. Subsequently, a set of vague characterized reactions occur which yield phosphate, formate (from C-1 of the ribos and 2-keto-4-methylthiobutyrate (αKMTB) [147, 148]. A magnesium and oxyg requiring bifunctional enolase-phosphatase is proposed to catalyse the dephosphorylati and oxidative cleavage of formate from 1-phospho-2,3-diketo-5-S-methylpentane [14 In the final step, αKMTB is reversibly transaminated in a reaction which (in mammali cells) preferentially uses glutamine or asparagine as an amino group donor to for methionine and the corresponding α-keto acid (α-ketoglutarate / α-ketoaspartate) [15 151]. Worthy of note, this last step in the recycling pathway has recently come und study as a potential site of drug intervention against *Trypanosoma brucei* due to t substrate preference of the trypanosomal enzyme for aromatic amino acids [152].

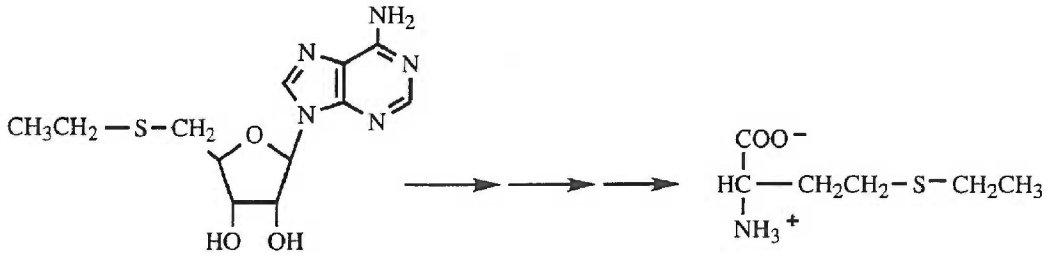
G. The Enzymes of Methionine Salvage as Chemotherapeutic Targets.

As described in the previous section, the catabolism of MTA to MTR-1-P occurs t either a one step (MTA phosporylase) or two step (MTA/SAH nucleosidase / MTR kinas process. Differences existing between the methionine salvage enzymes of mammali cells and those of a number of medically important parasitic protozoa, fungi, and bacter serve as sites of possible chemotherapeutic intervention [54, 56]. In general, drugs whi interfere in the recycling pathway can be predicted to act by one (or more) of thr possible mechanisms (Figure 1.3). By the first mechanism, drugs are metabolized toxic analogs of methionine and S-adenosylmethionine, which subsequently exert the effects by interfering with protein synthesis and biological methylation reactions [55 This is the proposed mode of action for 5'-deoxy-5'-ethylthioadenosine (ETA), one of t first analogs of MTA developed to target the recycling pathway. The second mode action involves agents which directly inhibit salvage enzymes. The resulting interference

Figure 1.3. Mechanisms of action of methythioadenosine and methylthioribo analogs.

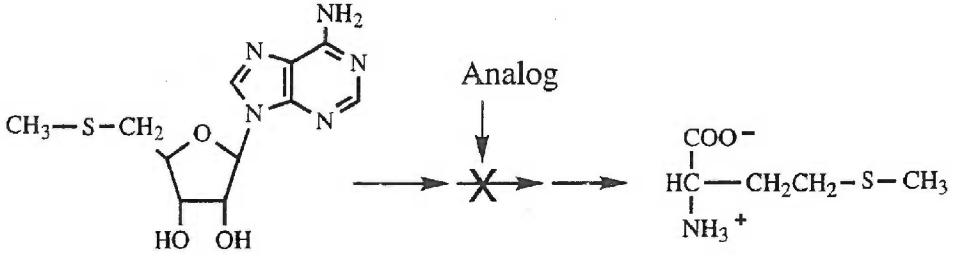
Modes of Action of Methionine Recycling Intermediate Analogs

(1) Conversion to toxic methionine analogs:



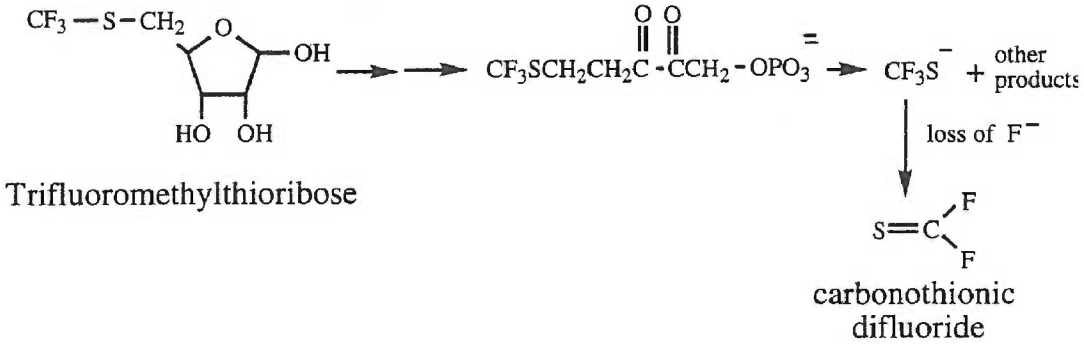
Ethylthioadenosine → Ethionine

(2) Inhibition of methionine recycling:



Methylthioadenosine → Methionine

(3) Generation of lethal intermediates:



Trifluoromethylthioribose → carbonothionic difluoride

in enzyme activity leads to a build up of catabolic intermediates, particularly MTA ar SAH, which in turn exhibit a strong inhibition of polyamine biosynthesis and methylatic reactions required for rapid cell proliferation [48, 67, 153]. The third possibility involv compounds which are catabolized by salvage enzymes to unstable intermediates, which turn decompose to toxic substances. Trifluoromethylthioribose (TFMTR) is proposed function in this manner. The action of MTR kinase on TFMTR generates the TFMTR-phosphate, which is converted to the 1-phospho-2,3-diketo-trifluoro-methylthiopenta intermediate. The presence of the trifluoromethylthio- group in the β position relative the keto moiety allows the spontaneous generation of carbonothionic difluoride, a high reactive cross-linking agent [126].

(i) MTA phosphorylase

For pathogenic organisms containing MTA phosphorylase (e.g. *Leishmania* ar *Trypanosoma* spp.), differences in the substrate specificities of the mammalian ar parasite enzymes have been explored as possible sites of drug design [112]. In 198 Koszalka and Krenitsky purified an MTA phosphorylase from *L. donovani* promastigot which exhibited a broader substrate specificity than its mammalian counterpart [110]. (particular note for drug development, the leishmanial enzyme recognized 2 deoxyadenosine, a trait not shared by human MTA phosphorylase [154]. Subseque work on the MTA phosphorylases from *Trypanosoma cruzi* , *T. brucei* , and *L. donova* demonstrated Michaelis constants for MTA that are essentially equivalent to human MT phosphorylase [111-113]. However, all the parasite enzymes catalyzed tl phosphorolysis of adenosine and 2'-deoxyadenosine with high efficiency as well. addition, the *T. brucei* enzyme utilized 2',3'-dideoxyadenosine as a substrate [113]. Th leaves open the possibility of selective delivery of toxic adenine analogs using tl corresponding adenosine, 2'-deoxyadenosine, or 2',3'-dideoxyadenosine derivati [112]. Other possibilities have been examined as well. An analog of MTA, 5'-deoxy-5

hydroxyethylthioadenosine (HETA) has been shown to be curative when administered mice infected with *Trypanosoma brucei* [155], and has advanced to the clinical trials stag (Dr. J. Sufrin, personal communication). HETA, which is a good substrate for th trypanosomal phosphorylase (but not the mammalian phosphorylase) [156], presumably converted to toxic methionine analogs, since its inhibitory effects *in vitr* against procyclic trpanosomes can largely be abrogated by the addition of methionine α-ketomethiobutyrate to the culture medium [155].

(ii) MTA/SAH nucleosidase & MTR kinase.

For pathogens containing the MTA/SAH nucleosidase/MTR kinase path, differenc in both substrate specificity and mechanism of action may be exploitable. In particula the nucleosidase is singly responsible for maintaining low levels of MTA and SAH compounds that have well documented inhibitory effects on methylation reactions [67 polyamine biosynthesis [48, 157], and cell proliferation [50, 158]. Numerous analogs MTA and SAH have been developed [65, 66, 156, 159-164], but relatively few repor exist of their effects on nucleosidase containing organisms. The analogs, 5'-deoxy-5 isobutylthioadenosine (SIBA), ETA, and HETA, have displayed modest inhibitor activities *in vitro* against *Plasmodium falciparum* [56, 165], with IC_{50} values generally the 20-200μM range. A series of 5'-monofluoro-, 5'-difluoro-, and 5'-trifluoro- analog of MTA and ETA have also been examined for *in vitro* antimalarial activity, with IC values generally seen in the 20-50μM range [56, 164]. The SAH analog, sinefungi showed the greatest *in vitro* inhibitory activity against the malarial parasite with an IC_{50} approximately 0.2μM [166]. However, it is unclear in this organism whether th compound is exerting its inhibitory effect by interfering with methionine/purine salvag or is acting as a protein methyl transferase inhibitor, as has been demonstrated *Leishmania* species [167]. Finally, a single report of the testing of a naturally occurri

xylosyl derivitative of MTA (from *Doris verrucosa*) against several bacterial species fail to demonstrate any significant antibacterial activity [168].

In contrast to microbial nucleosidases and phosphorylases, where the design substrate analogs must take into account possible interactions with the hc phosphorylase, MTR kinase appears to be an attractive target because an equivalc activity is not present in human cells. Analogs of MTR have been shown to have *in vit* antibacterial and antiprotozoal activity, with no demonstrable *in vitro* or *in vivo* inhibitc activity toward mammalian cells, even at high drug concentrations (5-10mM) [54, 5 116, 169]. The compound, TFMTR, has shown modest activity against *P. falciparı* (IC_{50} = 50μM) [55], and potent activity against *Klebsiella pneumoniae* (IC_{50} = 50nM [126]. Other more recently developed analogs, 5-(4-fluorophenylthio)ribose (PFPT and 5-(4-iodophenylthio)ribose (PIPTR), exhibit even greater antibacterial activiti against *K. pneumoniae* (IC_{50}'s = 0.0025-0.05nM) [170].

The remaining chapters of this thesis present studies on MTA/SAH nucleosidase a MTR kinase that were conducted to further explore the potential of these enzymes to ser as targets for rational drug design. The MTA/SAH nucleosidases from *Klebsie pneumoniae* and *Escherichia coli,* and the *Klebsiella pneumoniae* MTR kinase were us as model proteins for these investigations. Chapter 2 (paper #1) describes the purificati and characterization of the nucleosidase and kinase from *Klebsiella* cell lysates. Ami acid sequence information obtained for the *Klebsiella* MTA/SAH nucleosidase showed high degree of homology to the predicted translation product of *pfs*, an *E. coli* gene previously unknown function. The results of the cloning and expression studies of the *coli pfs* gene were used to confirm its identity as encoding MTA/SAH nucleosidase, a comprise the bulk of chapter 3 (paper #2). The next chapter (paper #3), outlines studi performed on the recombinant *E. coli* nucleosidase to test its ability to serve as an *in vit* model for drug design and understand in more detail the structural characteristics involv

in substrate and inhibitor recognition. Also contained in Chapter 4 is an initial kinet characterization of a truncated MTA/SAH nucleosidase which was engineered to study tl contribution of the first eight amino acid residues to substrate binding. Experimen describing attempts to clone the *Klebsiella* MTR kinase gene, preliminary antibacterial ar bone marrow cell toxicity testing of MTA analogs, and the development and use MTA/SAH monoclonal antibodies are contained in the appendices at the end of this thesi

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CHAPTER 2

Affinity Purification of 5-Methylthioribose Kinase and 5'-Methylthioadenosine/S-Adenosylhomocysteine Nucleosidase from *Klebsiella pneumoniae*

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Abbreviations: MTA, 5'-methylthioadenosine; MTR, 5-methylthioribose; SAM S-adenosyl-L-methionine; SAH, S-adenosylhomocysteine; DTT, dithiothreitc PAPTA, 5'-(p-aminophenyl)thioadenosine; PAPTR, 5-(p-aminophenyl)thioribos TFMTR, 5-(trifluoromethyl)thioribose; PIPTR, 5-(p-iodophenyl)-thioribose; CAP 3-(cyclohexylamino)propane-1-sulphonic acid.

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SUMMARY

Two enzymes in the methionine salvage pathway, 5-methylthioribose kinase (MT kinase) and 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTA/SA nucleosidase) were purified from *Klebsiella pneumoniae*. Chromatography using a nov 5'-(p-aminophenyl)thioadenosine/5-(p-aminophenyl)thioribose affinity matrix allowed t binding and selective elution of each of the enzymes in pure form. The molecular mas substrate kinetics and N-terminal amino acid sequences were characterized for each of t enzymes. Purified MTR kinase exhibits an apparent molecular mass of 46-50 kDa SDS/PAGE and S200HR chromatography, and has a K_m for MTR of 12.2 µM Homogeneous MTA/SAH nucleosidase displays a molecular mass of 26.5 kDa SDS/PAGE, and a K_m for MTA of 8.7 µM. Comparisons of the N-terminal sequenc obtained for each of the enzymes with protein-sequence databases failed to reveal a significant sequence similarities to known proteins. However, the amino acid sequen obtained for the nucleosidase did share a high degree of sequence similarity with t putative translation product of an open reading frame in *Escherichia coli*, thus providing tentative identification of this gene as encoding an MTA/SAH nucleosidase.

INTRODUCTION

5-Methylthioribose (MTR) kinase is not present in mammalian cells but is essential f methionine recycling in plants, as well as in numerous bacterial and protozoan speci (Scheme 1). Because of its prevalence in a number of bacterial and protozoan pathoge of humans, attention has turned to the enzyme as a target for the development of drugs selectively exploit or subvert microbial methionine metabolism [1-3]. It has be demonstrated that MTR kinase-containing organisms are selectively killed by analogs MTR [3-6]. Depending on their design, these analogs kill microbes by one or more of t following mechanisms: (1) direct inhibition of MTR kinase activity, thus preventi salvage of the energetically 'expensive' amino acid methionine; (2) conversion of the dr via MTR kinase and the salvage pathway into toxic derivatives of methionine or adenosylmethionine (SAM); and (3) conversion via MTR kinase to unstable intermediat which yield toxic products upon decomposition. One MTR analog, (trifluoromethyl)thioribose (TFMTR), acts as a subversive substrate of the methioni salvage pathway to yield an unstable intermediate which releases toxic carbonothic difluoride [171]. More recently, Winter et al. [6] have described 5-(iodophenyl)thioribose (PIPTR) which is at least 10000 times more potent that TFMT (e.g. IC_{50}'s of 2.5 pM and 40 nM versus *Klebsiella pneumoniae,* respectivel However, the precise mechanism by which this compound acts is not yet clear.

Further development of MTR analogs as selective antimicrobial agents would greatly facilitated by the availability of pure target enzyme which could be used to scre new compounds. In addition, N-terminal sequence data from purified enzyme wou provide valuable information required for cloning and expression of the correspondi gene. Because of its importance for future drug development efforts and for studies investigate the regulation of MTR kinase expression, we set out to purify MTR kinase

homogeneity. Critical to the mission of obtaining pure material was the development of novel substrate-affinity matrix, 5'-(*p*-aminophenyl)thioadenosine (PAPTA)/5-(aminophenyl)thioribose (PAPTR)-Sepharose. Herein we describe the use of this matrix to purify MTR kinase to homogeneity from a cell-free extract of *K. pneumoniae*, and the fortuitous co-purification of the enzyme 5'-methylthioadenosine (MTA)/S-adenosylhomocysteine (SAH) nucleosidase.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The clinical isolate of *K. pneumoniae* as well as the culture conditions used to maintain tl organism for these studies have been described previously [172].

Chemicals and substrates

S-Adenosyl-L-[*methyl*-^{14}C]methionine (^{14}C-SAM; 100 µCi/ml, 55 mCi/mmol) w purchased from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). 5 [*methyl*-^{14}C]-Methylthioadenosine (^{14}C-MTA) was synthesized from ^{14}C-SAM described by Schlenk [7]. 5-[*methyl*-^{14}C]Methylthioribose (^{14}C-MTR) was produc from ^{14}C-MTA by acid hydrolysis [8]. 5'-Tosyladenosine and 6-aminohexanoic acid N hydroxysuccinimide ester-Sepharose 4B were obtained from Sigma Chemical Corp. (S Louis, MO, U.S.A.). 4-Aminothiophenol was purchased from Aldrich (Milwaukee, W U.S.A.).

Synthesis of PAPTA

Solid sodium (8.8 mmol) was dissolved in 100 ml of reagent-grade methanol (Aldric with stirring in a round-bottomed flask. 4-Aminothiophenol (7.1 mmol) was th dissolved into the mixture, followed by the addition of 5'-tosyladenosine (7.1 mmo After flushing the vessel with nitrogen, it was tightly capped and allowed to stir for 2 da at room temperature. Conversion of the 5'-tosyladenosine to PAPTA was monitored b TLC on Whatman reverse-phase (C_2) plates using a solvent system composed acetonitrile/water (15:1). To complete the purification, the mixture was evaporated dryness and the residue extracted twice with a small volume of water. The remainii solid material (impure PAPTA) was recrystallized three times from methanol. The whi crystalline product was greater than 99% pure as judged by TLC. The identity of t compound was confirmed by MS (70 eV) and elemental analysis.

Synthesis of PAPTA/PAPTR affinity matrix

PAPTA–Sepharose was constructed by covalent linkage of the *p*-amino group of PAP] to 6-aminohexanoic acid N-hydroxysuccinimide ester–Sepharose 4B according to t manufacturer's protocol. Prior to coupling, 1 g of the dry resin was mixed overnight 20 ml of distilled water to yield approximately 3 ml of hydrated matrix. The matrix w then rinsed on a scintered glass filter with 400 ml of ice-cold 1mM HCl. PAPTA (60 m was dissolved in 6 ml of distilled water for 1 h in a boiling-water bath, followed by t addition of an equal volume of bicarbonate buffer (0.2 M $NaHCO_3$, pH 8.0/1 M Na(just before the addition of rinsed matrix. The ligand was allowed to react with t activated resin for 1 h at room temperature with constant agitation. Unbound ligand w removed from the resin by rinsing with 50 ml bicarbonate buffer. Unreacted sites we blocked by treatment with 15 ml of 1 M ethanolamine for 1 h at room temperatu Following this treatment, the matrix was rinsed extensively with five alternating wash (100 ml each) of acetate buffer (0.1 M sodium acetate, pH 4/0.5 M NaCl) and Tris buf (0.1 M Tris, pH 8/0.5 M NaCl). The PAPTA–Sepharose was subsequently rinsed w S200 buffer [10 mM imidazole, pH 6.8/100 mM NaCl/2mM dithiothreitol (DTT)/1% (v/ glycerol] and stored at 4°C until needed.

PAPTR-resin was synthesized by treating 2 ml of PAPTA–Sepharose with 5 ml pooled sample of partially purified *K. pneumoniae* MTA/SAH nucleosidase for 1 h 37°C with constant agitation. After nucleosidase treatment, the resin was placed in column (1 x 2 cm) and washed extensively with S200 buffer. The PAPTR resin w stored at 4°C in S200 buffer containing 0.2% azide.

Enzyme assays and kinetics

For enzyme purification purposes, MTR kinase activity was measured essentially described by Gianotti et al. [172]. Briefly, enzyme samples (1-10 µl) were incubated i 1 h at 37°C in 100 µl of reaction mixture containing 100 mM glycine (pH 9.5), 5 m

ATP, 20 mM $MgCl_2$, 5 mM DTT and 5 μM ^{14}C-MTR (0.029 μCi). The reaction w stopped by addition of 400 μl of ice-cold ethanol and centrifuged (10000 g for 5 min) sediment precipitated material. A 450 μl sample of the supernatant was applied to a 2 AG-1-X8 formate anion-exchange column (Bio-Rad). Unreacted substrate was remov by rinsing the column (3 x 5 ml) with 0.01 M sodium formate buffer (pH 5). Bound ^{14} MTR-1-phosphate was eluted with 8 ml of 0.75 M sodium formate buffer (pH 5). Af thorough mixing, a 2 ml sample of the eluate was added to 18 ml of EcoLume scintillati cocktail (ICN) and the radioactivity quantified on a Beckman LS3801 liquid-scintillati counter. For enzyme kinetic studies, the assay was performed as described above, wi ^{14}C-MTR concentrations ranging from 0.5 μM to 17.5 μM. Enzyme concentration a reaction duration were adjusted to limit conversion of substrate to less that 5%. Resu were plotted as double-reciprocal (Lineweaver-Burk) plots [9] and analyzed for kine parameters using the Leonora enzyme kinetics program [10].

MTA nucleosidase activity was measured by following the conversion of ^{14}C-M to ^{14}C-MTR, essentially as described by Della-Ragione et al. [11], with min modifications. The standard assay for following enzyme purification contained 25 μl sample, 190 mM imidazole (pH 7) and 37 μM ^{14}C-MTA (5 μCi/μmol), in a total reacti volume of 200 μl. Reactions were incubated at 37°C for 60 min and stopped by additi of 50 μl of ice-cold 3 M trichloroacetic acid. Precipitated material was removed centrifugation (10000 g for 10 min). A 200 μl sample of supernatant was applied to a ml AG50-X8 cation-exchange column (Bio-Rad; 100-200 mesh, hydrogen-ion form), a unbound ^{14}C-MTR eluted with 3 ml of distilled water directly into a 20 ml vial scintillation cocktail. For kinetic analysis the substrate concentration ranged from 2.5 μ to 20 μM. Enzyme concentration and reaction time were adjusted to limit substr hydrolysis to less than 5%.

Preparation of enzyme extracts and initial chromatography

Overnight cultures of *K. pneumoniae* grown at 37°C were diluted 100-fold into 16 litr of methionine-free defined medium [12]. Cultures were incubated at 37°C with vigoro agitation until mid-log phase (A_{545}≈0.6) and harvested by centrifugation (5000 g for 1 min). The resulting cell pellets (approx. 100 g wet weight) were stored at -20°C un needed. Unless otherwise stated, all subsequent steps in the enzyme purification schen were carried out on ice or in a 4°C refrigerated cold-room.

To prepare cell-free extracts, cell pellets were thawed and resuspended in 200 ml ice-cold ID buffer (10 mM imidazole, pH 6.8/2mM DTT). Cells were disrupted by fo passages through a French Pressure cell (SLM-Aminco) at 1104 x 10^2 kPa (160(lb./in^2). Cell lysates were centrifuged at 4°C (25000 g for 30 min) to remove cellul debris. For an initial enrichment of MTR kinase activity, dry ammonium sulphate (0.1 g/ml lysate) was stirred into the cell lysate over the course of 2 h. The lysate was stirr for an additional 1 h, followed by centrifugation (10000 g for 30 min) to remo precipitated material. The supernatant (containing MTR kinase activity) was then dialyz extensively against ID buffer.

Before affinity chromatography, the dialysate was subjected to DEAE (anic exchange) and S200HR chromatography. Briefly, the dialysate (400 ml) was loaded on a DEAE-Sepharose Cl-6B column (25 cm x 4 cm) at a flow rate of 0.5 ml/min. Tl column was washed with ID buffer (2 litres) until the A_{280} of the eluent was below 0.0 A linear gradient of 0-0.8 M NaCl in ID buffer (1.5 litre) was then applied to the colun at a flow rate of 0.27 ml/min. Fractions were collected every 15 min (~4 ml/fraction) a assayed for enzyme activity. Fractions containing high levels of MTR kinase activity we pooled and concentrated using Centriprep 10 ultrafiltration units (Amicon, Beverly, M U.S.A.) according to the manufacturer's specifications. The concentrated DEAE-purifi MTR kinase pool was then rechromatographed on the DEAE column using a 0-0.5

NaCl gradient. Fractions containing peak MTR kinase activity were pooled a concentrated as described above.

DEAE-purified MTR kinase (4 ml) was applied to a Sephacryl S200HR column (cm x 2.5 cm) equilibrated in S200 buffer. The flow rate was adjusted to 4 ml/h, with min fractions collected and assayed for enzymatic activity. An approximate molecul mass elution profile was assigned by performing a parallel separation with a mixture protein chromatographic standards (Pharmacia). Fractions containing peak levels of MT kinase activity were pooled, concentrated by ultrafiltration, and stored at 4°C for affini chromatography.

