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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PHYSIOLOGY AND BIOCHEMISTRY OF PURINE SALVAGE IN AN ARCHAEOBACTERIUM, *METHANOBACTERIUM THERMOAUTOTROPHICUM*

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

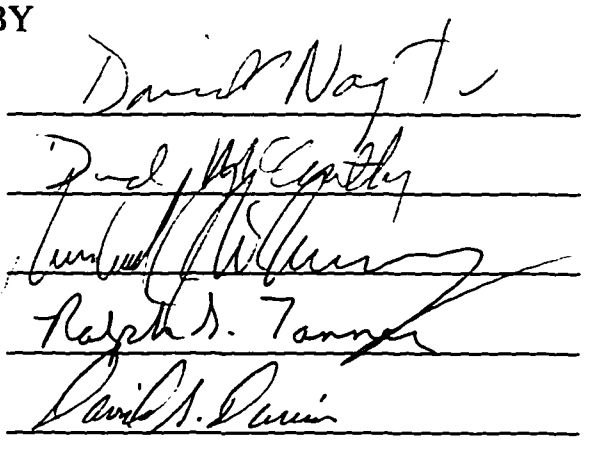
MANISH PAREKH
Norman, Oklahoma
1997

PHYSIOLOGY AND BIOCHEMISTRY OF PURINE SALVAGE IN AN ARCHAEOBACTERIUM, *METHANOBACTERIUM THERMOAUTOTROPHICUM*

A Dissertation APPROVED FOR THE

DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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To my parents and Ann Chen

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ABSTRACT

This dissertation describes a genetic and biochemical study of purine metabolism in Archaea and is expected to contribute to our knowledge about enzymes and pathways involved in salvage of purine bases. *Methanobacterium thermoautotrophicum* mutants spontaneously resistant to purine analogs were isolated. All mutants resistant to a hypoxanthine analog or a guanine analog were cross-resistant to analogs of the other base. These mutants lacked the ability to incorporate both purine bases (hypoxanthine or guanine), and were deficient in hypoxanthine and guanine phosphoribosyltransferase (HGPRTase) activities, suggesting that the organism contains a single enzyme capable of both activities.

Studies of the effect of temperature on stability of 100-fold purified HGPRTase from *M. thermoautotrophicum* suggested that this enzyme is the most thermostable of all known HGPRTases; upon incubation at 92°C followed by assay at 55°C, the half life of HGPRTase activities was 4 min without substrate $PRPP_i$ and greater than 23 min with it. The temperature optimum for methanogen HGPRTase was between 71 and 79°C, with most of its activity remaining at 88°C. Noteably, HGPRTase activities from the mesophiles *Escherichia coli* (eubacterium) and *Saccharomyces cerevisiae* (yeast) were also highly active at 92°C.

HGPRTase from *M. thermoautotrophicum* was purified greater than 1200-fold. The subunit molecular weight of HGPRTase was determined to be 28 kDa. 100-fold-purified enzyme was active over a broad pH range (optimum pH 9.9). The apparent K_m values for hypoxanthine, guanine, and $PRPP_i$ were 6.1 μM, 38.7 μM, and 86.3 μM, respectively. HGPRTase was stimulated by Mg^{2+}, Mn^{2+}, and Zn^{2+}, but inhibited by IMP and PP_i. Growth

in the presence of acetate but not purine bases yielded a two-fold increase in the levels of HGPRTase in extracts. The NH_2-terminal amino acid sequence of the peptide assigned to *M. thermoautotrophicum* HGPRTase was Met-Leu-Asp-Lys-Leu-Lys-Glu-Ser-Leu-Arg-Asn-Ser-Pro-Val-Ile-Lys-Lys-Gly-Glu-Tyr.

Viability studies of effects of starvation on *M. thermoautotrophicum* under different gas phases (H_2, N_2, and N_2:CO_2) suggested that it remained viable under N_2:CO_2, but lost viability much more rapidly under H_2 or N_2. Incubation of cells under N_2:CO_2 with inhibitors neomycin or 5-methyl tryptophan resulted in more rapid loss of viability than for cells without compound. This suggests that protein synthesis may be involved in survival of *M. thermoautotrophicum*.