PAPTR affinity chromatography

S200-purified MTR kinase (2 ml) was recycled five times over a 3 ml column of PAPT Sepharose. After the fifth application, the resin was washed with 20 ml each of: (1) buffer + 100 mM NaCl; (2) ID buffer + 500 mM NaCl; and (3) ID buffer + 2 M NaC MTR kinase activity was selectively removed by treatment with 20 ml of Mg/ATP buff (100 mM glycine, pH 9.5/5 mM ATP/20 mM $MgCl_2$) at 37°C. The column was wash with an additional 20 ml fraction of ID buffer +100 mM NaCl, and finally with 18 ml of low-pH buffer (100 mM glycine, pH 2.5) eluted directly into 2 ml of 1 M Tris (pH 8.(All elutions were concentrated by Centriprep 10 ultrafiltration and assayed for enzyma activity as described above.

Protein determination

Protein concentrations were determined using a Coomassie® Plus kit (Pierce, Rockfor IL, U.S.A.) according to the manufacturer's specifications. Absorbances were monitor at 595 nm with BSA used as a protein standard.

Protein analysis

SDS/PAGE was performed using a 12.5%-acrylamide resolving gel and 3%-acrylami stacking gel system [13] in a Hoefer Mighty Small II® apparatus. Electrophoresis w

typically conducted for 1 h at 100 V, followed by overnight staining of the gel in solution of 0.05% Coomassie Blue dissolved in 50% methanol/10% acetic acid. Resolv proteins were visualized following destaining in 50% methanol/10% acetic acid.

For electroblotting, proteins were resolved by SDS/PAGE on 10%-polyacrylami gels and equilibrated briefly in 3-(cycloyhexylamino)propane-1-sulphonic acid (CAP buffer (10 mM CAPS/10% methanol, pH11) [14]. Proteins were then transferred poly(vinylidene difluoride) membranes at 50 V for 35 min, using the CAPS buff system, in a water-cooled Hoefer TE series TransPhor electrophoresis chamber. Protei were visualized by brief (1 min) staining of the poly(vinylidene difluoride) membrane wi Coomassie Blue (0.1% in 50% methanol), destaining with 50% methanol/10% acetic aci followed by extensive rinsing in distilled water. Stained protein bands were excised a submitted to the Portland VAMC Core Molecular Biology facility for N-termin sequencing on an Applied Biosystems automated peptide sequencer.

RESULTS

Initial purification of MTR kinase

MTR kinase was initially purified from cell-free lysates of *K. pneumoniae* using succession of DEAE and S200HR chromatographic steps. Upon completion of siz exclusion chromatography, the enzyme had been purified approximately 1500-fold wi an overall yield of 55% (Table 2.1). An estimated molecular mass for MTR kinase of : kDa was obtained from S200HR chromatography by comparing the elution of MT kinase activity with the elution profile of proteins of established molecular mass.

PAPTA/PAPTR affinity chromatography

Chromatography of MTR kinase on the affinity matrix (Scheme 2) improved t purification factor to nearly 11000-fold with an 11% overall yield (Table 2.1). / demonstrated in Figure 2.1, most of the MTR kinase activity remained on the affini matrix even after treatment with 2 M NaCl, and was selectively eluted from the colun when treated under conditions which are known to be optimal for activity of the *Klebsiel* enzyme ('Mg/ATP' elution: 100 mM glycine buffer, pH 9.5/5 mM ATP/20 mM $MgCl_2$ mM DTT). Presumably, these conditions favor phosphorylation of the bound affini ligand and subsequent release of MTR kinase. A final elution of the column with 100 m glycine (pH 2.5) to elute remaining proteins from the matrix failed to yield any significa MTR kinase activity. However, subsequent analysis indicated that this fraction contain abundant MTA/SAH nucleosidase activity (Figure 2.1).

Enzyme analysis

SDS/PAGE analysis of fractions from the affinity resin revealed a single protein band wi a molecular mass of ~ 46 kDa in the 'Mg/ATP' elution (Figure 2.2, panel [A], lane B This value is consistent with the molecular mass predictions obtained by S200H chromatography. SDS/PAGE analysis of the final 100 mM glycine eluate (the fracti

containing the highest MTA/SAH nucleosidase activity) revealed a single protein ba with a relative molecular mass of 26.5 kDa (Figure 2.2, panel [B], lane D).

Kinetic analysis of homogeneously purified MTR kinase revealed a K_m for MTR 12.2 μM (Figure 2.3a). For purified MTA/SAH nucleosidase, a K_m for MTA of 8.7 μ was determined (Figure 2.3b). Both K_m values were extracted from double-reciproc plots using the Leonora enzyme kinetics program. N-terminal sequencing of both affinit purified enzymes allowed the identification of 19 of the first 20 amino acid residues for t MTR kinase, and 35 residues for MTA/SAH nucleosidase (Figure 2.4).

DISCUSSION

Methionine serves as a structural component of proteins as well as providing the initiat amino acid during protein synthesis [15]. Upon activation to SAM, it serves as a sour of methyl groups for a variety of transmethylation reactions, and as the source of prop amine groups for polyamine biosynthesis [16]. To meet the high demand for this ami acid, micro-organisms obtain methionine by *de novo* synthesis and through a variety salvage routes. Many organisms are able to salvage methionine from MTA, therel conserving the amino acid consumed during polyamine synthesis.

The salvage of methionine from MTA has been most extensively characterized in t gram negative bacterium, *K. pneumoniae* [17,18]. The present paper describes t purification to homogeneity of the first two enzymes involved in this cycle: MTA/SA nucleosidase and MTR kinase. At first glance, MTR kinase appears to be an ideal targ for anti-microbial chemotherapeutic drug development, since it has no correspondi human equivalent and recognizes a substrate that is not present in mammalian cel Previously, it has been shown in a number of biological systems that the presence of MT kinase can be selectively exploited by subversive substrates which are metabolized by t enzyme to toxic methionine analogs or to liberate highly reactive intermediates [1,19,20]

Because of the dual role of microbial MTA/SAH nucleosidase in regulati intracellular levels of both MTA and SAH, it also represents an attractive target for dr development. Elevated levels of SAH and MTA inhibit methylation reactions [21-24] a polyamine synthesis [25-27]. Nucleoside analogs which selectively inhibit t nucleosidase could act to kill invading micro-organisms by perturbing methylati processes and polyamine levels.

The primary goal of our study was to purify MTR kinase. Affinity chromatograp on PAPTR-Sepharose represented the key step in the purification of this enzyme

homogeneity. The choice of this particular affinity matrix was based on the remarkat affinity of the enzyme for various arylthio-substituted derivatives [6]. The resin w prepared by linking PAPTA to cyanogen bromide-activated Sepharose 4B, presumab yielding a mixed matrix in which the ligand is bound through the *p*-amino group of t phenyl side-chain or the 6-position amine of the adenine ring (or possibly both). T glycosidic bond was cleaved by the application of a partially purified preparation of *I pneumoniae* MTA/SAH nucleosidase to yield the final PAPTR matrix. Purification MTR kinase was achieved by applying selective assay-like elution conditions, i.e. pH 9 in the presence of ATP. We believe that under these conditions, MTR kinase is eluted the bound PAPTR ligand becomes phosphorylated. Despite rather harsh washi conditions, MTA/SAH nucleosidase applied to the column to generate the PAPTR liga remained bound to the matrix until it was eluted with an acidic pH buffer (100 m glycine, pH 2.5). Possibly, this strong retention of the nucleosidase to the matrix caused by the presence of non-hydrolysable ligand, due to the alternative linkage of t PAPTA via the 6-position amine of the adenine ring.

MTR kinase activity has been detected in a variety of bacteria, protozoa and plar [1,3,28-32]. The enzyme has been partially purified from *Enterobacter aerogenes* [2 *K. pneumoniae* [172], and seeds of *Lupinus luteus* [28]. Several investigators estimat the molecular mass of native MTR kinase to be in the range of 70 kDa, based on g filtration analysis [4,28]. Our own gel-filtration studies and SDS/PAGE analysis indica a monomer size nearer to 50 kDa. However, it should be noted that the elution profile activity from the S200HR column is quite broad, thus we cannot accurately assess t subunit make-up of the native enzyme. The substrate affinity displayed by the purifi kinase (K_m for MTR of 12.2 μM) is similar to the Michaelis constants determined l other investigators for partially purified enzymes [4,28,33].

MTA/SAH nucleosidase has previously been purified to homogeneity from *E. c* using an *S*-formycinyl homocysteine-Sepharose affinity column [11]. Della-Ragione al. [11] have characterized this enzyme extensively and report that it functions as monomer with a native molecular mass of 26.5 kDa. These observations are consiste with the results we obtained from SDS/PAGE analysis of homogeneously purified *pneumoniae* nucleosidase. However, our results indicate that the *Klebsiella* enzyme (*K* for MTA = 8.7 μM) is markedly different from MTA/SAH nucleosidase derived from *coli* and *L. luteus*, which exhibit much higher affinities for MTA (K_m = 0.4 μM for bot [11,34]. It is noteworthy that unlike *K. pneumoniae, E. coli* is incapable of methioni salvage from MTA since it lacks MTR kinase (M. K. Riscoe unpublished observation Instead, it has been reported that MTR produced in *E. coli* by the enzymatic cleavage MTA is exported [35,36]. Therefore, the observed differences in substrate affiniti displayed by MTA/SAH nucleosidases may be a reflection of the metabolism of MTA a disposition of its products, i.e. export versus salvage of MTR.

In conclusion, this study highlights a method for purifying MTR kinase a MTA/SAH nucleosidase to homogeneity from the pathogen *K. pneumoniae*. Sufficie material was obtained to allow the determination of the N-terminal amino acid sequence each of the enzymes. Comparing the 19 amino acid sequence found for MTR kinase sequences contained in SwissProt and PIR data banks failed to reveal any significa sequence similarities to other proteins or putative translation products. However, analy of the SwissProt database revealed a 95% identity between the *Klebsiella* MTA/SA nucleosidase N-terminal residues and a putative translation product of the *pfs* ge (accession no. P24247) reported upstream of the *dgt gene* in *E. coli* [37]. Based on t presence of conserved sequence motifs shared with known nucleoside phosphorylas investigators have predicted that the *pfs* gene product (calculated molecular mass ≈ 24 kDa) is a nucleosidase of unknown specificity [38]. Investigations into the identificati

of this region as encoding the MTA/SAH nucleosidase are currently under way in o laboratory.

Finally, it is hoped that the information presented in this article will facilitate t ultimate goal of cloning and sequencing the genes encoding MTR kinase and MTA/SA nucleosidase. Subsequent over-expression of the genes would provide large quantities both enzymes suitable for ongoing rational drug design and drug screening efforts.

ACKNOWLEDGMENTS

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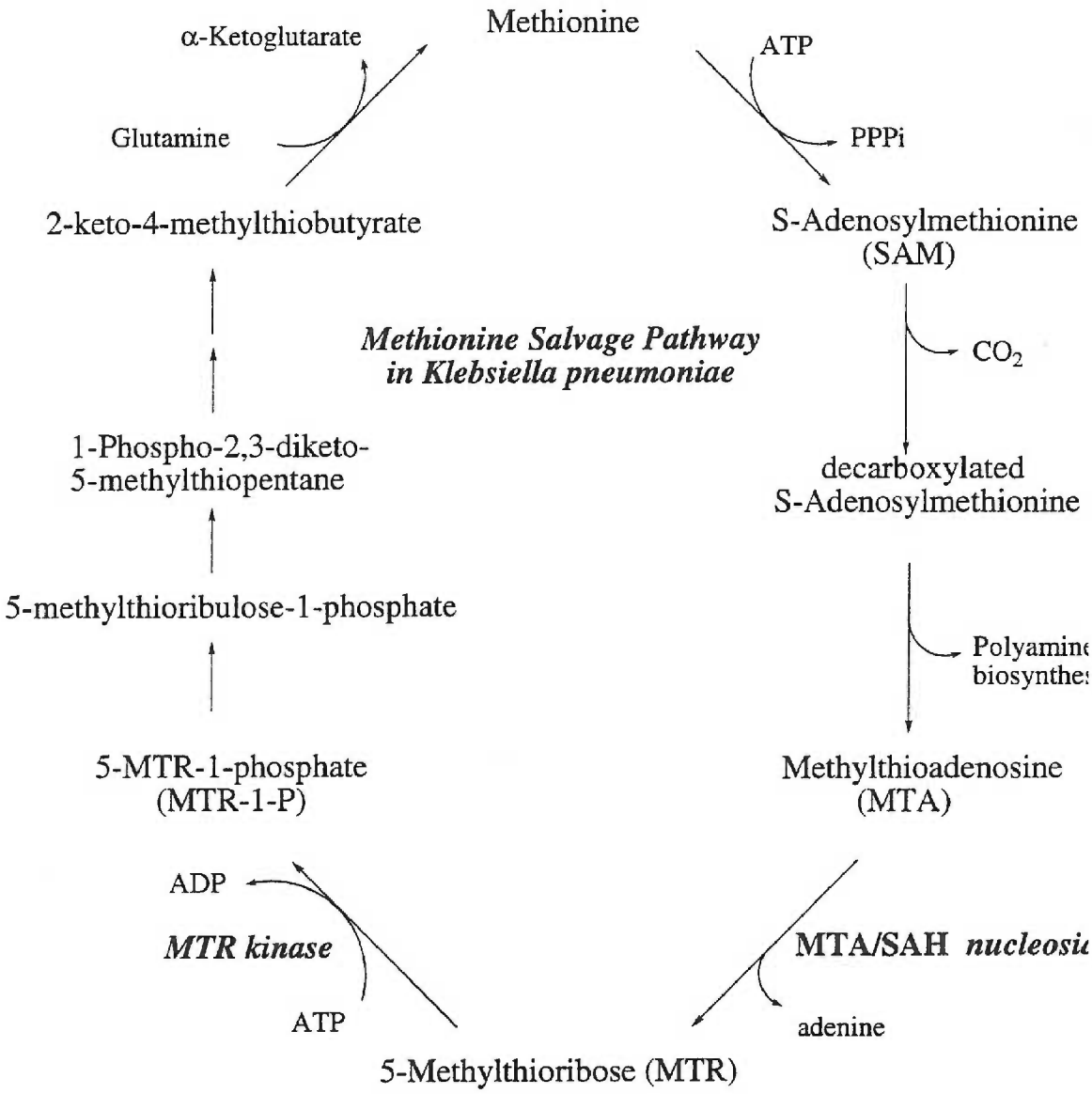
Table 2.1. Purification of MTR kinase from *K. pneumoniae*

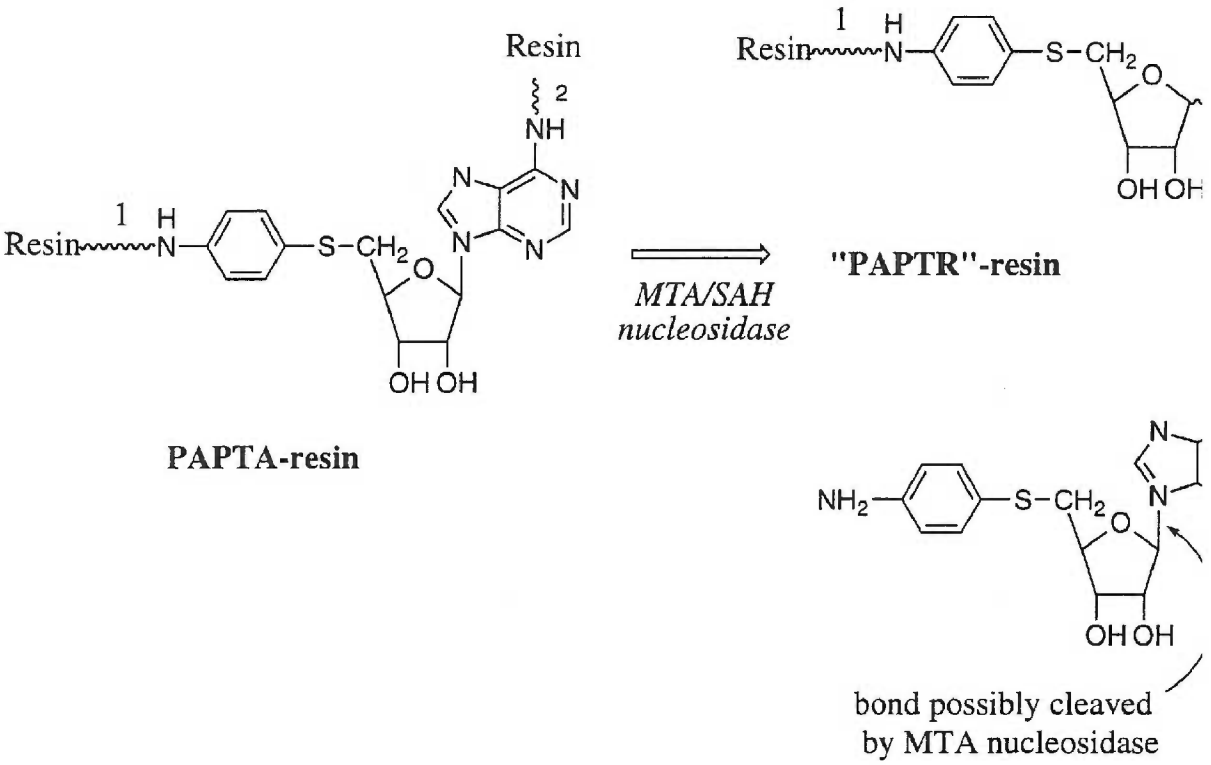
Purification step	Specific activity (pmol/min/mg protein)	Total protein (mg)	Yield (%)	Fold purification
Crude	7.93	1571	100	1
$(NH_4)_2SO_4$ (25% fraction supernatant)	440.2	125	451	55
DEAE-Sepharose (0-0.8M NaCl gradient)	1746.3	4.92	70	220
DEAE-Sepharose (0-0.5M NaCl gradient)	5283.5	1.34	58	666
S200HR	11634.5	0.58	55	1467
"PAPTR" affinity chromatography	85200	0.015	11	10744

FIGURE LEGENDS

Scheme 1. The methionine salvage pathway via MTA.


Methionine
α-Ketoglutarate
Glutamine
ATP
PPPi
2-keto-4-methylthiobutyrate
S-Adenosylmethionine
(SAM)
Methionine Salvage Pathway
in Klebsiella pneumoniae
CO2
1-Phospho-2,3-diketo-
5-methylthiopentane
decarboxylated
S-Adenosylmethionine
5-methylthioribulose-1-phosphate
Polyamine
biosynthes
5-MTR-1-phosphate
(MTR-1-P)
Methylthioadenosine
(MTA)
ADP
MTR kinase
ATP
MTA/SAH nucleosi
adenine
5-Methylthioribose (MTR)

Scheme 2. Schematic depicting the two possible linkages of PAPTA to Sepharose C 4B and the synthesis of PAPTR affinity matrix.



PAPTA-resin

"PAPTR"-resin

Figure 2.1. Elution of MTR kinase and MTA nucleosidase activity fr PAPTA/PAPTR affinity matrix.

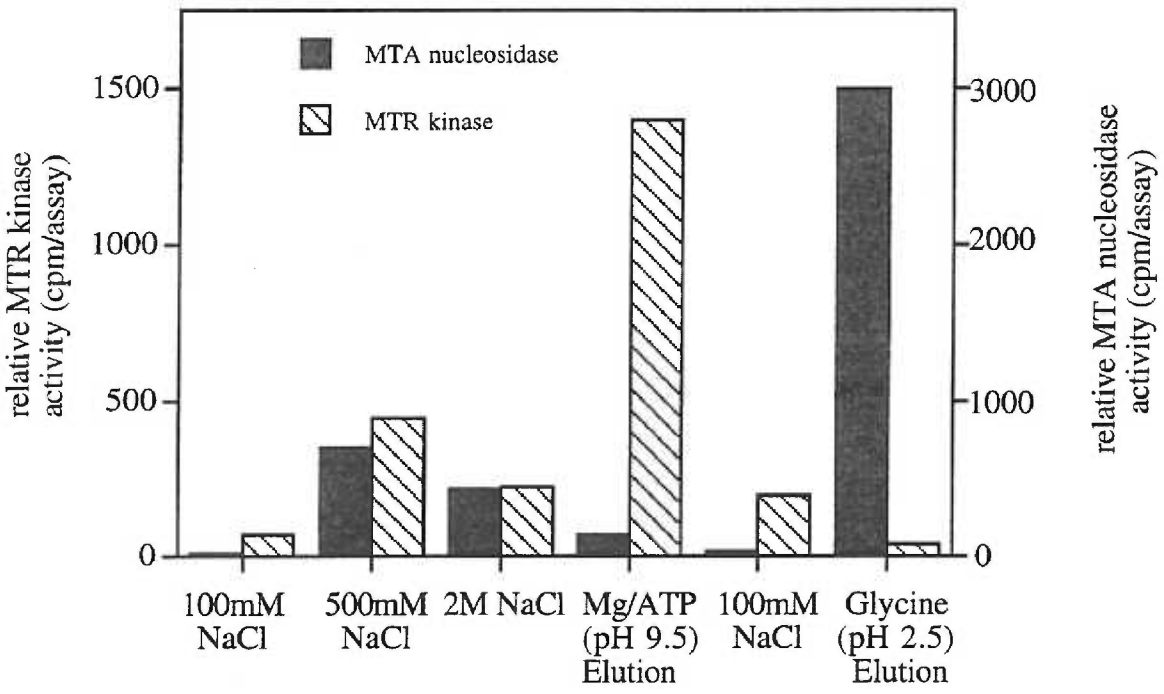

MTA nucleosidase
MTR kinase
relative MTR kinase activity (cpm/assay)
relative MTA nucleosidase activity (cpm/assay)
1500
1000
500
0
3000
2000
1000
0
100mM NaCl
500mM NaCl
2M NaCl
Mg/ATP (pH 9.5) Elution
100mM NaCl
Glycine (pH 2.5) Elution

Figure 2.2. SDS-PAGE analysis of affinity purified MTR kinase and M nucleosidase.

Panel A: **Lane A,** S200HR pool containing MTR kinase activity applied to the affini resin.

Lane B, "Mg/ATP, pH 9.5" elution containing purified MTR kinase.

Panel B: **Lane C,** S200HR pool containing MTA/SAH nucleosidase activity.

Lane D, "Glycine, pH 2.5" elution containing purified MTA/SAH nucleosidase.

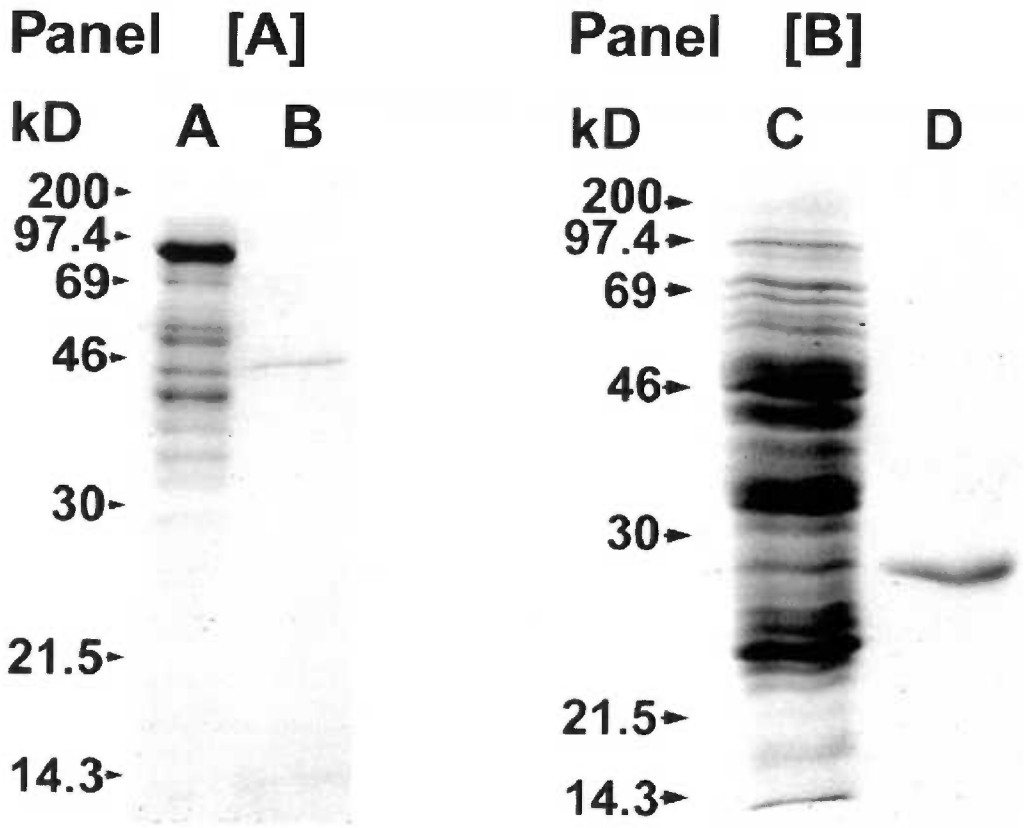
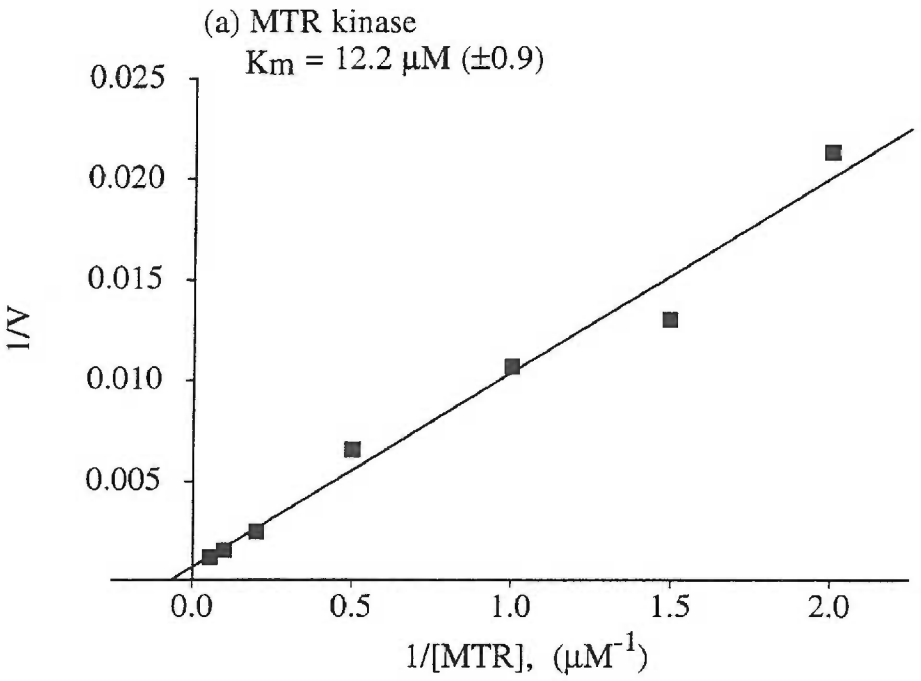

Panel [A]
kD
A
B
200
97.4
69
46
30
21.5
14.3
Panel [B]
kD
C
D
200
97.4
69
46
30
21.5
14.3

Figure 2.3. Lineweaver-Burke plots of (a) MTR kinase and (b) MTA/SAH nucleosid activity as a function of substrate concentration. Each point is the mean value of at lea experimental determinations. In MTR kinase kinetics, the concentration of ATP is fixe 5mM.


(a) MTR kinase
Km = 12.2 µM (±0.9)
0.025
0.020
0.015
0.010
0.005
1/V
0.0
0.5
1.0
1.5
2.0
1/[MTR], (µM-1)

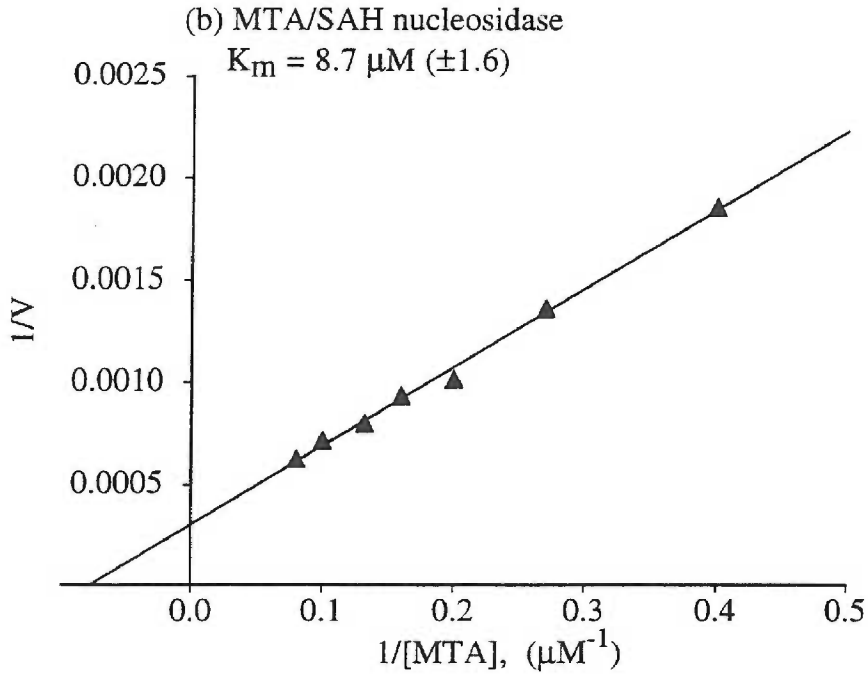

(b) MTA/SAH nucleosidase
Km = 8.7 µM (±1.6)
0.0025
0.0020
0.0015
0.0010
0.0005
1/V
0.0
0.1
0.2
0.3
0.4
0.5
1/[MTA], (µM-1)

Figure 2.4. Amino terminal amino acid sequences of affinity purified MTR kinase a MTA/SAH nucleosidase.

Protein	Residue #																														
	1				5					10					15					20					25					30			
MTR kinase	*	Q	Y	H	T	F	T	A	H	D	A	V	A	Y	A	Q	Q	F	A	G	-	-	-	-	-	-	-	-	-	-	-	-
MTA/SAH nucleosidase	M	K	I	G	I	I	G	A	M	E	E	E	V	T	L	L	R	D	K	I	E	N	R	Q	T	I	T	I	G	G	S	E	I

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CHAPTER 3

Cloning and Expression of *Escherichia coli* Methylthioadenosine/S-Adenosylhomocysteine Nucleosidase: Identification of the *pfs* gene product

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Running Title: Cloning of *E. coli* MTA/SAH Nucleosidase

Abbreviations: MTA, 5-methylthioadenosine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; MTR, 5-methylthioribose

Key Words: Methionine, recycling, salvage, MTA/SAH nucleosidase, MTR kinase, MTA phosphorylase, *pfs* gene

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SUMMARY

The enzyme 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (EC 3.2.2. is responsible for cleavage of the glycosidic bond in both 5-methylthioadenosine (MT and S-adenosylhomocysteine (SAH). Based on amino acid sequence analysis of tl enzyme from *Klebsiella*, we recently speculated that an open reading frame found in *coli* (designated *pfs*) encoded an MTA/SAH nucleosidase. To explore this possibili we amplified and cloned the complete *pfs* gene from *E. coli* genomic DNA. The ge was subsequently expressed as both a glutathione S-transferase fusion protein, and as full length protein under tryptophan regulation. The latter protein exhibited a molecul weight on SDS-PAGE of ~ 26KDa and a pI of ~5.0; consistent with values reported f the native *E. coli* enzyme. Kinetic studies on both recombinant forms of the enzyr revealed Michaelis constants for MTA of ~0.5μM, nearly identical to the report $K_{m[MTA]}$ for native enzyme. The enzymatic hydrolysis of MTA was strongly inhibit by the alternate substrate, SAH, but not significantly affected by the products of tl enzymatic reaction, adenine and methylthioribose. From this biochemical evidence, v confirm our original assignment of the *pfs* gene as encoding MTA/SAH nucleosidas The results of amino acid sequence comparisons of the protein databases are presente revealing a number of interesting homologies to other nucleoside cleaving enzymes.

INTRODUCTION

The nucleosides, 5'-deoxy-5'methylthioadenosine (MTA) and S-adenosylhomocystei (SAH), exert significant antiproliferative effects in many cellular systems and are believ to function as growth regulatory molecules (1-4). MTA is derived from adenosylmethionine (SAM) by several metabolic routes, and serves as a comm intermediate in the salvage of methionine and adenine (5,6). Quantitatively, most of t MTA in proliferating cells is derived from polyamine biosynthesis, where the nucleosi is produced stoichiometrically with spermidine and spermine. MTA acts as both a stro "feedback" inhibitor of bacterial and mammalian polyamine syntheses (7,8), as well as "suicide-like" inhibitor of mammalian SAH hydrolase (9,10). SAH is a coproduct a potent feedback inhibitor of SAM dependent methyltransferase reactions (11).

In enteric bacteria, MTA and SAH are cleaved by a single enzyme, MTA/SA nucleosidase (MTA/SAH'ase), which hydrolyzes the glycosidic bond of the tw nucleosides to yield adenine and the corresponding thiopentose (methylthioribose and ribosylhomocysteine) (Figure 3.1) (12). In contrast, MTA and SAH are catabolized mammalian cells by two distinct enzymes: MTA phosphorylase and SAH hydrola (13,14). Exploitation of potential differences in substrate specificity between t microbial and mammalian enzymes represents a promising opportunity for development selective antimicrobial agents. Structural analogs of MTA and SAH have bee synthesized which display effective antimicrobial activity both *in vitro* and *in vivo* (1 19).

Recently, we reported the purification to homogeneity of MTA/SAH'ase from ce lysates of a clinical isolate of *Klebsiella pneumoniae* (20). Amino terminal sequence da was obtained for the first thirty-five amino acids of the *Klebsiella* enzyme. A compute

assisted search of the protein sequence databases failed to reveal any signific homologies to known proteins. However, a high degree of homology (≈95%) w observed with the first thirty-five residues of the deduced translation product of the *E. c* *pfs* gene (an open reading frame of unknown function). In order to prove the identity *pfs* as encoding MTA/SAH'ase, and to investigate its potential as a chemotherapeu target, the *E. coli* gene was cloned using a PCR based strategy and subsequently ov expressed as a glutathione-S-transferase fusion protein and as the native enzyme unc tryptophan regulation. Biochemical analysis of the recombinant gene product allowed t definitive assignment of *pfs* as the MTA/SAH'ase gene.

EXPERIMENTAL PROCEDURES

Radiochemicals

S-Adenosyl-L-[methyl-^{14}C] methionine (100μCi/mL, 55mCi/mmol) was purchased fr American Radiolabeled Chemicals (St. Louis, MO). 5'-[methyl-^{14}C] Methylth adenosine (^{14}C-MTA) was synthesized from ^{14}C-SAM as previously described (6). [^{35}S] dATP was purchased from NEN-DuPont (Boston, MA).

Molecular biology reagents

Restriction endonucleases and DNA modifying enzymes were obtained from Boehring Mannheim Biochemicals (Indianapolis, IN) and from Life Technologies, In (Gaithersburg, MD). AmpliTaq DNA polymerase and other polymerase chain reacti reagents were purchased from Perkin-Elmer Corp. (Norwalk, CT). The Sequenase DN sequencing kit (version 6.0) was obtained from U.S. Biochemical Corp. (Clevelan OH). The *Taq* DyeDeoxy cycle sequencing kit was obtained from Applied Biosyster Inc. (Foster City, CA). Oligonucleotide primers for PCR and DNA sequencing we synthesized by Oligo's Etc. (Wilsonville, OR) and the VAMC Molecular Biology Co Facility (Portland, OR). Ampicillin, tetracycline, IPTG, and X-gal were obtained fro Sigma Chemical Corp. (St. Louis, MO).

Bacterial strains and plasmids

The *E. coli* strain XL-1 Blue was obtained from Stratagene and maintained on Luri Bertani (LB) agar supplemented with tetracycline (50μg/mL). Competent cells of *E. c* strains XL-1 Blue, Top10F', and GI724 were purchased from InVitrogen Corp. (LaJoll CA) or prepared as described by Maniatis, et al. (21) and stored as frozen aliquots -80°C until needed. PCR products were "TA" cloned (22) into pCR™ II (InVitrogen) ar

subcloned into either the pGEX5X-1 (Pharmacia) or pAL781 (InVitrogen) expressi vectors.

Polymerase Chain Reaction, cloning and sequencing of the *pfs* gene

The putative open reading frame (657bp) initially described by Wurgler and Richards (23) was amplified as a 967 bp EcoRI/NotI fragment using the primer set: 5'-CTC GA TTC TCT ATG AAA ATC GGC ATC ATT GGT GCA ATG G-3'(forward) and 5'-CT GCG GCC GCC AGG CAA TCA CCA GAT CGG G-3'(reverse). A crude cell lysate *E. coli* strain XLl-1 Blue was used as the source of chromosomal DNA. Briefly, a 1n sample of overnight culture was harvested by centrifugation (10,000xg / 10 mir resuspended in 100µl of sterile dH_2O and boiled for 15 min. The resulting lysate w centrifuged (10,000xg / 10min) to remove debris and a 1µl sample used for the PC reaction. Amplification of the DNA fragment was performed in an Applied Biosyster Thermocycler using an initial denaturation step (3min, 95°C) followed by 35 cycles denaturation (30sec, 95°C), annealing (3min, 60°C), and extension (2min, 72°C). A 10 aliquot of the PCR product was ligated into pCR™II and used to transform competent *coli* TOP10F' cells according to the manufacturer's specifications. Recombinants we chosen based on growth and blue/white color selection (white colonies = positi recombinants) on LB agar supplemented with ampicillin (100µg/ml) and X-gal (25µg/m Small scale plasmid minipreps were prepared from positive-appearing colonies by t alkaline lysis/PEG precipitation method (24), double digested with EcoRI/NotI (20uni ea. / 37°C / 2hrs), and subjected to agarose gel electrophoresis. A plasmid containing t complete 967bp EcoRI/NotI insert (designated pCRIImtan) was sequenced in bo directions by the dideoxynucleotide chain termination method (25). Discrepanci between the observed nucleotide sequence and the reported open reading frame we confirmed by sequencing an additional six independent pCR™II subclones across t disputed region using a Taq DyeDeoxy™ Terminator Cycle Sequencing kit. Cyc

sequencing reactions were analyzed on an Applied Biosystems automated Model 37:
DNA Sequencer (Foster City, CA).

Subcloning and expression of MTA/SAH nucleosidase in the pGEX5 system

The 967bp EcoRI/NotI fragment from clone pCRIImtan was resolved by agarose g
electrophoresis, spin-eluted from a gel slice, and directionally ligated in
EcoRI/NotI/shrimp alkaline phosphatase treated pGEX5X-1. Competent *E. coli* XL
Blue cells were transformed with the ligation mixture and positive recombinants selecte
by the growth of colonies on LB agar plates supplemented with ampicillin (100µg/ml) ar
tetracycline (12.5µg/ml). The orientation and maintenance of correct reading fran
relative to the glutathione-S-transferase (GST) fusion partner was confirmed by DN
sequence analysis using pGEX5X specific primers (Pharmacia). A plasmid clo
(designated p5Xmtan) was selected for further expression studies.

Expression and purification of the GST-nucleosidase fusion protein (GST-MTAI
was performed according to the manufacturer's protocol. Briefly, a 1ml sample of a
overnight culture of *E. coli* XL1-Blue cells containing p5Xmtan was used to inocula
200ml LB broth + ampicillin (100µg/ml). The culture was incubated in a 37°C shak
bath until the growth reached mid-log phase (OD_{595}≈0.6) and then induced by addition o
IPTG (1mM final conc.) for an additional 3hrs at 37°C. Induced cells were harvested b
centrifugation (5,000xg / 15min), washed once with PBS (pH 7.2), and resuspended
10ml ice cold PBS. Cell lysates were prepared by 4 passages of the sample through
French Pressure cell (SLM-Aminco) at 16,000psi, followed by centrifugation (25,000xg
30min / 4°C) to remove cellular debris. The lysate was applied to a 1ml glutathion
Sepharose column (Pharmacia), washed extensively with ice cold PBS (pH 7.2), and th
bound GST-MTAN selectively eluted with 10ml glutathione buffer (25mM glutathion
120mM NaCl, 100mM TrisHCl, pH7.5). The eluted material was concentrated wi

buffer exchange into PBS (pH 7.2) using a Centriprep 10 device (Amicon). The prot concentration of the final product was determined using the Coomassie Plus® ass (Pierce, Rockford, IL) and analyzed by SDS-PAGE on 12.5% polyacrylamide gels assess purity (26).

Subcloning and expression of native MTA/SAH nucleosidase in t pAL781 system

Two oligonucleotide primers, 5'CTC CAT ATG AAA ATC GGC ATC ATT GGT GC ATG G-3' (forward) and 5'CTC GGA TCC TTA GCC ATG TGC AAG TTT CTG CA CAG TGA C-3'(reverse) with engineered NdeI and BamHI restriction endonuclease si were used to amplify the 699bp native coding region (including termination codon) f MTA/SAH'ase from *E. coli* XL1-Blue cells. Conditions for PCR amplification and "T cloning of the product were as described above. The NdeI/BamHI digest fragment w ligated into NdeI/BamHI/shrimp alkaline phosphatase treated pAL781 (InVitrogen), a transformed into *E. coli* strain GI724. Positive recombinants were selected based growth in the presence of ampicillin (100μg/ml). A clone containing the plasmid pAL7 with the complete NdeI/BamHI insert (designated p781mtan) was chosen for furth studies.

Expression of MTA/SAH'ase was accomplished by preparing an overnight cultu of cells grown in RMG-Amp broth (1X M9 salts, 2% Casamino acids, 1% glycerol, 1m $MgCl_2$, 100μg/ml ampicillin) at 30°C. A 0.5ml aliquot of the overnight culture was us to inoculate 10ml of fresh induction medium (InVitrogen) and growth allowed to proce at 30°C until the $OD_{550} \approx 0.5$ (about 3.5 hrs). Expression was induced by addition tryptophan (100μg/ml final concentration) for 4hrs at 37°C. Cells were harvested b centrifugation (5,000xg / 15min) and lysed by treatment with the French Pressure cell described above. Induced MTA/SAH'ase (rMTAN) was initially purified b chromatofocusing on PBE94 resin (Pharmacia) equilibrated in 0.025M histidineH

buffer (pH 6.25) and eluted with Polybuffer74 (pH 4.0). Elution fractions containi nucleosidase activity were pooled and further purified on a monoclonal antibody affini resin specific for the enzyme (27). The affinity-purified material was concentrated wi buffer exchange into PBS (pH 7.2) using a Centriprep 10 device, and stored at 4°C un needed.

Enzyme assays and kinetics

MTA nucleosidase activity and inhibitor analysis was measured by following conversi of ^{14}C-MTA to ^{14}C-MTR, essentially as described by Ferro et al. (28) with min modifications. The standard assay for following enzyme purification contained 20µ sample, 50mM potassium phosphate (pH 7), 37µM ^{14}C-MTA (5µCi/µmol), and 0.5 bovine serum albumin in a total reaction volume of 200µL. Reactions were incubated 37°C for 15min and stopped by addition of 20µL 3M TCA. Precipitated material w removed by centrifugation (10,000xg / 10min). A 200µL sample of supernatant w applied to a 2mL AG50-X8 cation exchange column (BioRad, 100-200 mesh, H^+ forr and ^{14}C-MTR eluted with 3mL dH_2O directly into a 20mL vial of scintillation cockta For kinetic analysis the substrate concentration ranged from 0.4-10µM MTA. Enzyn concentration was set at 10 picograms per assay and the reaction time adjusted to lin substrate hydrolysis to <10%. Results were plotted as double reciprocal plots ar analyzed for kinetic parameters as described by Lineweaver and Burke (29) using tl Leonora enzyme kinetic analysis program (30). For inhibition analysis, the concentratic of MTA was set at 1µM and the concentration of inhibitor yielding a 50% reduction enzymatic activity determined from the average of at least 3 separate experiments.

RESULTS

The gene encoding a putative MTA/SAH nucleosidase was amplified from *E. c* genomic DNA using primers based on the reported open reading frame found upstrea from the deoxyguanosine triphosphate triphosphohydrolase (*dgt*) gene. Subsequent DN sequencing of the cloned 967 bp PCR product (containing the complete open readi frame) revealed several alterations (Figure 3.2) in the nucleic acid sequence original reported by Wurgler and Richardson (23). The first apparent alteration, a C—› transversion at position 45 does not change the encoded amino acid (leu). The appearan of an inserted guanosine residue at position 634 and 667 alters the reading frame near t 3' terminus of the gene, thereby eliminating the reported stop codon (TGA) at positi 658-660 and regenerating a termination signal (TAA) at position 697-699. These findin were confirmed by automated DNA sequencing of an additional six independent PC clones, and are in agreement with sequence data recently published by Fujita et al. (31 The revised open reading frame is 696 nucleotides long and encodes an additional amino acids. A calculated molecular weight of 24.35 kDa for the translation product this gene is in close agreement with the ~26-31kDa molecular weight reported for nati *E. coli* MTA/SAH nucleosidase (28,32).

The putative nucleosidase gene was first expressed as a glutathione S-transfera fusion protein (GST-MTAN) in the pGEX5X-1 system (Pharmacia) to allow faci separation of the recombinant protein from the native enzyme. SDS-PAGE analysis glutathione-Sepharose affinity purified fusion protein (Figure 3.3, panel A) revealed molecular weight for GST-MTAN of ~50kDa; consistent with the sum of the calculat molecular weight for the open reading frame (24.35kDa) fused to glutathione transferase (~26kDa). Sufficient fusion protein was purified to develop a panel

monoclonal antibodies specific for the nucleosidase which were employed in subseque enzyme purifications.

Using the pAL781 system (InVitrogen), rMTAN was expressed after induction wi tryptophan. Initial purification from bacterial lysates of over-expressed rMTAN w performed by chromatofocusing of the enzyme across a descending pH gradient of 6.: to 4.0. MTA/SAH'ase activity eluted between pH5.3 and 4.7, with peak activi occurring at ~pH 5.0, as predicted by the calculated pI of 4.93 based on the prima amino acid sequence. Material from the chromatofocusing column was further purified a monoclonal antibody resin specific for *E. coli* MTA/SAH'ase. Glycine eluates (p 2.5) of the monoclonal column contained homogeneous enzyme with an appare molecular weight of ~26kDa, as judged by SDS-PAGE analysis and Coomassie bl staining (Figure 3.3, panel B). Antibody column purifications yielded from 0.5-1mg nucleosidase per cycle, with a total yield of approximately 20mg rMTAN per liter induced culture.

Kinetic analysis of both GST-MTAN and rMTAN revealed Michaelis constants f MTA of 0.53μM and 0.45μM, respectively (Figure 3.4). The specific activity of GS MTAN was ~50μmoles MTA converted/min/mg enzyme, approximately half the activi found for rMTAN (116μmoles/min/mg); which is expected since the nucleosidase portic accounts for approximately half the total mass of the fusion protein. The substrate SA was an inhibitor of (^{14}C) MTA hydrolysis ([MTA] = 1μM) exhibiting an IC_{50} for th enzyme of 2.4μM (Table 3.1). The products of enzymatic hydrolysis of MTA, adenir (IC_{50} = 300μM) and methylthioribose (IC_{50} > 1mM), displayed only weak inhibitor activity, consistent with earlier reports for the native *E. coli* nucleosidase (28, 32

DISCUSSION

The open reading frame *pfs* was first identified in *E. coli* upstream of the *dgt* gene (2: We tentatively identified this gene as encoding MTA/SAH nucleosidase based on a hi sequence homology between the 35 amino terminal residues of the *Klebsiella* enzyme a: the putative translation product of *pfs* (20). DNA sequence analysis of the PC amplified *E. coli pfs* gene revealed several differences from the earlier reported op reading frame, the most significant of which is the presence of 2 additional guanosi residues at the 3' end of the gene which result in the extension of the open reading fra by 39 bases. A recent report by Fujita et. al (31) on the sequencing of *E. coli* genome the region of 2.4-4.1 min (110,917-193,643 bp) confirms this sequence for the *pfs* gen

Over-expression of the *pfs* gene product as a gst fusion protein and separation the resulting recombinant enzyme from native nucleosidase (based on affinity of t recombinant for glutathione) allowed the gene to be unambiguously identified as encodir MTA/SAH nucleosidase (E.C 3.2.2.9). A second tryptophan-inducible expressic system was used to produce large quantities of a native form of the enzyme (rMTAN) f use in x-ray crystallographic studies and rational drug design, including the developme of MTA/SAH analogs with selective antimicrobial properties.

In each case, the recombinant enzymes exhibited specific activities and Michael constants for MTA that are virtually identical to those reported in the literature for t native *E. coli* enzyme. Inhibition of MTA hydrolysis by SAH demonstrates that t enzyme has some flexibility in structural specificity for the 5' alkylthio side chain, an supports previous work which showed SAH was cleaved at approximately 35-40% of t rate of MTA (28,32). In contrast to mammalian MTA phosphorylase (33), the produc

of enzymatic cleavage of MTA are poor inhibitors of MTA/SAH'ase activity and probat do not play a significant role in the direct regulation of enzymatic activity.

Based on sequence similarities existing between various nucleoside phosphorylas nucleosidases, and phosphoribosyltransferases, Mushigian and Koonin recently predict that the *pfs* protein may belong to a general family of enzymes which catalyze t phosphorylytic or hydrolytic cleavage of N-glycosidic bonds of nucleosides a nucleotides (34). A search of the SWISSPROT database using the BLASTP progra (35) identified an additional *pfs* protein homologue reported for *Haemophilus influenz* (36,37) which contains a 57% sequence identity (73% identity with conservati substitutions), and presumably is the MTA/SAH nucleosidase from this organism (Figu 3.5). The regions of highest identity should be useful in designing PCR primers investigate the nucleosidase gene in other organisms.

Two smaller regions of sequence homology (amino acid regions 23-90 and 16 197) are found between MTA/SAH'ase and the purine nucleoside phosphorylase (*deo* gene product; inosine preferring PNP'ase) of *E. coli* and *H. influenzae* . Within the regions, the homology between the bacterial PNP'ases and MTA/SAH'ase approximately 50% with conservative substitutions. By comparison, MTA/SAH'a sequence shows only one short region (a.a. 69-91) of high homology (73% wi conservative substitutions) to the human and murine PNP'ases (a.a. 109-130). particular note, crystallographic studies of the human PNP'ase have shown this region be part of the active center of the enzyme, with the backbone amido group of alanine 11 forming a hydrogen bond to the 3' hydroxyl group of the ribose moiety (38 Presumably, sequence homologies indicate that this region functions similarly in t recognition of the ribose moiety by MTA/SAH'ase.

Similar to the *deoD* gene products, the bark storage precursor proteins of popla (Bsp A,B) (39,40) also display two regions of ≥50% homology with MTA/SAH'ase (a.

regions 25-80 and 189-212). While specific functions are not known for the Bsp's, t sequence similarities to MTA/SAH'ase and other purine nucleosidases suggests a role nucleoside metabolism. Lastly, MTA/SAH'ase shares only one short region of homolo to the *E. coli* AMP nucleosidase (a.a. 161-197; 62% identity with conservati substitutions). However, the precise contribution of residues in this region towa substrate recognition remain to be elucidated.

Interestingly, the BLASTP search failed to identify any large regions of identi existing between the MTA/SAH nucleosidase and either the human MTA phosphoryla (MTAP'ase) (41) or adenosylhomocysteine hydrolase (42) sequences, although th share common substrates. A search alignment using the MacVector Pustell protein matr program did reveal two small regions (a.a. 1-8 and 84-94) of >80% homology (wi conservative substitutions) between the *E. coli* MTA/SAH'ase and human MTAP'a (Figure 3.5). In particular, the appearance of the (M/V)KIGIIG(A/G) sequence at or ne the amino terminal end of each of the proteins is intriguing. It is tempting to speculate th this sequence may confer specificity for the 5' alkylthio group of the nucleoside. Tl second homologous region exists near to residues we suspect to be involved recognition and binding of the ribose portion of the substrate (based on homology human PNP), and may represent extensions of these structures. Other possib interactions existing between the nucleosidase and its substrate await the results mutational analyses and x-ray crystallographic studies currently underway in th laboratory.

ACKNOWLEDGMENTS

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Table 3.1. Inhibition of recombinant *E. coli* MTA/SAH nucleosidase (rMTAN) activity. The IC_{50} values were determined in the presence of 1μM MTA.

Compound	**IC_{50} (μM)**
S-adenosylhomocysteine (SAH)	2.4
Adenine	300
Methylthioribose (MTR)	>1000

FIGURE LEGENDS

Figure 3.1. The metabolism of MTA and SAH.

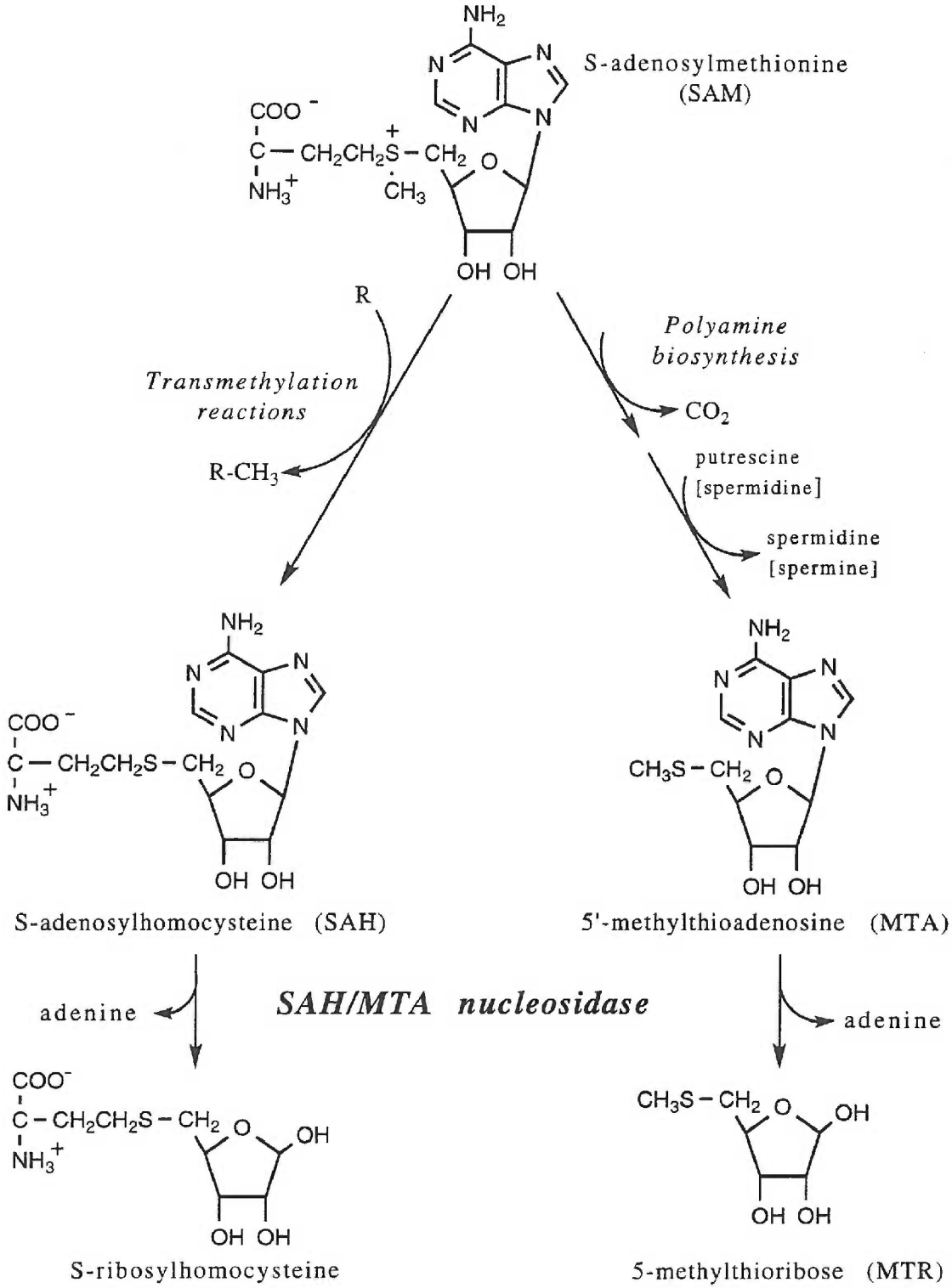

S-adenosylmethionine
(SAM)
R
Transmethylation
reactions
R-CH3
Polyamine
biosynthesis
CO2
putrescine
[spermidine]
spermidine
[spermine]
S-adenosylhomocysteine (SAH)
5'-methylthioadenosine (MTA)
adenine
SAH/MTA nucleosidase
adenine
S-ribosylhomocysteine
5-methylthioribose (MTR)

Figure 3.2. The nucleotide sequence of *E. coli* MTA/SAH nucleosidase shown wi the annotated deduced amino acids. Stretches of underlined nucleotides correspond portions of PCR primers used to amplify the gene sequence. Individual nucleotides th differed from the initial reported sequence of *pfs* are printed in bold underlined typescri

```
      ATG AAA ATC GGC ATC ATT GGT GCA ATG GAA GAA GAA GTT ACG CTG
  1   M   K   I   G   I   I   G   A   M   E   E   E   V   T   L

      CTG CGT GAC AAA ATC GAA AAC CGT CAA ACT ATC AGT CTC GGC GGT
 16   L   R   D   K   I   E   N   R   Q   T   I   S   L   G   G

      TGC GAA ATC TAT ACC GGC CAA CTG AAT GGA ACC GAG GTT GCG CTT
 31   C   E   I   Y   T   G   Q   L   N   G   T   E   V   A   L

      CTG AAA TCG GGC ATC GGT AAA GTC GCT GCG GCG CTG GGT GCC ACT
 46   L   K   S   G   I   G   K   V   A   A   A   L   G   A   T

      TTG CTG TTG GAA CAC TGC AAG CCA GAT GTG ATT ATT AAC ACC GGT
 61   L   L   L   E   H   C   K   P   D   V   I   I   N   T   G

      TCT GCC GGT GGC CTG GCA CCA ACG TTG AAA GTG GGC GAT ATC GTT
 76   S   A   G   G   L   A   P   T   L   K   V   G   D   I   V

      GTC TCG GAC GAA GCA CGT TAT CAC GAC GCG GAT GTC ACG GCA TTT
 91   V   S   D   E   A   R   Y   H   D   A   D   V   T   A   F

      GGT TAT GAA TAC GGT CAG TTA CCA GGC TGT CCG GCA GGC TTT AAA
106   G   Y   E   Y   G   Q   L   P   G   C   P   A   G   F   K

      GCT GAC GAT AAA CTG ATC GCT GCC GCT GAG GCC TGC ATT GCC GAA
121   A   D   D   K   L   I   A   A   A   E   A   C   I   A   E

      CTG AAT CTT AAC GCT GTA CGT GGC CTG ATT GTT AGC GGC GAC GCT
136   L   N   L   N   A   V   R   G   L   I   V   S   G   D   A

      TTC ATC AAC GGT TCT GTT GGT CTG GCG AAA ATC CGC CAC AAC TTC
151   F   I   N   G   S   V   G   L   A   K   I   R   H   N   F

      CCA CAG GCC ATT GCT GTA GAG ATG GAA GCG ACG GCA ATC GCC CAT
166   P   Q   A   I   A   V   E   M   E   A   T   A   I   A   H

      GTC TGC CAC AAT TTC AAC GTC CCG TTT GTT GTC GTA CGC GCC ATC
181   V   C   H   N   F   N   V   P   F   V   V   V   R   A   I

      TCC GAC GTG GCC GAT CAA CAG TCT CAT CTT AGC TTC GAT GAG TTC
196   S   D   V   A   D   Q   Q   S   H   L   S   F   D   E   F

      CTG GCT GTT GCC GCT AAA CAG TCC AGC CTG ATG GTT GAG TCA CTG
211   L   A   V   A   A   K   Q   S   S   L   M   V   E   S   L

      GTG CAG AAA CTT GCA CAT GGC TAA GTCACTGTTCAGGGCGCTGGTCGCCCC
226   V   Q   K   L   A   H   G   *

ATGTCTTTTCTTGCGCCACTGTGGCTCAACGCCGCGCCGCGCGTCACGCTTTCTCCCGC
CAACACTGAACTTGCCTTTGCCGCCGGGATCACGCCGGTTGGGGTCAGCAGCTATTCCG
ACTATCCTCCACAAGCGCAAAAGATTGAGCAGGTTTCCACCTGGCAGGGGATGAATCTG
GAACGCATTGTCGCGCTGAAACCCGATCTGGTGATTGCCTGGC
```

Figure 3.3. Expression and purification of recombinant *E. coli* MTA/SA nucleosidase.

Panel A. Coomassie stained SDS-PAGE of GST-MTAN purification fractions. **STD** 10kDa molecular weight ladder (BRL); **Uninduced** = 5µg crude lysate proteins fro p5Xmtan containing XL1-Blue cells grown in the absence of IPTG; **Induced** = 5µ crude lysate proteins from p5Xmtan containing XL1-Blue cells following three hou induction with IPTG (1mM); **GSH purified** = approx. 5µg homogeneous GST-MTA purified by glutathione-resin affinity chromatography.

Panel B. Coomassie stained SDS-PAGE of rMTAN purification fractions. **STD** = lo molecular weight protein standard (BioRad); **Crude** = 5µg crude lysate proteins fro p781mtan containing GI724 cells following four hours induction with tryptoph (100µg/mL); **C.F.** = 5µg chromatofocusing peak proteins; **MAb Affinity** = 5µ rMTAN purified on monoclonal antibody affinity resin. **df** = dye front.

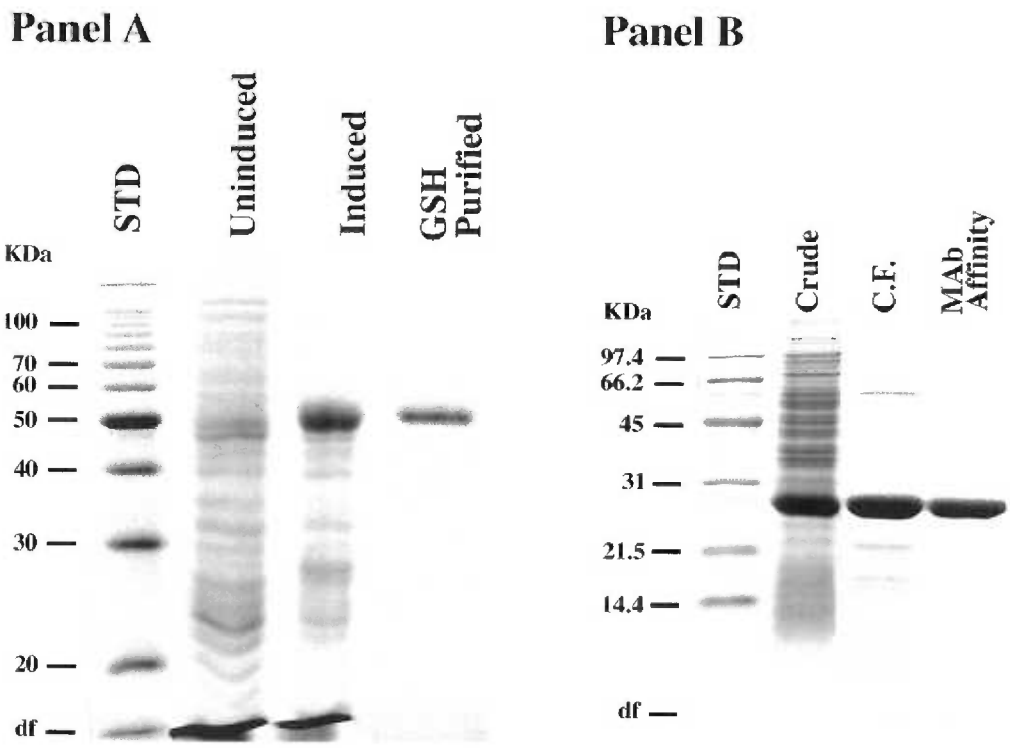
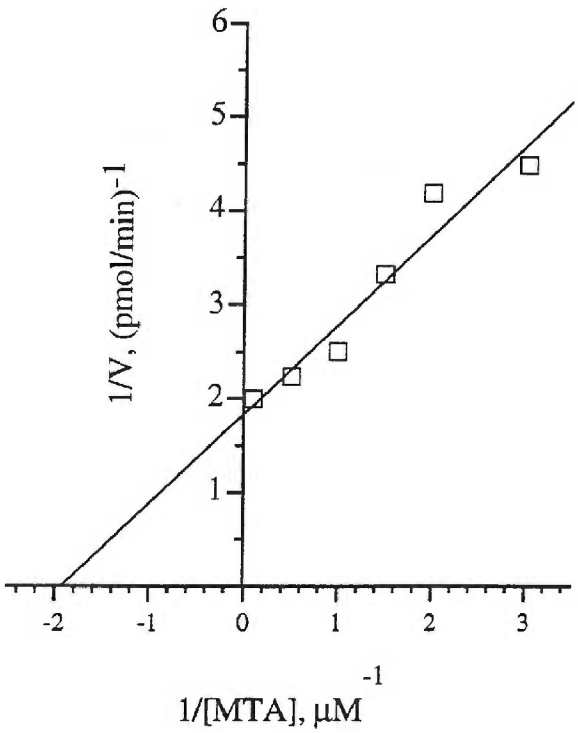

Panel A
STD
Uninduced
Induced
GSH Purified
KDa
100
70
60
50
40
30
20
df
Panel B
STD
Crude
C.F.
MAb Affinity
KDa
97.4
66.2
45
31
21.5
14.4
df

Figure 3.4. Double reciprocal plots of (A) GST-MTAN and (B) rMTAN activity as function of MTA concentration. Each point represents the mean value of thr experiments.

A.GST-MTAN
Km = 0.53μM (±0.12)



B. rMTAN
Km = 0.45μM (±0.07)

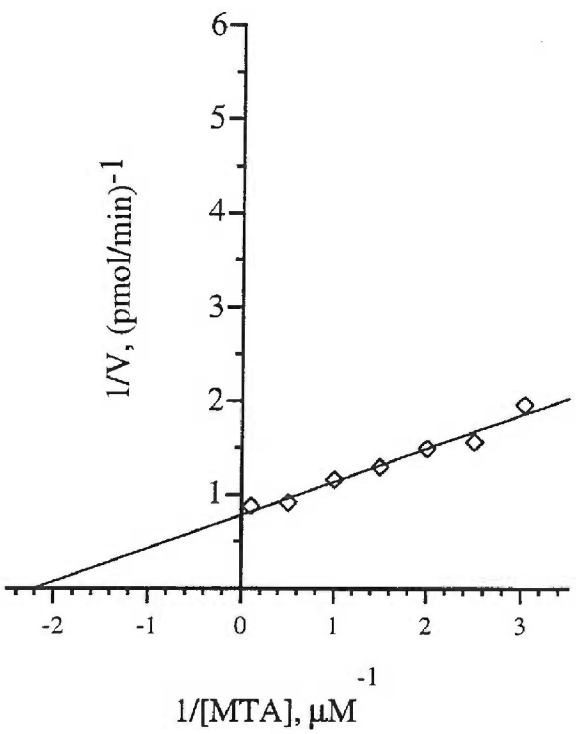


Figure 3.5(A) Sequence alignments of the *E. coli* MTA/SAH'ase with the highe scoring protein sequences found in a BLASTP search of the SWISSPROT databa (release 32.0 Dec. 1995). Identical and conserved amino acids are shown; dots represe non conserved amino acids. Numbers found immediately to the left and right of t amino acid sequence correspond to the first and last residues of the respective prote listed in the row. PFS_ECOLI, hypothetical protein, proposed here to be MTA/SA nucleosidase (Swiss-Prot Accession no. P24247); PFS_HAEIN, hypothetical MTA/SA nucleosidase homolog from *Haemophilus influenzae* (Swiss-Prot Accession no. P45113 MTAP_HUMAN, MTA phosphorylase (GenBank Accession no. U22233 DEOD_ECOLI, purine nucleoside phosphorylase (inosine phosphorylase, Swiss-Pr Accession no. P09743); DEOD_HAEIN, bark storage protein B precursor from popla (Swiss-Prot Accession no. Q09117); BSPA_POPDE, bark storage protein A precurs from poplars (Swiss-Prot Accession no. Q07469); PNPH_HUMAN, purine nucleosic phosphorylase (Swiss-Prot Accession no. P00491); PNPH_MOUSE, purine nucleosic phosphorylase (Swiss-Prot Accession no. P23492); AMN_ECOLI, AMP nucleosida (Swiss-Prot Accession no. P15272).

*The sequence comparison of MTA/SAH'ase and MTA phosphorylas (MTAP_HUMAN) was performed using the MacVector Pustell protein matrix program.

A.

```
PFS_ECOLI      1   MKIGIIGAME EEVTLLRDKI ENRQTISLGG CEIYTGQLNG
PFS_HAEIN      1   MKIGIVGAM. QEV.ILKN.M .DR....V.. ..IF.GKING
MTAP_HUMAN*   10   VKIGIIGG
DEOD_ECOLI    38                          RE..NV.G ...FTG...G
DEOD_HAEIN    48                                   FTG...G
BSPB_POPDE    56                             SV.I.G ...HSG.LNG
BSPA_POPDE    56                             SV.I.G ...HSG.LNG

PFS_ECOLI     41   TEVALLKSGI GKVAAALGAT LLLEHCKPDV IINTGSAGGL
PFS_HAEIN     41   .DVALLQSGI GKVAAAIG.T .LLQ..KPD. VINTGSAGGV
DEOD_ECOLI    56   .KISVM..GM G..S.SI... .LI..... II..GS.G.V
DEOD_HAEIN    55   .KISIM..GM G..S.SI.A. .LI..... II..GS.G.V
BSPB_POPDE    72   S.I..VKTG. ..V..A.... ILL..... VI..GNAG.L  1
BSPA_POPDE    72   S.I..VKTG. ..V..A.... ILL..... VI..GNAG.L  1
PNPH_HUMAN   109                               D. LV.T.AAGGL  1
PNPH_MOUSE   109                               E. LV.T.AAGGL  1

PFS_ECOLI     81   APTLKVGDIV VSDEARYHDA DVTAFGYEYG QLPGCPAGFK  1
PFS_HAEIN     81   A..LKVGDIV ISDE.RYHDA DVTAFGYE.G QLP..PA.F.  1
MTAP_HUMAN   102      IQ.GDIV I.DQ                              1
DEOD_ECOLI    96   .P.VKL.DVV I                                 1
DEOD_HAEIN    95   ...VKV.DVI I                                 1
PNPH_HUMAN   121   .P..EVGDIM L                                 1
PNPH_MOUSE   121   .P..EVGDIM L                                 1

PFS_ECOLI    121   ADDKLIAAAE ACIAELNLNA VRGLIVSGDA FINGSVGLAK  1
PFS_HAEIN    121   SD.KL...AQ ....K...S. .RGLI.SGDS FIN....IAQ  1

PFS_ECOLI    161   IRHNFPQAIA VEMEATAIAH VCHNFNVPFV VVRAISDVAD  2
PFS_HAEIN    161   IK.DFP.... VEMEATAIA. VCY.FNVPFV VVRAISD..D  2
DEOD_ECOLI   177           L. VEMEA..I.. V...F....L .I..VSD     2
DEOD_HAEIN   176           L. VEMEA..I.. V...Y....L .I..VSD     2
BSPB_POPDE   266                                 FV V.Q.VSNVA.  2
BSPA_POPDE   266                                 FV V.Q.VSNVA.  2
AMN_ECOLI    391   LR.N..RAVA IDMES..IA. ..Y.F.VPY. .L..VSD     4

PFS_ECOLI    201   QQSHLSFDEF LAVAAKQSSL MVESLVQKLA HG          2
PFS_HAEIN    201   .KA.MSFEEF L.LAAKQSS. LV..MI.RL              2
BSPB_POPDE   277   E.S..S...Y LA                                2
BSPA_POPDE   277   E.S..S...Y LA                                2
```

Figure 3.5(B) Comparison of % homologies between aligned proteins from Figı 3.5(A), with and without conservative substitutions.

B.

Protein:	Amino Acids	(MTA/SAH'ase A.A.)	% Identity	% Positive w/conservative substitutions
PFS_HAEIN	1-229	(1-229)	57%	73%
MTAP_HUMAN	10-17	(1-8)	62%	88%
	102-112	(84-94)	45%	82%
DEOD_ECOLI	38-106	(33-91)	24%	52%
	177-205	(169-197)	34%	48%
DEOD_HAEIN	48-105	(44-91)	25%	53%
	176-204	(169-197)	31%	48%
PNPH_HUMAN	109-131	(69-91)	47%	73%
PNPH_MOUSE	109-131	(69-91)	43%	73%
BSPB_POPDE	56-111	(35-80)	28%	50%
	266-289	(189-212)	41%	62%
BSPA_POPDE	56-111	(35-80)	28%	50%
	266-289	(189-212)	41%	62%
AMN_ECOLI	391-427	(161-197)	35%	62%

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CHAPTER 4

Characterization of Recombinant *Escherichia coli* 5'-Methylthioadenosine / S-Adenosylhomocysteine Nucleosidase: Analysis of Enzymatic Activity and Substrate Specificity

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Abbreviations: MTA, 5'-methylthioadenosine; SAH, S-adenosylhomocysteine; SAM S-adenosylmethionine; rMTAN, recombinant MTA/SAH nucleosidase; rMTAN-8, truncated recombinant MTA/SAH nucleosidase; MTAPase, MTA phosphorylase; MTR methylthioribose; PCR, polymerase chain reaction; cAMP, adenosine 3',5'-monophosphate; Mab, monoclonal antibody; amp^r, ampicillin resistant; ECL, enzyme chemiluminescence.

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SUMMARY

Recombinant *E. coli* 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (E 3.2.2.9) was used to study the potential for this enzyme to serve as a target f chemotherapeutic intervention. An examination of the parameters required for enzyma activity indicate that the nucleosidase functions over a broad range of pH and temperatu with acidic conditions and temperatures of 37-45°C being optimal. Analogs of 5 methylthioadenosine and adenosine were assessed as potential enzyme inhibitors and provide details regarding substrate specificity and reaction mechanism. The 5'-arylth analog, 5'-(*p*-nitrophenyl)thioadenosine, was the most potent enzyme inhibitor studie with a K_i of 20nM. A mutant of the nucleosidase lacking the first 8 amino acids w engineered to determine the contribution of these conserved residues toward enzyr specificity. The truncated enzyme exhibited a $K_{m[MTA]}$ of 1.43μM, approximately 3 fc higher than the K_m reported for the full-length nucleosidase.

INTRODUCTION

The worldwide emergence of drug resistant microbial pathogens necessitates the contin development of novel chemotherapeutic agents (1,2). The identification of metabo differences existing between the pathogen and the host cell would allow for t development of drugs which specifically inhibit microbial growth while leaving the hum host unaffected. One such metabolic difference occurs in the mechanism by which ce catabolize methylthioadenosine (MTA) and S-adenosylhomocysteine (SAH), byproduc of S-adenosylmethionine (SAM) dependent polyamine biosynthesis (3) and methylati reactions (4). MTA and SAH both inhibit critical cellular processes including protein a DNA methylation (5), polyamine biosynthesis (6,7), cAMP metabolism (8) and cytoki secretion (9). Targeted inhibition of the breakdown of these regulatory nucleosides microbial pathogens would therefore exert selective deleterious effects.

In mammalian cells, MTA is catabolized in a reversible reaction to methylthioribos 1-phosphate (MTR-1-P) and adenine by a specific MTA phosphorylase (MTAPase) (1(Adenine enters the purine salvage pathway (11,12), while MTR-1-P is recycled in a seri of steps to methionine and SAM (13,14). SAH is not a substrate for MTAPase, but degraded to adenosine and homocysteine by SAH hydrolase (15).

In contrast, many microbial pathogens utilize a single nucleosidase to hydroly both MTA and SAH to adenine and the corresponding thio-sugar, methylthioribo (MTR) and ribosylhomocysteine, respectively (16,17). The nucleosidase has be purified from several bacterial and plant species (18-22). Unlike MTAPase, t nucleosidase reaction is essentially irreversible, with the *E. coli* enzyme displayi Michaelis constants for MTA (K_m=0.43μM) and SAH (K_m=4.3) that are an order magnitude lower than the kinetic values for the corresponding mammalian enzym

(23,24). Such differences in substrate affinity and specificity suggest that t nucleosidase may be amenable to chemotherapeutic intervention.

The gene encoding the *E. coli* enzyme has recently been cloned and expressed our laboratory (25). In the present study, the potential of purified recombinant MTA/SA nucleosidase (rMTAN) to serve as a target for drug development is explored. A numb of physical parameters (pH, buffer, reaction temperature) were studied to determine ide reaction conditions. In addition, a series of MTA and adenosine analogs were assessed inhibitors of the enzymatic reaction. The results provided clues to the enzyme reacti mechanism and requirements for substrate/inhibitor recognition. Lastly, based on ami terminal sequence similarities between the bacterial nucleosidase and human MT phosphorylase, a mutant of the *E. coli* nucleosidase gene was constructed which lack the codons for the first 8 amino acids of the enzyme. The truncated nucleosida (rMTAN-8) was expressed, and the contribution of these residues toward substra affinity examined.

MATERIALS AND METHODS

Radiochemicals

S-Adenosyl-L-[methyl-^{3}H] methionine (^{3}H-SAM, 0.5 mCi/ml, 56.1 Ci/mmol) w purchased from American Radiolabeled Chemicals (St. Louis, MO). 5'[methyl-^{3}H Methylthioadenosine (^{3}H-MTA) was synthesized from ^{3}H-SAM as described by Schle (26).

Chemical and molecular biology reagents

Alkyl-substituted analogs of MTA, 5-trifluoromethylthioribose, and 5-(*p*-iodopheny thioribose were synthesized as previously described (5,36,39,41). Aryl-substitut analogs of MTA, 5'-methylthiotubercidin, 5'-methylthioinosine, 5'-(purine)thioadenosine, and 5'-methylselenoadenosine from Dr. R. Winter, Interlab In (Lake Oswego, OR). Carbocyclic MTA was obtained from the laboratory of Dr. J Borchardt (Dept. Pharmaceutical Chemistry, University of Kansas). All oth compounds tested were purchased from Sigma (St. Louis, MO). Restricti endonucleases and DNA modifying enzymes were obtained from Boehringer Mannhei Biochemicals (Indianopolis, IN). AmpliTaq DNA polymerase and other PCR reager were purchased from Perkin-Elmer Corp. (Norwalk, CT). Oligonucleotide primers we synthesized by the Portland VAMLC Molecular Biology Core Facility.

Bacterial strains

Escherichia coli strain XL1-Blue was obtained from Stratagene (La Jolla, CA) a maintained on Luria-Bertani (LB) agar supplemented with tetracycline (50µg/ml Competent cells of *E. coli* strains Top10F' and GI728 were purchased from InVitrog Corp. (La Jolla, CA) and stored at -80°C until needed.

Enzyme reaction conditions

Over-expressed recombinant wild type MTA/SAH nucleosidase (rMTAN) was purified previously described (25). The standard nucleosidase assay which follows conversion ^{3}H-MTA to ^{3}H-MTR contained 50mM potassium phosphate (pH 7), 1μM ^{3}H-MT (500μCi/μmol), and 0.5% bovine serum albumin in a total reaction volume of 180μ Reactions were initiated by the addition of the 20μl rMTAN (10 picograms) and incubat at 37°C for 15 min. Reactions were terminated by the addition of 20μL 3M TCA a precipitated protein removed by centrifugation (10000xg/ 10min). A 200μL sample supernatant was applied to a 2mL AG50-X8 cation exchange column (BioRad, 100-2 mesh, hydrogen form) and ^{3}H-MTR eluted with 3mL dH_2O directly into a 20mL vial EcoLume scintillation cocktail. To measure the effect of pH on enzyme activity, reacti mixtures contained 50mM potassium phosphate buffer adjusted to a pH value rangi from 4.5 to 9.5. Reactions were otherwise run as described above. The effect different buffers (at 50mM, pH7.0) was measured in similar fashion. The effect temperature on enzyme activity was assayed in reaction mixtures containing 50m potassium phosphate (pH 7.0) equilibrated to the desired temperature prior to addition enzyme.

Inhibitor analysis

The effect of nucleoside analogs on enzyme activity was measured using the standa nucleosidase assay ([MTA] = 1μM) supplemented with various concentrations inhibitor. IC_{50} values were determined from nonlinear regression analysis of inhibit concentration versus % inhibition of enzyme activity. Inhibition constants (K_i's) we derived form measurements of the effect of two fixed amounts of inhibitor across a ran of ^{3}H-MTA concentrations (0.4μM-10μM). Reaction time was adjusted to limit substra conversion to less than 10%. Michaelis constants ($K_{m\ app}$) were calculated from doub

reciprocal plots (27) using the Leonora enzyme kinetics program (28). K_i values we obtained from Dixon plots ([inhibitor] vs. v^{-1}).

Development of mutant MTA/SAH nucleosidase (rMTAN-8)

The truncated nucleosidase gene (lacking codons for the first 8 amino acids) w constructed by PCR using oligonucleotide primers with engineered NdeI and Bam restriction endonuclease sites: 5'-TCT CAT ATG GAA GAA GAA GTT ACG (forward) and 5'-TCT GGA TCC TTA GCC ATG TGC AAG TTT CTG-3' (reverse). 675bp PCR product was obtained from *E. coli* XL1-Blue chromosomal DNA using t protocol developed for amplification of the full-length gene (25). The PCR product w ligated into pCR™II (InVitrogen) and transformed into competent *E. coli* TOP10F' ce according to the manufacturer's specifications. Small scale plasmid minipreps we prepared from positive-appearing colonies (white, amp^r) by the alkaline lysis meth (29), double digested with NdeI/BamHI (10units ea./37°C/12hrs), and subjected agarose gel electrophoresis. A gel isolated NdeI/BamHI fragment was ligated into t tryptophan inducible expression vector pAL781 (InVitrogen), and transformed into *E. c* strain GI728 according to the manufacturer's protocol. Tryptophan induction a purification of rMTAN-8 was accomplished as previously described (25).

Analysis of rMTAN-8

The protein concentration of purified enzyme was determined using the Coomassie Plu assay (Pierce, Rockford, IL). Enzyme preparations were electrophoretically separated denaturing 12% polyacrylamide gels (30) and either stained with Coomassie blue stain, western blotted to nitrocellulose (31) to assess yield and purity. Nitrocellulc immobilized proteins were detected by incubation for 2hrs with diluted hybridoma c culture supernatants (1:5 in PBS) containing the anti-nucleosidase antibody ML R8B2.4.1 (32) followed by extensive washing in PBS. The blot was then exposed diluted goat anti-mouse Ig-HRP conjugate (1:3000 in PBS) for 1hr, washed extensive

and bound antibody detected using the Amersham ECL kit according to t manufacturer's specification. The kinetics of MTA cleavage by rMTAN-8 were assess as described above.

RESULTS AND DISCUSSION

Analysis of reaction conditions

As shown in Figure 4.1A, recombinant full-length MTA/SAH nucleosidase exhibit activity across a broad pH range. Enzyme activity was moderately improved under aci conditions, which may simply reflect a higher degree of protonation at N-7 of the puri ring of MTA, such that it resembles the proposed transition state (21). Alternative increased protonation of residues within the enzyme may enhance substrate recognitic A pH of 7 was selected for all further studies since it approximates the average activ seen across the range of conditions tested and allows comparison to prior work in the fi (18,21).

The recombinant nucleosidase functioned equally well in potassium phospha HEPES, or MES buffers (Figure 4.1B), confirming earlier reports that the activity of t enzyme is hydrolytic rather than phosphorylytic (16). Activity was moderat suppressed in imidazole buffer, and severely restricted (~80%) in Tris buffer. Inhibiti by Tris has been previously noted for the native enzyme (18).

The effects of temperature on reaction rate are presented in Figure 4.1C. T recombinant enzyme functions optimally in the range of 37-45°C, with signific substrate conversion seen even at 4°C. Activity is also seen at higher temperatur however the enzyme is rapidly inactivated (~90%) after incubation at 55°C for 10 n (data not shown).

Inhibitor analysis

The results of inhibitor testing of a variety of nucleoside analogs toward MTA hydroly by the recombinant full-length enzyme are found in Table 4.1. SAH was only moderat inhibitory (IC_{50} = 2.4μM) consistent with previous reports of reduced enzyme reactiv

for this alternative substrate (35-42% of maximal cleavage) (21,23). Similarly, analo with simple 5'-alkylthio modifications (2-4 carbons) proved to be good nucleosida inhibitors (IC_{50}'s=0.58-1.1μM). Several of these compounds (ETA, PTA, BTA, IBT are substrates for the native enzyme, with decreasing substrate activity correlated increasing alkyl chain length (21,23). The notable exception is the reduced inhibitc activity of dimethylthioadenosine (DMTA, IC_{50}=6.8μM), presumably due to the presen of the positively charged sulfonium ion center.

Aryl-substituted analogs of MTA were found to be the best nucleosidase inhibitc tested. 5'-Phenylthioadenosine, 5'-(*p*-fluorophenyl)thioadenosine, and 5'-(*p*-brom phenyl)thioadenosine were moderately good inhibitors, with IC_{50}'s in the same range simple alkyl-substituted analogs of MTA. *Para* substitutions of the phenyl ring w chloro- (PClPhTA), iodo- (PIPhTA), or amino- (PAPhTA) groups substantially improv inhibitory activity with IC_{50} values of ~0.2-0.37μM.

The most potent inhibitor studied was (*p*-nitrophenyl)thioadenosine (PNO_2PhT IC_{50} = 0.13μM). Interestingly, the nitro ($-NO_2$) group functions as an isoster of carbox groups in biological systems (33). Thus, it is possible that the nitro moiety PNO_2PhTA, resembles the carboxyl group of SAH, and is involved in hydrogen bondi in the substrate binding site of the enzyme. The Dixon plot for PNO_2PhTA (Figure 4. demonstrates that it acts as a competitive inhibitor with a K_i of 20nM. This is similar the inhibition exhibited by formycinyl analogs: 5'-chloroformycin (K_i = 32nM), methylthioformycin (K_i = 28nM) and S-formycinylhomocysteine (K_i = 9.7nM) report previously (21,23). These compounds represent the most potent analogs reported to d for the nucleosidase and highlight leads for future inhibitor design.

The study of other MTA analogs provided additional insight into the relati contribution of various portions of the substrate toward enzyme recognition. Our resu showed a much lower IC_{50} value for methylthioinosine (MTI) with the recombin

enzyme (IC_{50} = 30μM) than had been reported for the native enzyme (IC_{50} > 500μ
(21). Regardless of this discrepancy, both results indicate the importance of the
position amino group in substrate recognition. Modifications to the ribose portion of
molecule also reduced inhibitory activity, as is evident with carbocyclic MTA (IC_{5C}
27μM). Similar findings reported for acyclic analogs of SAH suggest that an intact rib
ring is necessary for enzyme recognition (21).

Other substitutions in the MTA molecule were better tolerated. Moderate enzy
inhibitors were obtained when the sulfur atom was replaced with selenium to yield
methylselenoadenosine (MSeA, IC_{50} = 4.5μM); and when purine (linked via C-6) w
substituted for the methyl group to form 5'-(6-purinothio)adenosine (PurTA, IC_{50}
4.7μM). The effect of alterations to the purine base was explored with the 7-dea
analog, methylthiotubercidin (MTT). MTT exhibited an IC_{50} value of 3.3μM and a K_i
0.75μM against the recombinant enzyme. The nucleosidase reportedly does not cle
MTT, indicating a stronger involvement for N-7 in catalysis than substrate recogniti
(21). This result is consistent with the view that protonation of N-7 is required
attaining the transition state in hydrolysis of the glycosidic bond of purine nucleosi
(34,35).

Substrate specificity was further investigated by examining the inhibitory activity
adenosine and related analogs. Adenosine displayed no discernible activity even at hi
concentrations (IC_{50} >500μM). Formycin A (8-aza-9-deaza adenosine) was a mu
better inhibitor (IC_{50} = 57μM), probably due to the protonation of N-7 at neutral
which allows it to resemble the transition state. However, the presence of a carb
carbon bond between C-1 of the sugar and C-9 in the pyrazole ring prevents formycir
from being cleaved (21,23). Halogenation of the 5' carbon also dramatically impro
the inhibitory activity of adenosine toward the nucleosidase. 5'-Chloroadenosine (IC_{5}

9.8μM), an inhibitor of human MTAPase (36), exhibited a ~50 fold increase in inhibitc activity relative to adenosine.

Adenine and methylthioribose (MTR), products of the enzymatic cleavage of MT were both poor inhibitors of the enzyme (IC_{50}'s = 305μM and >1000μM, respectivel indicating they do not contribute significantly to regulation of nucleosidase activity. T analogs of MTR, 5-trifluoromethylthioribose and 5-(*p*-iodophenyl)thioribose, were al inactive toward the nucleosidase, supporting the previous assertion that these are speci inhibitors of MTR kinase (37-41).

Mutational Analysis

A high degree of homology exists between the first eight amino acids of *E. c* MTA/SAH nucleosidase (MKIGIIGA) (25) and a similar region in human M phosphorylase (VKIGIIGG) (42,43). With very little other sequence homology existi between these two enzymes, we postulated that this region may be involved in M binding, possibly conferring some specificity for the sulfur group at the 5' position of t nucleoside. To test this, a truncated nucleosidase (designated rMTAN-8) was engineer which lacked the MKIGIIGA sequence. Highly purified rMTAN-8 was obtain following immunoaffinity chromatography using an anti-nucleosidase antibody affin resin (Figure 4.3).

Kinetic analysis of rMTAN-8 (Figure 4.4) showed a reduced affinity for M ($K_{m\ [MTA]}$ = 1.5μM) compared to the full-length recombinant ($K_{m\ [MTA]}$ = 0.5μM) (2 and native *E. coli* enzyme ($K_{m\ [MTA]}$ = 0.43μM) (21). The truncated enzyme was al more sensitive to inhibition by adenosine ($IC_{50} \approx$ 65μM) compared to rMTAN (IC_{50} 500μM), suggesting a loss of substrate specificity. Further elucidation of the molecu interactions involved in substrate binding, particularly those imparting specificity for t sulfur atom of MTA and SAH, await the results of x-ray crystallographic studies curren being pursued in this laboratory.

Conclusion

Purified recombinant *E. coli* MTA/SAH nucleosidase was examined by a number different approaches to further characterize its ability to serve as a chemotherapeutic targ The nucleosidase appears to function well across a broad range of pH, temperature, a buffer conditions, with the notable exception of Tris which effects a clear inhibition enzyme activity. An analysis of over 20 nucleoside analogs provided insight into t structural characteristics important for substrate/inhibitor recognition and catalysis; a suggests future modifications that may yield even more potent enzyme inhibitors. particular, a 6-position amino group, intact ribose, and an uncharged sulfur atom attach to the 5' position of the sugar appeared to be important for good substrate/inhibi activity. The PNO_2PhTA analog acted as an extremely potent competitive inhibitor (K_i 20nM), comparable to the best inhibitors (formycinyl and tubercidinyl derivatives) of t enzyme on record. Nucleoside analogs incorporating both a (ρ-nitrophenyl)thi substitution at the 5' position of the sugar and formycinyl- or tubercidinyl- replacemei for the base would likely yield non-hydrolyzeable analogs with even greater potency f the enzyme. A mutant nucleosidase was developed to explore the contribution of the fi 8 amino acids of the enzyme to substrate binding. Kinetic studies of the truncated enzyı showed a reduced affinity for MTA. Concomitantly, the increased susceptibility rMTAN-8 to adenosine inhibition provides a preliminary indication of the involvement the deleted amino terminal residues in substrate specificity.

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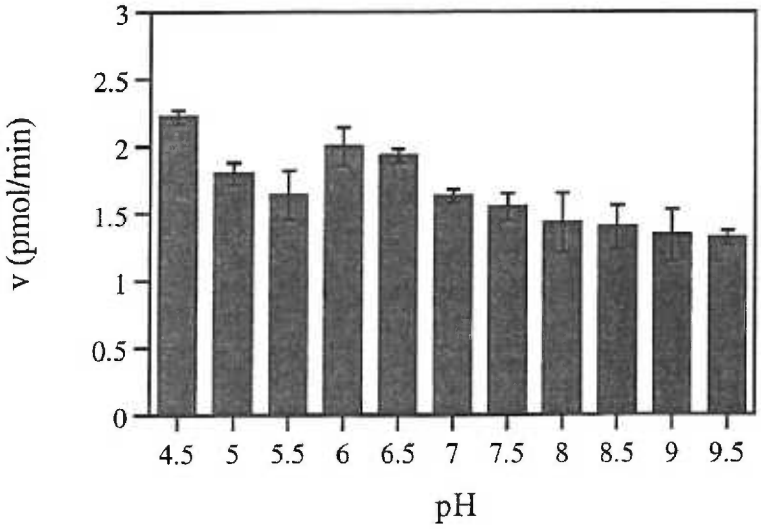
Table 4.1. Inhibitor analysis of MTA/SAH nucleosidase. The IC_{50} valu (concentration yielding 50% inhibition) were calculated at an MTA concentration 1.0μM. Values reported represent the average of 2-3 experiments, with individual poir determined in triplicate.

Compound	Abbreviation	IC_{50} (μM)	K_i (μM)
Substrates			
5'-Methylthioadenosine	MTA	1	-
S-Adenosylhomocysteine	SAH	2.4	-
Alkyl-substituted MTA analogs			
5'-Ethylthioadenosine	ETA	0.9	-
5'-Dimethyl(+)thioadenosine	DMTA	6.8	-
5'-Propylthioadenosine	PTA	0.58	-
5'-Isopropylthioadenosine	IPTA	1.1	-
5'-Butylthioadenosine	BTA	0.68	-
5'-Isobutylthioadenosine	IBTA (SIBA)	0.74	-
Aryl-substituted MTA analogs			
5'-Phenylthioadenosine	PhTA	0.88	-
5'-(*p*-Fluorophenyl)thioadenosine	PFPhTA	0.9	-
5'-(*p*-Chlorophenyl)thioadenosine	PClPhTA	0.2	-
5'-(*p*-Bromophenyl)thioadenosine	PBrPhTA	0.85	-
5'-(*p*-Iodophenyl)thioadenosine	PIPhTA	0.22	0.17
5'-(*p*-Aminophenyl)thioadenosine	PAPhTA	0.37	-
5'-(*p*-Nitrophenyl)thioadenosine	PNO2PhTA	0.13	0.02
Other MTA analogs			
5'-Methylthiotubercidin	MTT	3.3	0.75
5'-Methylthioinosine	MTI	30	-
5'-Purinothioadenosine	PurTA	4.7	-
carbocyclic MTA	cMTA	27	-
5'-Methylselenoadenosine	MSeA	4.5	-
Other nucleosides / bases			
Adenosine	Ado	>500	-
Erythro-9-(2-hydroxy-3-nonyl)adenine	EHNA	>100	-
Formycin A	FormA	57	10
5'-Chloroadenosine	5ClAdo	9.8	-
Inosine	Ino	>100	-
Adenine	Ade	305	300
Sugars			
5-Methylthioribose	MTR	>1000	-
5-Trifluoromethylthioribose	TFMTR	>1000	-
5-(*p*-Iodophenyl)thioribose	PIPTR	>1000	-

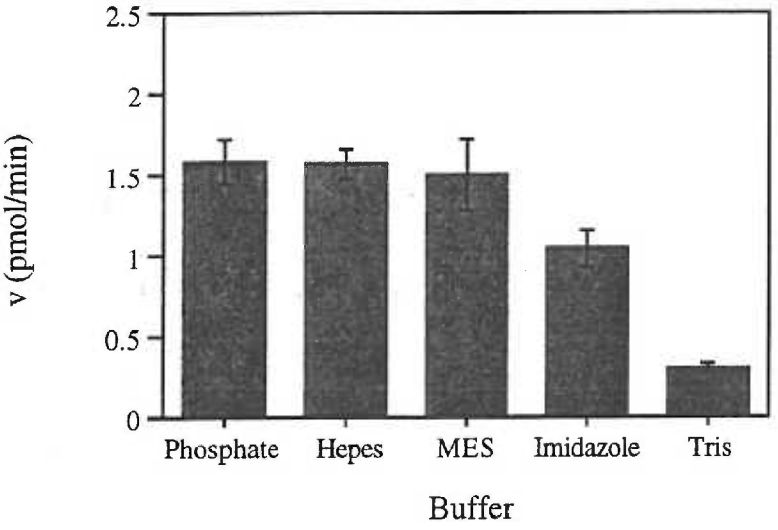
FIGURE LEGENDS

Figure 4.1. Effect of (A) pH; (B) buffer; and (C) temperature on MTA cleaving activi of purified recombinant full-length nucleosidase (rMTAN). Experiments were perform as described in Materials and Methods. Each bar represents the average of thr experimental determinations ± standard error.

A.


v (pmol/min)
3
2.5
2
1.5
1
0.5
0
4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5
pH

B.


v (pmol/min)
2.5
2
1.5
1
0.5
0
Phosphate Hepes MES Imidazole Tris
Buffer

C.

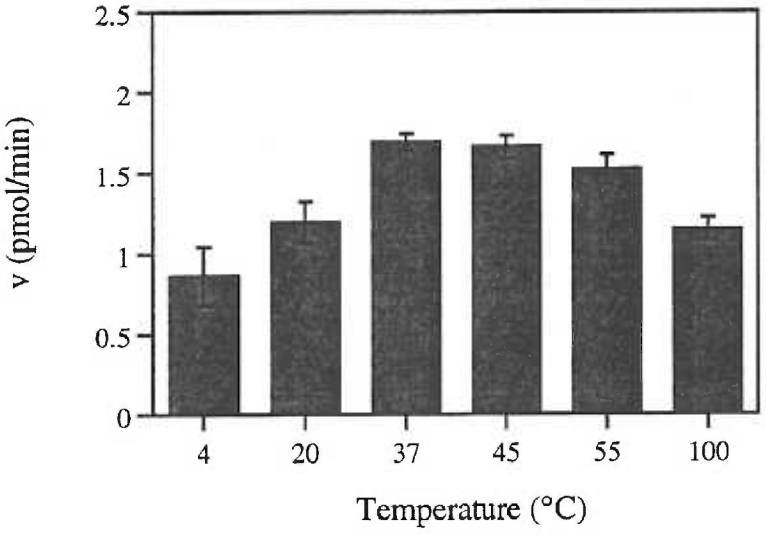

v (pmol/min)
2.5
2
1.5
1
0.5
0
4 20 37 45 55 100
Temperature (°C)

Figure 4.2. Dixon plot of the inhibitory activity of 5'-(p-nitrophenyl)thioadenosi (PNO2PhTA) on MTA cleavage. Inset: PNO2PhTA (nM) vs. apparent K_m. Poin represent the average of 3 experimental determinations.

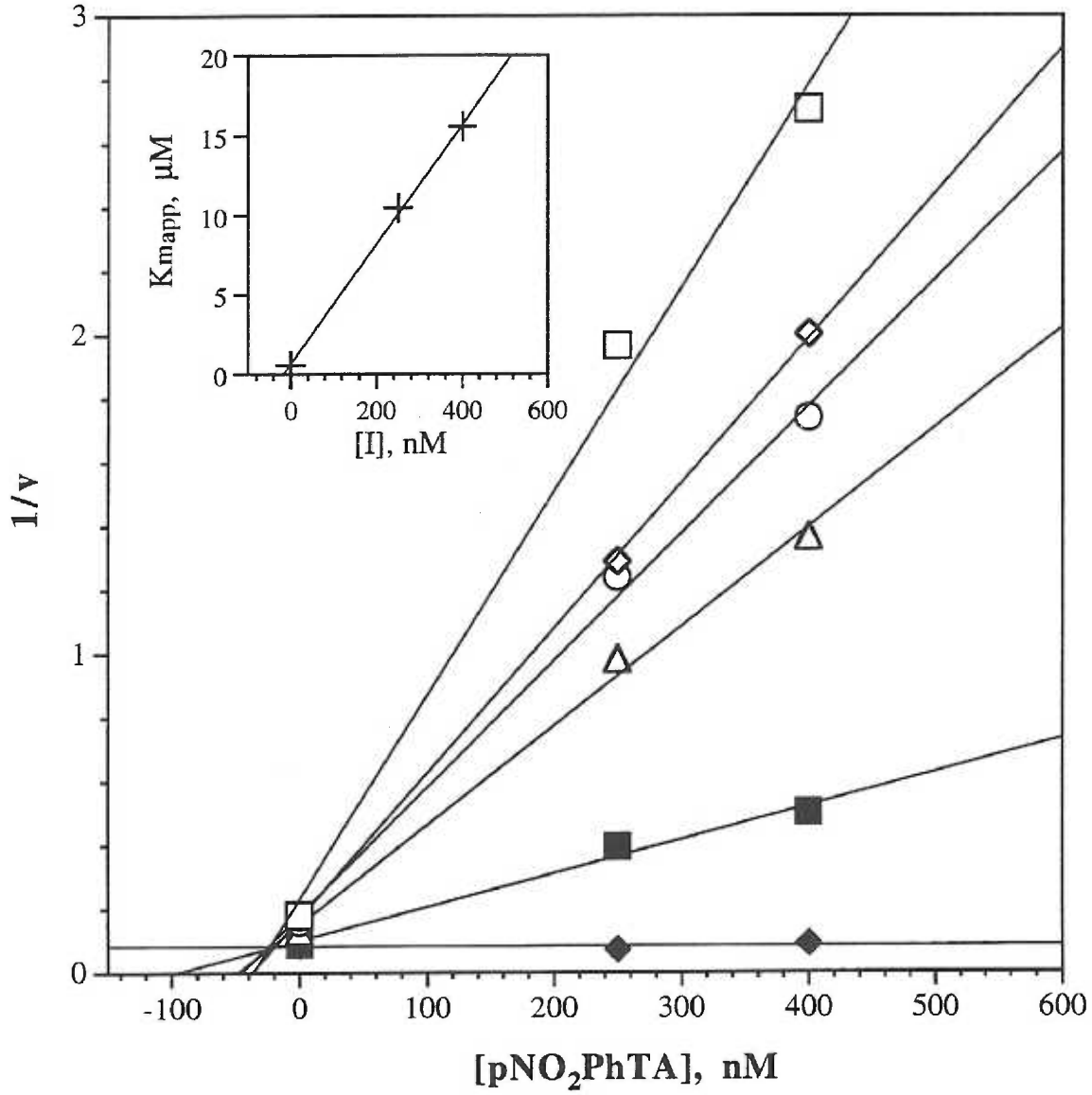

3
2
1
0
1/v
-100
0
100
200
300
400
500
600
[pNO2PhTA], nM
20
15
10
5
0
Kmapp, µM
0
200
400
600
[I], nM

Figure 4.3. Panel A: Coomassie Blue stained SDS-PAGE gel of purified full-leng rMTAN (lane 1) and truncated rMTAN-8 (lane 2). In each case the lane contained 5μ protein. Molecular masses of protein standards are reported on the left side of the pan (df = dye front). **Panel B**. Immunoblot analysis of rMTAN (1μg, lane 1) and rMTAN (1μg, lane 2) using Mab R8B2.4.1.

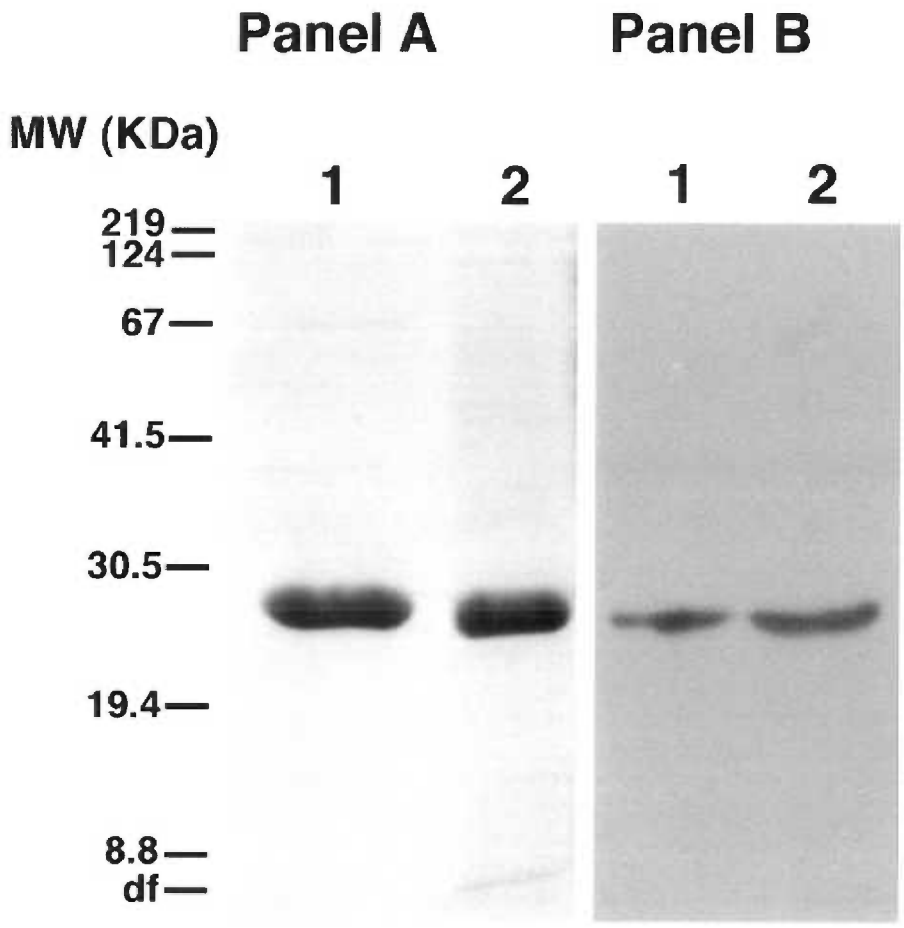
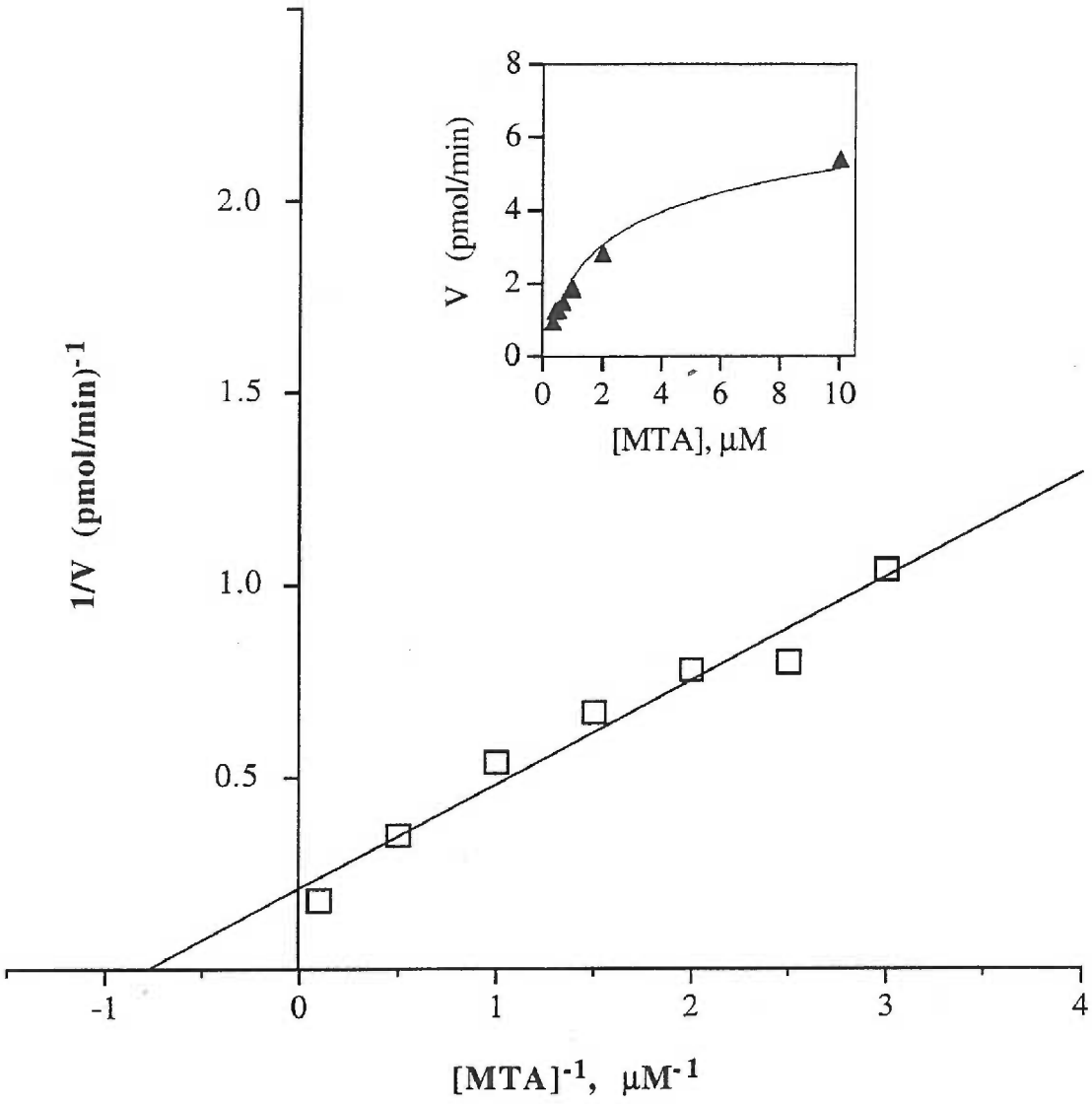

Panel A
Panel B
MW (KDa)
1
2
1
2
219
124
67
41.5
30.5
19.4
8.8
df

Figure 4.4. MTA kinetics for truncated rMTAN-8. Double reciprocal plot of the initi velocity [v (picomoles/min)] versus MTA concentration (μM). Inset: Hill plot of initi velocity versus MTA concentration.


1/V (pmol/min)$^{-1}$
2.0
1.5
1.0
0.5
-1
0
1
2
3
4
[MTA]$^{-1}$, μM^{-1}
V (pmol/min)
8
6
4
2
0
0
2
4
6
8
10
[MTA], μM

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The enzymes involved in methionine recycling differ significantly between huma and a number of microbial pathogens in terms of their mechanism of action and substra specificity. The main objective of this thesis was to explore the potential of the methioni salvage pathway as a site for chemotherapeutic intervention. To accomplish this I purifi and characterized two key microbial enzymes in the pathway, MTA/SAH nucleosidase a MTR kinase, and attempted to clone their respective genes. For the purposes of o study, the enteric bacteria *Klebsiella pneumoniae* was chosen as an initial source of the enzymes due to its significance as a nosocomial pathogen [1], and because of its historic use as a model system for studying methionine salvage from MTA [2-6].

The purification to homogeneity of MTA/SAH nucleosidase and MTR kinase fro *Klebsiella* allowed us to perform an initial characterization of these enzymes in terms their substrate kinetics and molecular weight (Chapter 2). Of particular importance, t amino terminal amino acid sequence was determined for both the nucleosidase (residues) and the kinase (19 residues). The amino acid sequence for the *Klebsie* nucleosidase showed a high degree of homology to the putative translation product of open reading frame (*pfs*) in *E. coli* which we predicted to encode this enzyme [6].

A second objective of this thesis was to clone and express the genes for MTA/SA nucleosidase and MTR kinase. MTR kinase presents an ideal target in many bacteri protozoal and plant systems, since an equivalent enzymatic activity is not present mammalian cells. In addition, a number of MTR analogs have been synthesized a tested which display potent antimicrobial properties, but do not exhibit cytotoxic antiproliferative activities for human cells [7-10]. Unlike the nucleosidase, the init

amino acid sequence for the *Klebsiella* MTR kinase did not appear to be homologous any known peptides or translation products in the combined protein databases. Therefor the strategy for cloning the kinase gene was to screen a *Klebsiella* genomic λgt1 expression library using a degenerate oligonucleotide probe based on the known peptic sequence and codon bias for this organism (Appendix A). Initial attempts at cloning tl MTR kinase gene have not been encouraging, although work on one of the identifi clones (MTRK#5) still appears hopeful. Continued analysis of this clone remains priority for the near future. Other possible avenues for the successful cloning of this ge are presented and discussed in Appendix A.

Subsequent work focused on cloning and expression of the *E. coli pfs* gene, whi allowed us to prove the hypothesis that it encoded the MTA/SAH nucleosidase (Chapter [11]. The *pfs* gene was amplified via PCR using primers based on the open readi frame reported in GenBank [12]. Sequencing studies of the cloned PCR product reveal a number of discrepancies from the reported *pfs* sequence, the most important of whi was the presence of two additional guanosine residues near the 3' end of the gene whi effectively extended the open reading frame by 13 codons. Analysis of 6 addition independent PCR clones confirmed our results, as did recent information deposited in GenBank for the DNA sequence located at the 2.8-4.1 minute region on the *E. c* chromosome [13]. Functional identification of the *pfs* gene as encoding MTA/SA nucleosidase was demonstrated by expression as both a glutathione-S-transferase fusi protein and as a tryptophan inducible full length recombinant. Kinetic studies conduct on affinity purified recombinant nucleosidases provided Michaelis constants for MTA th were in close agreement with the ~0.5μM values reported in the literature for partial purified preparations of the native *E. coli* enzyme [11, 14-16].

MTA analogs and other compounds were tested as potential inhibitors of t recombinant nucleosidase (rMTAN) in an effort to further define the molecul

characteristics responsible for substrate recognition and enzyme activity (Chapter 4). It noteworthy that the nucleosidase reaction is essentially irreversible, and the product adenine and methylthioribose, do not appear to exert a significant allosteric or competiti inhibition on enzyme activity [15, 17]. Adenine and methylthioribose display IC_{50}'s f enzyme activity (at 1μM MTA) of 300μM and >1000μM, respectively. In contrast, t mammalian MTA phosphorylase catalyzes a readily reversible reaction, which is inhibit by low micromolar concentrations of the products, adenine and methylthioribose-phosphate [16, 18]. From the results of inhibitor analysis (Table 4.1), a number generalities regarding substrate recognition can be gleaned that may be useful in the desi of additional compounds:

1. A wide variety of 5'-alkylthio- side chains (1-4 carbons) can be recognized by the enzyme. This lack of specificity is not surprising since the nucleosidase functions to cleave the glycosidic bond of both MTA (5'-methylthio- side chain) and SAH (5'-[3-amino-3-carboxy]propylthio- side chain).

2. As is evident by the decreased IC_{50} of 5'-dimethylthioadenosine (IC_{50} = 6.8μM) relative to 5'-ethylthioadenosine (IC_{50} = 0.9μM), a positively charged sulfonium ion decreases the recognition of the substrate molecule.

3. Similarly, substitutions of the 5'-methylthio- group with a 5'-methyl-seleno- (IC_{50} = 4.5μM), 5'-purinothio- (IC_{50} = 4.7μM), 5'-chloro- (IC_{50} = 9.8μM), and 5'-hydroxy- (IC_{50} = >500μM) side chains decreased the substrate recognition. This indicates that a) the sulfur atom is the best 5' substituent, b) there may be limits to the type of bulky side chain that can be attached to the sulfur, and c) substitutions on the 5' position with strongly electron withdrawing groups (i.e. 5'-chloro-) increase recognition of the substrate relative to weaker groups (i.e. 5'-hydroxy-).

4. Several of the nitrogen atoms in the nucleoside base are important for recognition and catalysis. Deamination of position 6 of the purine ring to yield methylthioinosine, dramatically reduces substrate binding (MTI, IC_{50} = 30μM). The 7-deaza derivative, 5'-methylthiotubercidin, is a moderate inhibitor (IC_{50} = 3.3μM, K_i = 0.75μM) and is non-hydrolyzeable [15], indicating N-7 is probably more important to catalysis than substrate binding. Similarly, the non-hydrolyzeable 8-aza-9-deaza adenosine analog, formycin A (IC_{50} = 57μM), was a much better inhibitor than adenosine (IC_{50}>500μM), possibly due to protonation of N-7 of the formycinyl base at physiological pH. The results for MTT and formycin A support earlier assertions that protonation of N-7 is important for reaching the transition state leading to hydrolysis of the glycosidic bond [19]. 3-deaza analogs of MTA were unavailable for testing, but this position of the molecule has also been implicated in substrate recognition [15, 18].

5. The first evidence of the inhibitory activity of MTA analogs containing aryl and halogenated aryl side chains is presented in Chapter 4. Of these compounds, 5'-phenylthioadenosine, 5'-(*p*-fluorophenyl)thioadenosine, and 5'-(*p*-bromophenyl)thioadenosine behaved in a manner similar to simple alkyl substuted MTA analogs. 5'-(*p*-Chlorophenyl)thioadenosine (IC_{50} = 0.2μM), 5'-(*p*-iodophenyl)thioadenosine (IC_{50} = 0.22μM, K_i = 0.17μM), and 5'-(*p*-aminophenyl)thioadenosine (IC_{50} = 0.2μM), all display inhibitory activity on par with recent reports for 5'-monofluoromethylthioadenosine (MFMTA) and 5'-trifluoromethyl-thioadenosine (TFMTA) [20]. A final compound, 5'-(*p*-nitrophenyl)thioadenosine (IC_{50} = 0.13μM, Ki = 0.02μM) is one the best inhibitors ever tested for the enzyme. Whether these arylthio analogs act as better inhibitors due to increased interactions between the halogenated aryl

side chain and amino acid residues in the substrate binding site, or because the 5' substitutions act as strong electron withdrawing agents to destabilize the carboxonium-like transition state predicted to be involved in the depurination of MTA [19, 20] is unknown, but will be addressed in the future.

MTA/SAH nucleosidase appears to be an attractive target owing to its presence a junction between both methionine and purine metabolic pathways, and the variety inhibitory effects that can result when MTA and SAH concentrations are elevated [21, 2 Disappointingly, the aryl-substituted MTA analogs which were potent nucleosida inhibitors *in vitro*, did not prove to exert particularly strong antibacterial activities, a several displayed significant antiproliferative effects against bone marrow cells (s Appendix B). In this regard, our results mimic those noted for xylos methylthioadenosine, the only report that has surfaced so far on the antibacterial effect an MTA analog [23]. The reasons for the poor antibacterial properties of the compounds are unknown, but may relate to inefficient transport of the analogs or ra intracellular catabolism. In some of the earliest work in the MTA field, Stanley Shap demonstrated in 1953 that methionine auxotrophs of *Aerobacter (Enterobacter) aeroge* could grow when cultures were supplemented with MTA, indicating that uptake of t thioether occurs [24]. Radiotracer studies have shown that MTA is able to gain entry i a variety of lower eukaryotes, including *Ochromonas* [25, 26], and *Candida* [27-29]. sytematic study on the uptake of radiolabelled MTA in bacteria has not been perform and remains an obvious area for further study. In contrast, there is significant evide for the rapid transport of MTA and its analogs into mammalian cells, although mechanism of entry is in dispute [30-34].

In the future, a number of approaches may be pursued in the development a testing of MTA analogs. Initially, it may be more fruitful to simply test the *p*

substituted arylthioadenosine compounds as antiprotozoal and antineoplastic agents, arena in which the antiproliferative effects of MTA and SAH analogs have be extensively studied [35-41] Indeed, MTA catabolizing enzymes (MTA/SA nucleosidase, MTA nucleosidase, or MTA phosphorylase) appear to be particularly go sites for drug intervention in the parasitic protozoa, since they lack *de novo* puri biosynthesis, and are thus reliant on exogenous salvage and efficient recycling of the compounds [8, 41-46]. Some of the most promising uses of MTA and its analogs th far have been in the treatment of experimental *Trypanosoma brucei* infections of mi [40], and as *in vitro* potentiating agents for drugs which interfere with polyami biosynthesis (e.g. difluoromethylornithine, DFMO)[47]. Other *in vitro* studies ha demonstrated that MTA analogs can act as antimalarial [39, 41, 48-51] and antileishman agents [52], albeit at concentrations that are relatively high (20-300μM). We propose test our substituted arylthioadenosine analogs in the future on cultures of *P. falciparum*, that their antimalarial effects relative to these previous reports can be determined.

Our *in vitro* study of nucleosidase inhibitors, and work by other investigato suggests a series of additional compounds which should be synthesized and their me tested as nucleosidase inhibitors. Since replacement of the methylthio group of MTA wi a *para* (nitro, chloro or iodo)phenylthio moiety yields a better inhibitor, and substitution the adenine with a tubercidinyl or formycinyl derivative produces a nonhydolyzeat substrate, compounds which combine these two characteristics are worthy of stud Other compounds which mimic nucleoside transition states predicted for the hydrolysis the glycosidic bond (e.g. amidrazones and dihydroxylated pyrrolidines), may be valuat nucleosidase inhibitors [53-5], particularly if they are derivatized to contain a 5'-alkylth group to improve specificity for the enzyme.

In addition, much of the work presented in this thesis has greatly enhanced c ability to undertake a more "rational" approach to drug design. The cloning of the fu

length recombinant *E. coli* MTA/SAH nucleosidase gene in a tryptophan inducible syste has made possible the expression of large quantities of protein (rMTAN) required f crystallography studies currently being pursued with collaborators. Aiding in this wor the development of a panel of anti-nucleosidase monoclonal antibodies (Appendix C) h led to the construction of an affinity column for use in the preparations of highly pu enzyme needed for the aforementioned studies. The long term goal of this investigation the development of a three dimensional structure for the nucleosidase which can serve as template for screening chemical databases in search of non-nucleoside inhibitors. A compounds that are identified by this approach will be analyzed to determine if th exhibit improved enzyme inhibitory activity and therapeutic properties (better antibacteri activity and pharmacokinetics) relative to the MTA analogs that have developed so far.

Another area of proposed study resulting from the work on MTA/SAH nucleosida and MTA analogs concerns an investigation of characteristics of MTA metabolis occuring in many neoplasms. Toohey first reported nearly two decades ago that son malignant murine cells lacked MTA phophorylase (MTAP) activity [56, 57]. MTA deficiency has since been associated with a long list of human malignancies includi acute lymphoblastic leukemia (ALL) [58-68], gliomas [67, 69, 70], non-small cell lu cancer [67, 71], pancreatic cell carcinoma [72], breast cancer [67, 73], liver cancer [6 melanoma [67], and chondrosarcoma [74, 75]. The location of the MTAP gene has be assigned to chromosome 9 [76], in a region of the short arm containing the interferon ge cluster (9p21-22) which is subject to frequent gene deletions and translocations [64, 7 Recent work has proposed that numerous human malignancies arise due to the loss of o (or more) of two tumor suppressor genes located on chromosome 9, p15 and p16, wi concomittant deletion of the adjacent MTAP gene [66, 78-80].

The consequence of the loss of MTA phosphorylase activity is the inability recycle purines and methionine from MTA [59, 81]. This metabolic deficiency may

chemotherapeutically exploitable since neoplastic cells also show a greater dependence c these nutrients for growth and proliferation [59, 81-85]. For patients identified as havir MTAP deficient tumors it may be possible to design more specific chemotherapeut regimens, since these neoplasms are more susceptible to agents which inhibit the *de no* synthesis of purines and methionine (e.g. methotrexate, azaserine, alanosine, etc. particularly when MTA is supplied as the only source of recycleable material for the compounds [58, 70, 71, 74, 75, 80, 86].

In response to MTAP deficiency, the intracellular levels of MTA rise, however, hig concentrations are prevented by secretion of the thioether into the surroundings [62, 6 87, 88]. One approach that may be valuable to investigate is the development compounds which interfere with the process of MTA secretion or export. The resultir accumulation of MTA would be expected to lead to a variety of deleterious events MTAP deficient (i.e. neoplastic) cells, including inhibition of spermine synthesis [89, 9(DNA and protein methylation [91-93], and SAH catabolism [68, 94].

A related avenue of study addresses the effects of MTA secretion on the immu system. MTA has been shown to interfere with natural killer cell activity [95], inhit lymphocyte proliferation [96, 97],and to decrease tumor necrosis factor (TNFα) secretic by mononuclear cells [98]. Thus, one could speculate that MTA secretion by MTA deficient tumor cells might aid in their ability to escape local immune surveillance. In th regard, the availability of the recombinant MTA/SAH nucleosidase may be of interest an "enzyme therapy", allowing MTA inhibition of the immune response to be overcom The use of the nucleosidase could be modelled after the use of L-asparaginase, an enzyr which is proposed to act by limiting the level of L-asparagine available for tumour c growth, and has become a therapeutic mainstay in the treatment of acute lymphoblas leukemia [99, 100].

In summary, the work presented in this thesis was conducted in order to bet understand the properties of two enzymes in the methionine salvage pathway, MTA/SA nucleosidase and MTR kinase. These enzymes are of interest due to th chemotherapeutic potential in the treatment of microbial diseases of humans and livesto My studies resulted in the first-ever homogeneous purification and amino termi sequence information obtained for these two enzymes on record. Work on the cloni and expression of the *E. coli* MTA/SAH nucleosidase has allowed the broad aims of t thesis to be realized. Investigations into the inhibitory activity of a series of MTA analo and the production of a truncated nucleosidase have provided insight into the fact involved in substrate recognition and catalysis, and highlight a number of additio compounds that are worth investigating as enzyme inhibitors in the future.

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APPENDIX A

Attempted Cloning of the *Klebsiella pneumoniae* MTR Kinase Gene.

Summary

Several attempts were made at cloning the MTR kinase gene from a *Klebsiel pneumoniae* genomic library. Two lambda clones (designated MTRK#4 and MTRK# were isolated which hybridized to a 256-fold degenerate oligonucleotide (29-mer) prol designed on the basis of the amino-terminal amino acid sequence that we reported for tl native MTR kinase (Chapter 2, Biochemical Journal paper) and the predicted codon bi for *Klebsiella* (codon bias reference). Automated nucleic acid sequence analysis of one the clones (MTRK#4) failed to show any homology to the original oligonucleotide prol used to screen the library, despite a positive hybridization on a Southern blot. BLASTN search [1] of the combined non-redundant databases (GenBank +EME +DDBJ +PDB) revealed that the 2,748 bp MTRK#4 sequence represented the *Klebsiel* equivalent of regions of the *E. coli* and *Haemophilus influezae* genome which enco rnpA (ribonuclease P), "o548" (unknown function), and thdF (thiophene & fur oxidation protein). The homology scores ranged from 64-89% over 400-1000 l stretches of DNA ($\rho \leq 2.8e^{-162}$). Additional library screening and sequence analysis the MTRK#5 clone are currently being pursued.

Introduction

The methionine recycling pathway enzyme, MTR kinase (EC 2.7.1.100), is interest because it is present in a number of medically important pathogens, but absent humans (see Tables 1.3-1.5). Thus, this enzyme serves as a possible site f chemotherapeutic intervention [2-4]. Analogs of MTR which specifically inhibit t kinase have been shown to be effective antimicrobial agents [5-7], and act synergistical with compounds (e.g. azaserine, proparylglycine, triazole) which block *de no* methionine biosynthesis [8]. In order to study the enzyme more thoroughly, we attempt to clone the corresponding gene from a *Klebsiella* genomic library. The long term gc was to overexpress the gene product for use in x-ray crystallographic studies and ratior drug design.

Experimental Procedures

Radiochemicals and Oligonucleotide Probes- [γ-^{32}P] ATP was purchased from NE DuPont (Boston, MA). The three oligonucleotides used in this study (see Table A. "TB" (256-fold degenerate, 29-mer), "ZP-1" (16-fold degenerate, 17-mer), and "ZP- (8-fold degenerate, 17-mer) were purchased from Oligo's, Etc. (Wilsonville, Ol Radiolabelled (^{32}P) oligonucleotide probes ($12x10^6$cpm/100pmol oligo) were synthesiz using T4 polynucleotide kinase (Life Technologies, Inc., Gaithersburg, MD) by stand end-labelling procedures [9], and purified by by spin-elution on G25 columns (5'-3' In Boulder, CO). X-ray film (XOMAT-AR) for autoradiographs was purchase fr Kodak/IBI (New Haven, CT).

Molecular Biology Reagents- Restriction endonucleases and DNA modifying enzyn were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and from L Technologies, Inc. (Gaithersburg, MD). The *Taq* DyeDeoxy cycle sequencing kit v obtained from Applied Biosystems, Inc. (Foster City, CA). Oligonucleotide primers

DNA sequencing were synthesized by the VAMC Molecular Biology Core Facili (Portland, OR). Ampicillin, tetracycline, IPTG, and X-gal were obtained from Sigr Chemical Corp. (St. Louis, MO).

Bacterial strains and plasmids- Competent cells of *E. coli* strains XL-1 Blue a Top10F' were purchased from InVitrogen Corp. (LaJolla, CA) or prepared as describ by Maniatis, et al. [10] and stored as frozen aliquots at -80°C until needed. The *E. c* strain Y1090r- was supplied by CloneTech (Palo Alto, CA). The plasmid vect pGEM4Z, was obtained from Pharmacia (Alameda, CA).

Library screening- A λgt11 *Klebsiella pneumoniae* genomic library containing Ecol fragments (7 kbp average insert size) was purchased from Clonetech (Palo Alto, CA With no reported MTR kinase nucleic acid sequences yet available in the gene databas the probe used to screen the library was an end-labelled 256-fold degener oligonucleotide ("TB") based on the amino-terminal amino acid sequence elucidated f the native MTR kinase [11], and the predicted codon bias for *Klebsiella* [12]. The libra was plated on *E. coli* strain Y1090r$^-$ host cells according to the manufacture specifications. Duplicate nylon filter plaque lifts were made from 5 plates (150mm dian each containing 1-5x10^4 plaques) and probed with the end-labelled oligonucleotide for hours at 42°C in hybridization buffer containing: 6x SSC, 5x Denhardts reagent, 30 formamide, 20mM sodium phosphate, 0.4% SDS, and 100μg/ml denatured shear salmon testes DNA. Following hybridization, the filters were rinsed extensively prewarmed (42°C) wash buffer (2xSSC, 0.1% SDS), and subjected to autoradiograph Five possible clones were selected based on the autoradiography results of the prima filter screens. Of these, two clones (designated MTRK#4 and MTRK#5) appear positive after secondary and tertiary filter screens, and were selected for further mappi and subcloning.

Subcloning and mapping studies- Samples of lambda MTRK#4 and MTRK#5 pha DNA were prepared from plate lysates according to standard procedures [9]. Purifi DNA was subjected to digestion with EcoRI restriction endonuclease and the inse resolved by electrophoresis (100V, 1hr) on a 0.7% agarose gel (+1μg/ml ethidiu bromide) in TAE buffer. Gels containing DNA fragments were denatured (0.5N NaO 1.5M NaCl, 1hr), neutralized (1M Tris, pH 8.0, 1.5M NaCl, 1hr), then transferred nylon membranes using 20xSSC and an Appligene vacuum blotter (Pleasanton, CA Filters were hybridized to the MTR kinase probe ("TB") using the conditions describ for the library screening. For subcloning, the DNA from clones MTRK#4 and MTRK was either digested with EcoRI or double digested with EcoRI and BamHI restricti endonucleases, and the fragments resolved by agarose gel electrophoresis. Desir fragments were recovered from the gel by spin elution through 0.45μM filtration devic (PGC Scientific, Gaithersburg, MD) and ligated into appropriately digested pGEM- using T4 ligase (Boehringer Mannheim) for 16 hours at 14°C. Ligation mixtures we transformed into competent *E. coli* strain XL-1 Blue or TOP10F' cells. Recombinar were chosen based on growth and blue/white color selection (white colonies = positi recombinants) on LB agar supplemented with ampicillin (100μg/ml) and X-gal (25μg/m Plasmids were prepared from positive-appearing colonies by alkaline lysis [9], a analyzed for the presence of the appropriate sized insert following restriction endonuclea digestion and agarose gel electrophoresis.

Initial restriction endonuclease maps were prepared by digestion of either t original λgt11 clone DNA, or various plasmid subclones with a number of otl restriction endonucleases according to the manufacturers specifications, followed agarose gel electrophoresis. To localize more precisely the region of the insert whi hybridized to the initial probe, restriction fragments of lambda clone and plasmid subclo digests were vacuum blotted to nylon membranes as previously described, and prob

with a mixture of (^{32}P) end-labelled oligonucleotides (ZP-1 & ZP-2) in hybridizati buffer (6xSSPE, 1% SDS) for 24 hours at 42°C. Blots were rinsed extensively a autoradiographed overnight at -80°C.

DNA sequence analysis- Plasmids containing inserts derived from lambda clo MTRK#4 were sequenced using a Taq DyeDeoxy™ Terminator Cycle Sequencing k Cycle sequencing reactions were analysed on an Applied Biosystems automated Mo 373A DNA Sequencer (Foster City, CA). Sequence data were analyzed using t MacVector and Assemblylign programs (Kodak/IBI).

Results and Discussion

Two lambda clones, designated MTRK#4 and MTRK#5, were isolated from screen of the *K. pneumoniae* λgt11 library using a 256-fold degenerate oligonucleoti (29mer) probe. The DNA from each clone yielded insert fragment sizes of approximat 2.7 kbp (MTRK#4) and 5 kbp (MTRK#5), which hybridized to the initial probe Southern blots (see figure A.1).

Southern blot analysis of EcoRI/BamHI digested MTRK#4 and MTRK#5 DI allowed the region hybridizing to the probe to be further localized (see figure A. EcoRI/BamHI digestion of MTRK#4 DNA yielded two insert fragments of ~900 a ~1800 bp, the larger of which hybridized to the probe (figure A.2, lane 2). EcoRI/Bam digestion of MTRK#5 DNA also yielded two insert fragments, ~2000 bp and ~3000 bp length, the larger of which hybridized to the probe (figure A.2, lane 6). The regi hybridizing to the probe in MTRK#4 DNA was further localized by double digestion w EcoRI and various other restriction endonucleases (figure A.2, lanes 3-5). MTRK#4 a MTRK#5 DNA inserts were ligated into pGEM4Z as EcoRI and EcoRI/Bam fragments. Subclones of MTRK#4 were selected for further investigation due to th smaller size. The presence of desired EcoRI and EcoRI/BamHI fragments in pGEM

subclones was confirmed by Southern blot analysis which shows hybridization only to t clones containing either the complete (p4Z-λ4-8) or 1800 bp (p4Z-B3-100) inserts (Figu A.2, lanes 9,11), but not to a clone (p4Z-C3X) containing only the 900 bp insert (la 10).

The results of Southern blot analysis presented in Figure A.2 (+ other data n shown) were used to assemble a preliminary restriction map (Figure A.3A Furthermore, from Figure A.2, the area complementary to the oligonucleotide probe (i. the beginning of the MTR kinase gene) was deduced to exist between the BamHI a NcoI restriction sites of MTRK#4 DNA. However, nucleic acid sequence analysis of t MTRK#4 subclones containing this region (p4Z-λ4-8 & p4Z-B300) failed to reveal a homology to the probe (Figure A.3B). Sequencing of the entire clone also failed identify any other regions homologous to the probe or capable of encoding the amino ac sequence known for the first 20 residues of the *Klebsiella* MTR kinase. Instead, t results of a BLASTN search of the combined non-redundant databases (GenBa +EMBL +DDBJ +PDB) indicate that the MTRK#4 sequence represents the *Klebsie* equivalent of a portion of the 81.5-84.5 min. region of the *E coli* genome (64-89 homology over 400-1000 bp comparisons of DNA, $\rho \leq 2.8e^{-162}$). This region contai the 3' end of rnpA (ribonuclease P), the complete "o548" gene (putative 60kD prote unknown function) and the 5' end of thdF (a protein involved in oxidation of thiophe and furan). Thus, MTRK#4 represents a false positive, probably due to the relative non-stringent conditions used in the initial library screening. An additional screening the *Klebsiella* library under more stringent conditions (48°C, 6xSSC, 30% formamic has failed to yield any additional phage clones.

The phage clone MTRK#5 remains a possibility for containing the MTR kin gene. The ~5 kbp EcoRI fragment (complete insert) from MTRK#5 has been subclon into pGEM4Z, as have two smaller (~2 kbp & ~3 kbp) EcoRI/BamHI fragments. T

MTR kinase probe does hybridize to the larger EcoRI/BamHI fragment (Figure A.2, la 6), but whether this also represents a false positive remains to be seen. Future work this clone will include mapping and hybridization studies conducted under more stringe conditions to determine if a smaller fragment containing regions homologous to the pro can be generated.

Beyond work on the MTRK#5 sequence, other additional studies are planned. lysate of *Klebsiella pneumoniae* cells (from 16L of culture) has been prepared to purs purification of additional native MTR kinase. Once purified, NH_2-terminal amino ac sequencing will be performed on a sample of the protein in an attempt to identi additional residues beyond the 19 which are known. This information may allow oligonucleotide probe containing less degeneracy to be developed. Alternatively, t added amino acid sequence may allow the design of a better oligonucleotide primer pair use in PCR amplification of a short stretch of the kinase gene. This PCR product cou subsequently be used to probe the *Klebsiella* genomic library. As an aside, this approa has been attempted using the oligonucleotides ZP-1 and ZP-2, but failed to yield a positi result, possibly because the primers were designed to regions so close together that t product (57 bp) would be difficult to resolve from primer artefacts by agarose electrophoresis.

In the event that the above measures fail to yield the MTR kinase gene, it may necessary to generate our own *Klebsiella* library. It is worthy of note that *Klebsie* MTR kinase activity appears to be down regulated when high levels of methionine present in the culture medium [13]. This regulation probably occurs via the action of methionine aporepressor (metJ gene product), which binds upstream methion regulatory sequences (when activated by S-adenosylmethionine), to block transcription a number of methionine biosynthetic genes [14-16] Thus, the possibility exists tha cDNA "subtraction" library could be synthesized in which the cDNA from *Klebsiella* c

grown in methionine deficient medium are first hybridized with biotinylated mRNA fro methionine replete cells to remove cDNA's of undifferentiated origin. The net resu should be a library enriched for cDNA's representing genes expressed under methioni limiting conditions. An alternate (or complementary) approach would be to generate phage display library, and enrich for MTR kinase gene containing clones by affini chromatography on the PAPTA/R resin described in chapter 2 of this thesis [11].

Table A.1. MTR kinase oligonucleotides.

Oligo	Sequence 5' 3'	M.P.
TB	CAGTA(C/T)CA(C/T)AC(C/G)TT(C/T)AC(C/G)GC(C/G)CA(C/T)GA(C/T)GC	54°C[a]
ZP-1	CAGTA(C/T)CA(C/T)AC(C/G)TT(C/T)AC	54°C[b]
ZP-2	CC(C/G)GC(G/A)AACTACTA(G/C)GC	61°C[b]

[a] Conditions: 6X SSC / 30% formamide.
[b] Conditions: 6X SSC / 0% formamide.

Figure Legends

Figure A.1. Agarose gel electrophoresis and Southern blot analysis of lamb MTRK#4 and MTRK#5 clones.

Panel A: 0.7% Agarose gel in TAE, 100V/1hour. Lane 1: lambda Hind III molecul weight standard; lane 2: undigested MTRK#4 DNA; lane 3: EcoRI digested MTRK DNA; lane 4: undigested MTRK#5 DNA; lane 5: EcoRI digested MTRK#5 DN Approximately 5μg of DNA were loaded in each lane.

Panel B: Autoradiogram (exposed for 16 hours at -80°C) of Southern blot of the g from (A), probed with ^{32}P-labelled oligonucleotide "TB".

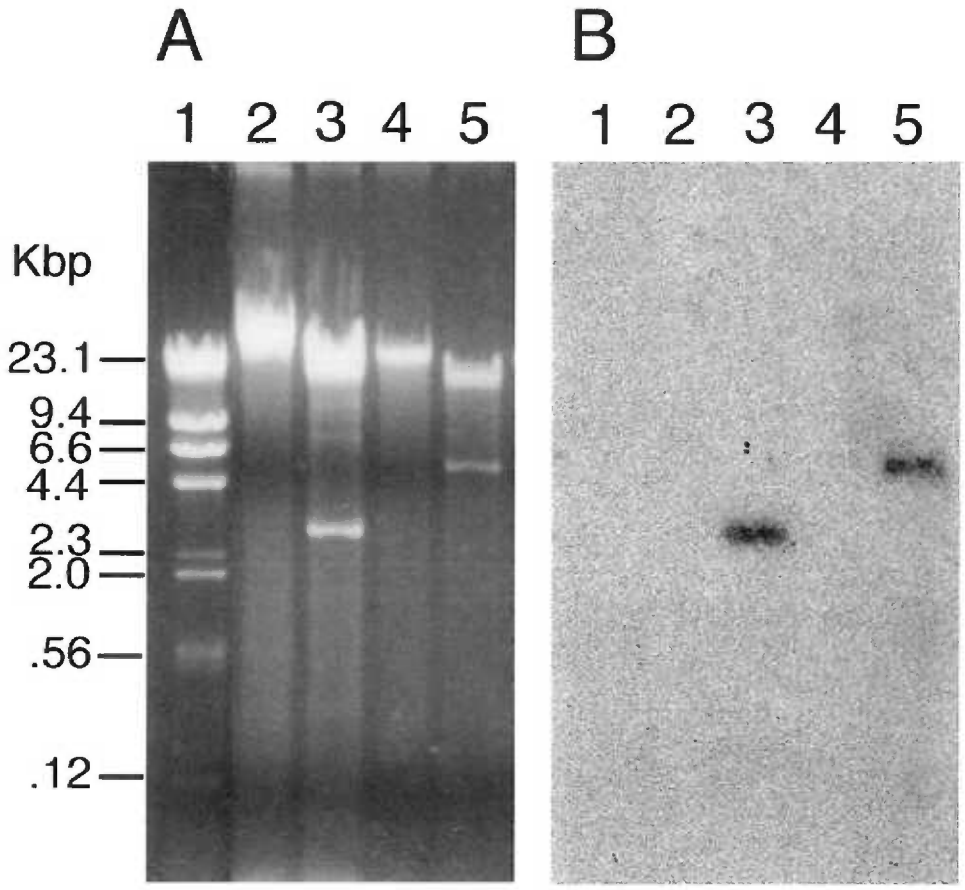

A
B
1 2 3 4 5
1 2 3 4 5
Kbp
23.1
9.4
6.6
4.4
2.3
2.0
.56
.12

Figure A.2. Agarose gel electrophoresis (0.8% Agarose gel in TAE, 50V/150 mi and Southern blot analysis of restriction endonuclease digested lambda-MTRK# lambda-MTRK#5, and subclone pGEM4Z-MTRK#4 DNA.

Panel A: 0.8% Agarose gels in TAE, 50V/150 min. lane 1: lambda HindIII molecu weight ladder; lane 2: EcoRI/BamHI digested MTRK#4 DNA; lane 3: EcoRI/Hind digested MTRK#4 DNA; lane 4: EcoRI/NcoI digested MTRK#4 DNA; lane 5: EcoRI/S digested MTRK#4 DNA; lane 6: EcoRI/BamHI digested MTRK#5 DNA; lane 7: lamb HindIII molecular weight ladder; lane 8: EcoRI/BamHI digested pGEM4Z; lane EcoRI/BamHI digested p4Z-λ4-8 (contains the complete MTRK#4 insert); lane 1 EcoRI/BamHI digested p4Z-C3X (contains a ~900 bp fragment of MTRK#4); lane 1 EcoRI/BamHI digested p4Z-B3-100 (contains a ~1800 bp fragment of MTRK#4).

Panel B: Autoradiogram (exposed for 16 hours at -80°C) of Southern blots of gels fro panel A, probed with ^{32}P-labelled oligonucleotides "ZP-1" and "ZP-2".

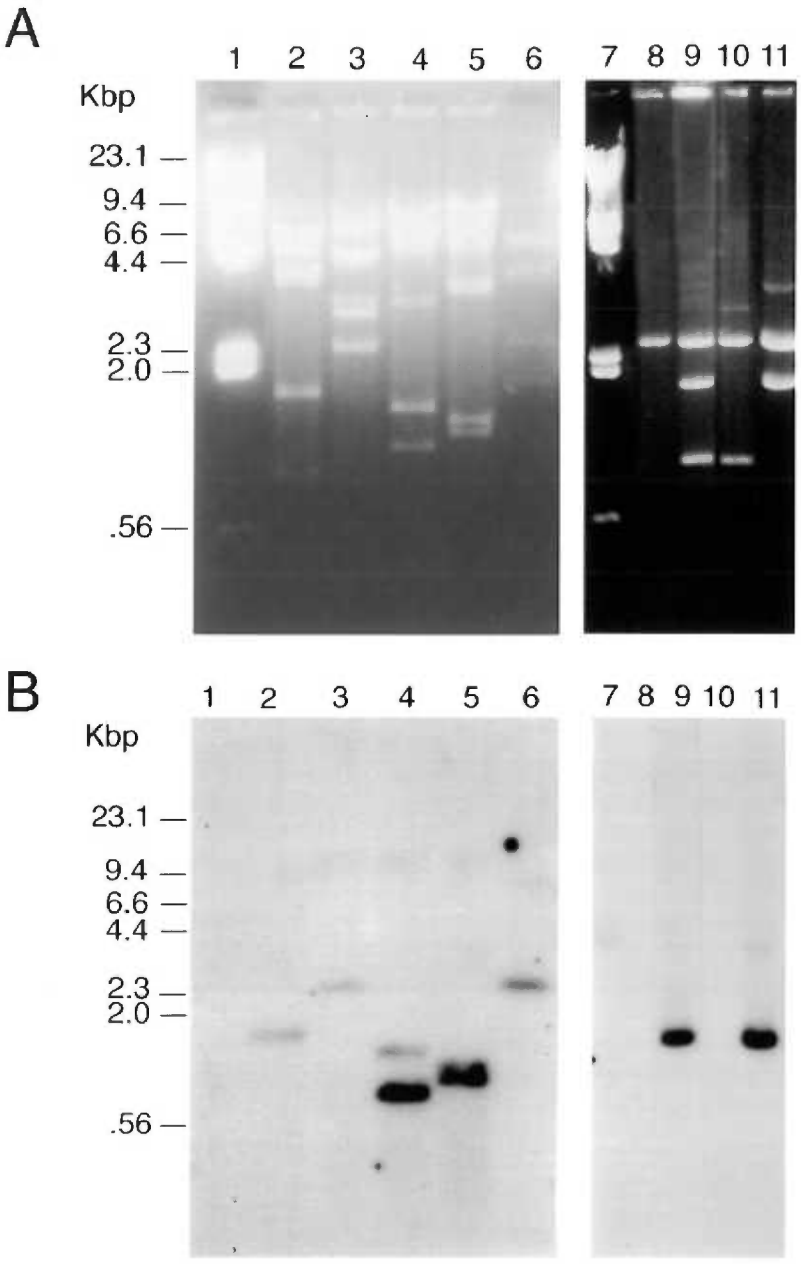
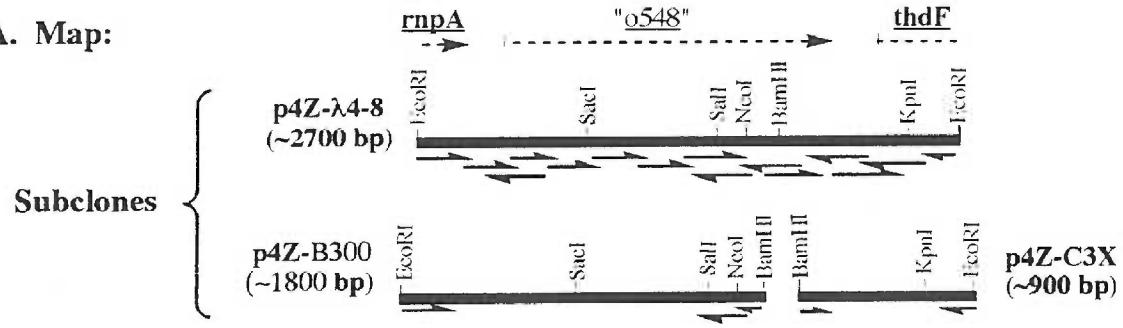

A
1 2 3 4 5 6 7 8 9 10 11
Kbp
23.1
9.4
6.6
4.4
2.3
2.0
.56
B
1 2 3 4 5 6 7 8 9 10 11
Kbp
23.1
9.4
6.6
4.4
2.3
2.0
.56

Figure A.3. Restriction map and sequence analysis of MTRK#4 insert.

A. Diagram of sequencing strategy for pGEM4Z subclones of MTRK#4. Small arrov indicate the region of sequencing primers used for reactions. The location of homologoı *E. coli* genes are indicated by dashed arrows (----->).

B. Complete sequence of the MTRK#4 insert. Uppercase, underlined sequenc represent open reading frames in *Klebsiella* corresponding to *E. coli* genes: mpA (end), "o548" (complete), and thdF (5' end).

A. Map:



B. Sequence:

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GCAATCGGATTAAACGTCTGACGCGTGAAAGTTTTCGTTTGCGTCAACATGAACTCCCGCCAATGGAT 1
TTCGTGGTGGTGGCGAAAAGAGGGGTTGCCGACCTCGATAACCGTGCTCTCTCGGAAGCGTTGGAAAA
ATTATGGCGCCGCCATTGTCGCCTGGCTCGCGGGTCCTGAtcggcctgattcgagtttatcagcgcct 3
gattagtccgctactcgggccgcattgtcgtttcaccccaacctgttctcaatacggaattgaggcct
tacgcaggtttggagtgataaaaggcagttggttgacgatgaaacgcgtattaaaatgccacccttta 4
caccctggtggtgacgatcccgtcccgcctggaccatttgataccagagaacactaacgATGGATTCG
CAACGCAATCTTCTTATCATCGCTTTGTTGTTCGTGTCTTTCATGATCTGGCAAGCCTGGGAGCAGGA 5
CAAAAATCCGCAGCCCCAGCAGCAGACCACGCAGACTACGACCACAGCAGCGGGTAGCGCCGCCGACC
AGGGCGTACCGGCCAGTGGCCAGGGGAAACTGATTACGGTTAAAACCGACGTGCTTGAGCTGACTATC 6
AACACCAACGGTGGCGATATTGAGCAGGCGCTGCTTCTGGCGTATCCCAAAACGCTGAAATCGACCGA
ACCGTTCCAGTTACTGGAAACCACGCCGCAGTTTGTCTACCAGGCGCAGAGCGGCTTAACCGGCCGTG 8
ACGGTCCGGATAACCCGGCAAACGGCCCGCGTCCGCTGTACAACGTCGATAAAGAGGCGTTTGTGTTG
GCCGATGGCCAAGATGAGCTCGTTATCCCGCTGACCTACACTGACAAAGCCGGCAACGTCTTCACCAA 9
AACCTTCACCCTGAAGCGCGGTGGCTATGCGGTGAACGTGGGTTACAGCGTGCAGAATGCCAGCGAGA
AGCCTCTGGAAGTCTCGACCTTCGGTCAGCTGAAGCAGACCGCTGCGCTGCCGACCAGTCGCGATACG 10
CAGACCGGTGGCCTGTCCACGATGCATACTTTCCGTGGCGCCGCGTTCTCCACTGCGGATTCGAAATA
CGAAAAATATAAATTCGATACCATTCTGGATAACGAAAACCTGAACGTCAGCACCAAAAACGGTTGGG 12
TTGCCATGCTGCAGCAGTACTTCACCACCGCATGGGTGCCGCGGAATAACGGGACGAATAACTTCTAC
ACCGCCAACCTCGGCAACGGCGTTGTCGCTATTGGCTACAAATCGCAGCCGGTACTGGTGCAGCCAGG 13
CCAGACCGACAAACTGCAGAGCACGCTGTGGGTCGGCCCGGCTATTCAGGACAAAATGGCTGCCGTTG
CGCCGCACCTGGATCTGACCGTCGACTACGGCTGGCTGTGGTTCATCTCCCAACCGCTGTTCAAGCTG 14
CTGAAATTCATCCACAGCTTCCTCGGCAACTGGGGCTTCTCGATCATCGTTATCACCTTTATCGTTCG
TGGCATCATGTACCCGCTGACCAAAGCGCAGTACACCTCCATGGCGAAGATGCGCATGCTGCAGCCGA 16
AGATTCAGGCCATGCGTGAGCGTCTGGGCGACGATAAACAACGTCAAAGCCAGGAGATGATGGCGCTG
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TGCGCTGTACTACATGCTGAGCGCCTCGGTTGAACTGCGTCATGCGCCGTTTATCCTGTGGATCCACG
ACCTGTCTGCTCAGGACCCGTACTACATCCTGCCGATCATCATGGGCGCGACCATGTTCTTCATCCAG 19
AAGATGTCGCCGACCACCGTGACCGACCCGATGCAGCAGAAGATCATGACCTTTATGCCGGTCATCTT
CACGGTGTTCTTCCTGTGGTTCCCGTCTGGCCTGGTGGTGTACTACATCGTCAGCAACCTGGTCACCA 20
TTATTCAGCAGCAGCTGATTTACCGTGGTCTGGAGAAACGTGGCCTGCATAGCCGCGAGAAGAAGAAA
TCCTGAtactcttcattcttcaagccgcagatgcgttggcttcattagttcgccccagtcacttacta 21
cagtaagctcctggggcctcactaacttgccgcctttctgcaacttgaattatttcgagtatctacgg
tagcgttaatgccagagaaggcggtcaatggaccgccttttttacatctacatagagagtcaccATGA 23
GCCATAACGACACTATCGTCGCCCAGGCAACCCCTCCGGGACGCGGGGGTGTGGGCATCCTGCGTATC
TCCGGCCTTAAGGCGCGCGACGTCGCGCAGGCGGTGCTGGGCAAGCTGCCGAAGCCGCGCTATGCCGA 24
CTACCTGCCGTTCAACGACGTTGACGGTACCCCGCTGGATCAGGGGATTGCGCTGTGGTTCCCCGGGC
CGAACTCCTTTACCGGGGAAGATGTGCTTGAGCTGCAGGGCCACGGCGGCCCGGTCATTCTCGACCTG 25
CTGCTTAAACGTATTCTGACCCTGCCGGGCCTGCGCATCGCCAGGCCGGGTGAGTTTTCCGAGCGCGC
GTTCCTCAACGACAAGCTCGATCTGGCGCAGGCAGAGGCCATCGCCGACCTTATCGACGCCAGTTCAG 27
AGCAGGCGGCGCGCTCGGCGCTgaattc 27

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APPENDIX B

Antibacterial Effects and Toxicity Testing of MTA and MTA Analogs.

A number of MTA analogs (figure B.1), initially studied as inhibitors of MTA/SA nucleosidase [1], were examined in an effort to correlate enzyme-inhibitory effect wit antibacterial activity. In addition, the effect of these analogs against isolated low densit bone marrow cells was investigated to gain a preliminary insight into their possib toxicity against eukaryotic cells.

The antibacterial activities of MTA and its analogs were measured against cultures *E. coli* (ATCC #25922), a standard reference strain used for antibiotic testing. Assay were conducted in 96-well plates containing minimal media [2] supplemented with 1 non-essential amino acids and vitamins (Gibco/BRL) (Min^{++} media) and variou concentrations of drug (final conc.= 0-100μM). *E. coli* cells from an overnight cultu (grown in Min^{++} media) were diluted in Min^{++} to yield 1-2 x 10^3 cells/10μl. Assay were initiated with the addition of 10μl diluted cells/ well (200μl total volume/ well), ar incubated with gentle agitation at 37°C until the $OD_{540} \approx 0.3$. Optical density readin were performed on a Bio-Tek microplate autoreader (Model EL309). Inhibitory effec were determined by comparing average optical density readings for treated wells untreated controls. Drug concentrations required to yield a 50% reduction in grow (IC_{50}) were estimated by non-linear regression analysis. Estimated IC_{50} values ar effects of 100μM drug concentrations on the growth of E. coli cells are presented in Tab B.1.

The effect of MTA and various analogs were measured on bone marrow ce derived from healthy adult volunteers. Low density bone marrow cells (CFU-GM) we

isolated by density gradient centrifugation on ficoll, and plated in semi-solid agaro medium containing various concentrations of drug, supplemented with human placen conditioned media (HPCM) to stimulate colony formation [3]. Plates (~$2x10^5$ CFU GM's/ 50mm plate) were incubated in a humidified 37°C incubation chamber containi 5% CO_2/95% air. Colony formation was assessed on day 12 by inspection of the plat under a dissecting microscope. Inhibition of HPCM stimulated growth was calculated b comparing the average colony number present on drug-treated versus untreated plate IC_{50} values were estimated from non-linear regression analysis of the averages of 3 experiments (Table B.2).

The results of these studies demonstrate that only MTT ($IC_{50} \approx 55\mu M$) exhib significant antibacterial activity. The remaining compounds were not particularly effecti against *E. coli*, in spite of generally good inhibitory activities (IC_{50}'s <1μM) agai MTA/SAH nucleosidase (Chapter 4). 5'-(*p*-Nitrophenyl)thioadenosine, a nucleoside wi potent *in vitro* inhibitory activities against the enzyme (K_i = 20nM), exerted significa activity against *E. coli* only at high concentrations (41% inhibition at 100μM). T physiological cause for the insensitivity of *E. coli* to MTA analogs is unclear, but m reflect poor cellular transport of these compounds, or rapid hydrolysis by the nucleosida upon entry into the cell.

Human bone marrow cells were generally more sensitive than *E. coli* to t thionucleosides tested here. The effects of MTA, MTT, and pIPhTA, were particula striking with IC_{50} values in the 30-45μM range. MTA is known to be transported i mammalian cells [4, 5], where it can exert inhibitory effects on (a)SAH hydrolase [6], DNA and protein methylation [7-9], and (c) polyamine biosynthesis [10, 1 Presumably, MTA in our experiments is inhibiting HPMC stimulated GM-CI proliferation by one or all of these mechanisms. MTT is probably functioning similarly MTA, as well as acting as an inhibitor MTA phosphorylase [12, 13]. The effects of

para substituted aryl-thionucleosides presented here have not been studied previousl

Table B.1. Sensitivity of *E. coli* strain #25922 to MTA and MTA Analogs*.

Drug	Abbrev.	Inhibitory Effect on Growth	
		% Inhibition at 100μM	Estimated IC_{50} (μM)
5'-Methylthioadenosine	MTA	22 %	>100
5'-Methylthiotubercidin	MTT	73 %	55
5'-Aryl-substituted MTA analogs:			
5'-Phenylthioadenosine	PhTA	37 %	>100
5'-(*p*–Fluorophenyl)thioadenosine	pFPhTA	32 %	>100
5'-(*p*–Chlorophenyl)thioadenosine	pClPhTA	17 %	>100
5'-(*p*–Bromophenyl)thioadenosine	pBrPhTA	21 %	>100
5'-(*p*–Iodophenyl)thioadenosine	pIPhTA	13 %	>100
5'-(*p*–Aminophenyl)thioadenosine	pAPhTA	7 %	>100
5'-(*p*–Nitrophenyl)thioadenosine	pNO2PhTA	41 %	>100

*Values represent the average of 2-3 experiments (individual points determined triplicate).

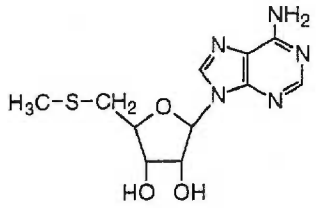
Table B.2. Sensitivity of HPMC Stimulated Bone Marrow Cells (CFU-GM) to MTA and MTA Analogs*.

Drug	Abbrev.	Inhibitory Effect on Growth	
		% Inhibition at 100μM	Estimated IC_{50} (μM)
5'-Methylthioadenosine	MTA	78 %	35
5'-Methylthiotubercidin	MTT	58 %	30
5'-(6-Purino)thioadenosine	PurTA	16 %	>100
5'-Aryl-substituted MTA analogs:			
5'-Phenylthioadenosine	PhTA	16 %	>100
5'-(*p*–Fluorophenyl)thioadenosine	pFPhTA	23 %	>100
5'-(*p*–Chlorophenyl)thioadenosine	pClPhTA	60 %	72
5'-(*p*–Bromophenyl)thioadenosine	pBrPhTA	67 %	84
5'-(*p*–Iodophenyl)thioadenosine	pIPhTA	83 %	45
5'-(*p*–Aminophenyl)thioadenosine	pAPhTA	21 %	>100
5'-(*p*–Nitrophenyl)thioadenosine	pNO2PhTA	45 %	>100

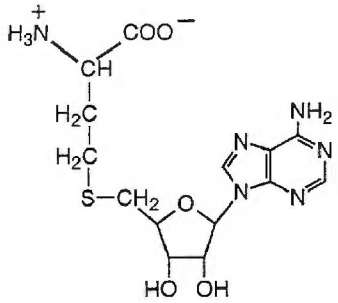
*Values represent the average of 3-4 experiments (individual points determined triplicate).

Figure B.1. The chemical structures of MTA, SAH and related analogs.

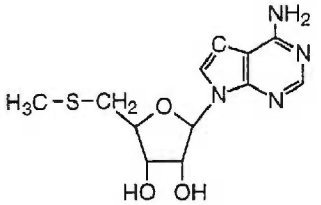
MTA, SAH & Analogs



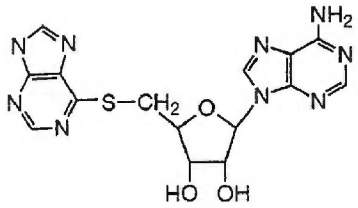
MTA



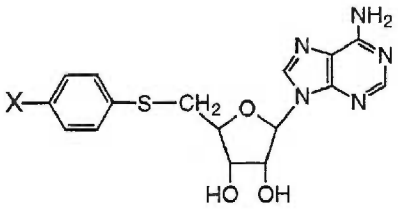
SAH



MTT



Purinothioadenosine (PurTA)



***para*-substituted Phenylthioadenosine**

Compound	Abbrev.	X
5'-Phenylthioadenosine	**PhTA**	H
5'-(ρ-Fluorophenyl)thioadenosine	**pFPhTA**	F
5'-(ρ-Chlorophenyl)thioadenosine	**pClPhTA**	Cl
5'-(ρ-Bromophenyl)thioadenosine	**pBrPhTA**	Br
5'-(ρ-Iodophenyl)thioadenosine	**pIPhTA**	I
5'-(ρ-Aminophenyl)thioadenosine	**pAPhTA**	NH_3^+
5'-(ρ-Nitrophenyl)thioadenosine	**pNO$_2$PhTA**	NO_2^-

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APPENDIX C

Development of Anti-MTA/SAH Nucleosidase Specific Monoclonal Antibodies & Immunoprecipitation Studies.

Monoclonal antibodies specific for *E. coli* MTA/SAH nucleosidase (MTAN) we developed in order to provide additional tools for use in protein purificatio immunoprecipitation, and expression library screening. Purified recombinant glutathion S-transferase-MTAN fusion protein (GST-MTAN, Chapter 3)[1] in Freunds comple antigen (CFA) was used as the antigen for the initial immunization of five Balb/C mic Mice were boosted by immunization with gst-MTAN in Freunds incomplete antigen (IF/ on days 30 and 60. The development of an immune response was followed by seru reactivity on Western blots of GST-MTAN. Ten days after the final immunizatio spleens from immunized animals were harvested and hybridomas developed l polyethylene glycol fusion of splenic lymphocytes to Sp2/0 myeloma cells [2, 3 Hybridomas were cloned into medium supplemented with epithelial cell grow supplement (ECGS) [4]. Clones secreting antibodies specific for the nucleosidase we selected based on ELISA results against GST-MTAN (and lack of reactivity against GS alone).

Eight hybridomas were selected after several isolations by limiting dilution (Tab C.1). Antibody isotyping using a Hyclone® (Logan, UT) mouse sub-isotyping ELIS kit revealed that the eight monoclonals represented four different immunoglob subclasses (IgA, IgG_1, IgG_{2A}, and IgG_{2B}). The clone R8B2.4.1 (isotype IgG_2 showed good reactivity for the nucleosidase by both ELISA and Western blotting (Figu 4.3B), and was selected for further expansion and antibody purification. R8B2.4

antibodies from hybridoma cell culture supernatants were purified by affini chromatography on protein A-Sepharose, and immobilized on CNBr-activated Sepharo by cross-linkage with dimethylpimelimidate [5]. Immobilized R8B2.4.1 was used f immunoaffinity chromatography of recombinant MTA/SAH nucleosidase (rMTAN) a truncated enzyme (rMTAN-8) (Chapter 4).

The possible utility of R8B2.4.1 in purification of nucleosidases from a number organisms was examined by immunoprecipitation studies performed with the immobiliz antibody. Cell lysates (1-3 mg protein) from eight different organisms were incubat with 50µl R8B2.4.1-beads for 24 hours at 4°C. After extensive washing, bou nucleosidase was eluted with low pH buffer (100mM glycine, pH 2.5), neutralized wi Tris, and enzyme activity (hydrolytic or phosphorylytic) assayed using the standa protocol (Chapter 4). The results in Figure C.1 demonstrate that the monoclonal antibo may be useful in the purification of nucleosidases from a number of bacterial and low eukaryotic species. All five bacterial species (*E. coli*, *S. typhimurium*, *K. pneumonia E. aerogenes*, *S. aureus*) showed large increases in enzyme specific activity in the affini eluates. In addition, the antibody was able to precipitate enzyme activity from lysates two nucleosidase-containing protozoans, *Entamoeba invadens* and *Ochromon malhamensis*.. In contrast, the antibody failed to immunoprecipitate enzyme activity fro lysates of *Candida albicans*, despite it having the highest specific acitivity in the cru lysate. It should be noted that *Candida albicans* was the only MTA phosphoryla containing species tested.

The results of the immunoprecipitation study indicate that the antibody develop against the *E. coli* enzyme cross-reacts significantly with epitopes on the other microb nucleosidases, supporting the possibility that the nucleosidases from the vario organisms contain highly conserved regions. The lack of immunoreactivity exhibited f the fungal phosphorylase is not entirely unexpected considering the differences betwe

the primary and quarternary structures reported for MTA phosphorylases and MTA/SA nucleosidases [1, 6-12]. MTA phosphorylases have generally been reported to exist homotrimers, with near neutral isoelectric points (pI≈6.8), whereas MTA/SA nucleosidase functions as a monomer with an acidic isoelectric point (pI≈5.0). In additio relatively small amino acid sequence homologies are apparent between the human MT phosphorylase and *E. coli* nucleosidase (Chapter 3).

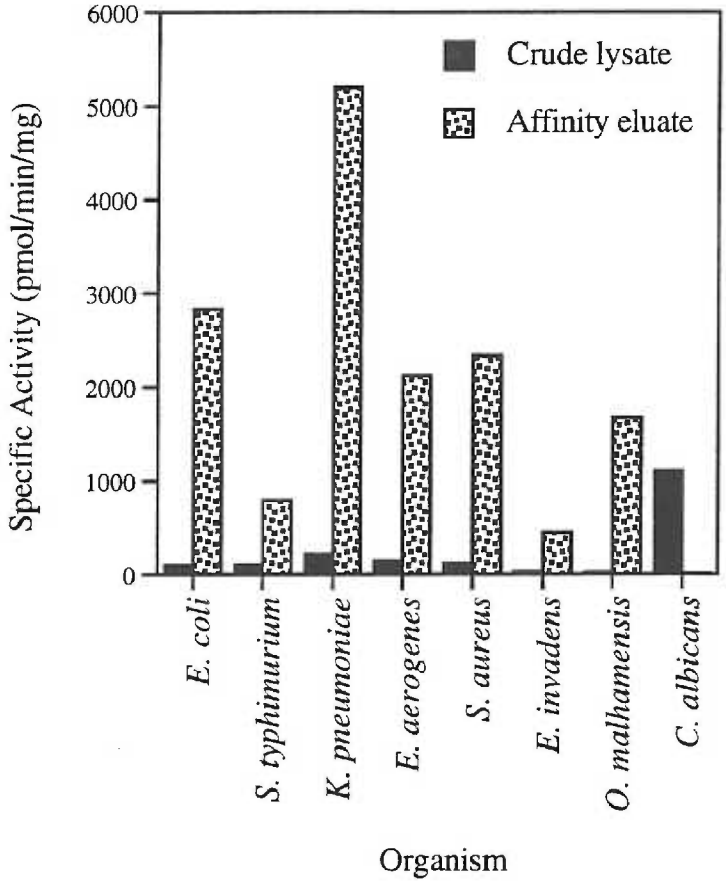
In summary, monoclonal antibodies were developed against the *E. coli* MTA/SA nucleosidase which show some promise in being useful in the purification and study nucleosidases from a variety of other microbes. Initial indications are that tl monoclonals will not recognize MTA phosphorylases, although only one antibody (eight) and one phosphorylase (from *Candida*) were examined. Future efforts with tl antibodies generated here will focus on exploring their ability to be used in screeni expression libraries of various nucleosidase-containing pathogens.

Table C.1. Properties of monoclonal antibodies developed against E. coli MTA/SA nucleosidase.

MAb Designation	Isotype	Anti-gst reactivity	Anti-nucleosidase reactivity		
		ELISA	ELISA	Western	Immuno-precip.
R8B2.4.1	IgG_{2A}	-	+	+	+
R3D4.6.1	IgG_{2B}	-	+	+	nt
R3D4.6.3	IgG_{2B}	-	+	+	nt
R3D4.6.6	IgG_{2B}	-	+	+	nt
R1C4.6.4	IgA	-	+	+/-	nt
K6D6.3.2	IgG_1	-	+	+/-	nt
K7A2.2.5	IgG_1	-	+	+	nt
K7B3.6.5	IgG_1	-	+	-	nt

(+) positive reactivity, (−) negative reactivity, (+/−) weak reactivity, (nt) not tested

Figure C.1. Immunoprecipitation of nucleosidase activity with immobilized R8B2.4 antibody. *E. coli*: *Escherichia coli*; *S. typhimurium*: *Salmonella typhimurium*; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. aerogenes*: *Enterobacter aerogenes*; *S. aureus*: *Staphylococcus aureus*; *E. invadens*: *Entamoeba invadens*; *O. malhamensis*: *Ochromonas malhamensis*; *C. albicans*: *Candida albicans*.


Specific Activity (pmol/min/mg)
6000
5000
4000
3000
2000
1000
0
Crude lysate
Affinity eluate
E. coli
S. typhimurium
K. pneumoniae
E. aerogenes
S. aureus
E. invadens
O. malhamensis
C. albicans
Organism

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