PREFACE

Comparison of 16S rRNA sequences has led to the division of the living world into three domains: Eucarya, Eubacteria, and Archaea. The archaeal domain consists of organisms whose 16S rRNA sequence homologies cluster together in a group that is as different from eubacteria as it is from eucaryotes. The archaea are extremophiles and share many biochemical properties in addition to rRNA sequence similarities (Kandler and Zillig, 1986; Jones *et al.*, 1987). Within the archaea, according to Stetter (1996) are two kingdoms: *Crenarchaeota* and *Euryarchaeota*, which are internally consistent for rRNA sequence homologies and for physiological similarities. The *Crenarchaeota* group consists of the extreme thermophiles, and the *Euryarchaeota* group consists of extreme halophiles and methanogenic archaea. Archaea and archaeobacteria will be used in this document throughout as synonyms. Members of archaea occur in environmental habitats that are extreme, such as high salt, high temperature, and strict anaerobic conditions. The *Crenarchaeota*, extreme thermophiles (also known as hyperthermophiles) can be isolated from geothermal habitats such as sulfur-rich hot springs, and submarine thermal waters. Recently, presence of archaea belonging to *Crenarchaeota* group was detected in marine environments based upon analysis of 16S ribosomal RNA gene sequences from the biomass (DeLong, 1992; Fuhrman *et al.*, 1992). Except for genera *Sulfolobus*, *Metallosphaera*, *Acidianus*, and *Pyrobaculum*, all hyperthermophiles known to date are anaerobic (Stetter, 1996). Halophiles are aerobic or facultative bacteria that commonly occur in salt lakes, industrial plants that produce salt, and salted food such as salted fish. Methanogens occur in anaerobic habitats such as marshes, swamps, pond and

lake mud, marine sediments, the intestinal tract of humans and animals, the rumen of cattle, and anaerobic sewage digesters in sewage-treatment plants.

Methanogens are able to utilize a limited number of substrates for growth. Methanogenic archaea obtain energy for growth by oxidizing compounds such as H_2 or formate, and utilize the electrons thus generated to reduce CO_2 with the formation of methane gas (CH_4). The methyl group of acetate is also reduced to CH_4 by some species. Besides being able to utilize a very limited range of substrates, methanogens require nickel, iron, and cobalt as trace metals for growth. These bacteria play an important role in the carbon cycle and the environment and in sewage plants for anaerobic treatment of organic wastes (Ferry, 1993). CH_4 formation is the terminal step in carbon flow in anaerobic habitats, and CH_4 escaping into the environment can serve as a carbon and energy source for aerobic methanotrophic bacteria. CH_4 is also implicated as one of the greenhouse gases.

Methanogens are known to contain unique coenzymes, including Coenzyme M and Coenzyme F_{430}, that have not been found in other bacteria (Ferry, 1993). Together with these two examples, there are unusual tetrahydromethanopterin derivatives and deazaflavins that are involved in reactions of methanogenesis and anabolic metabolism in these organisms. The methanopterins are similar to tetrahydrofolate coenzymes and the deazaflavins are structurally analogous to flavins but functionally more like nicotinamide or ferredoxin cofactors than flavins. Catabolic metabolism results in ATP synthesis via proton and sodium gradients and also produces one-carbon intermediates to be utilized in anabolic reactions. Methanogens also differ from true bacteria with respect to membrane and cell wall structure. Unlike true bacteria, membrane lipids in methanogens are composed of polyisoprenoids ether-linked to glycerol or other carbohydrates,

and cell walls in these organisms are devoid of peptidoglycan containing muramic acid. Excellent reviews on methanogens are available by Jones *et al.* (1987) and by Ferry (1993). Additional references can be found in the following chapters.

Among many aspects of archaeal biology to be studied, nucleobase metabolism can yield comparative information among archaea, bacteria, and eucaryotes. In particular, purine and pyrimidine nucleotide metabolism in Archaea is of great interest. The biochemical pathways involved in nucleotide metabolism in Archaea can be compared with those in the domains Eubacteria and Eucarya for the purpose of evolutionary studies. Purine and pyrimidine utilization in other organisms has been exploited in chemotherapy, genetics, and been informative biochemically. In salvage reactions, cells recover preformed bases which are incorporated into nucleotide pools and then polynucleotides. When analogs are taken in and metabolized (e.g. flurouracil, mercaptopurine), normal host functions are disrupted in a variety of ways. There has been an interest in targeting HGPRTases directly to control schistosomal (Yuan *et al.*, 1992) and protozoal infections in humans (Allen and Ullman, 1994), and indirectly for treatment of certain cancers in humans (Elion, 1989). Very few studies in this area are reported for members of Archaea. Knox and Harris (1988) reported isolation of spontaneous mutants of *Methanobacterium* sp. strain FR-2 resistant to some of the pyrimidine analogs. Studies on pyrimidine metabolism have also been reported in *Methanobacterium thermoautotrophicum* (Teal and Nagle, 1986; Nagle *et al.*, 1987) and *Sulfolobus acidocaldarius* (Grogan and Gunsalus, 1993), an extremely thermophilic archaeon. It is important to note that very little is known about purine and pyrimidine salvage metabolism in hyperthermophiles, and especially halophiles.

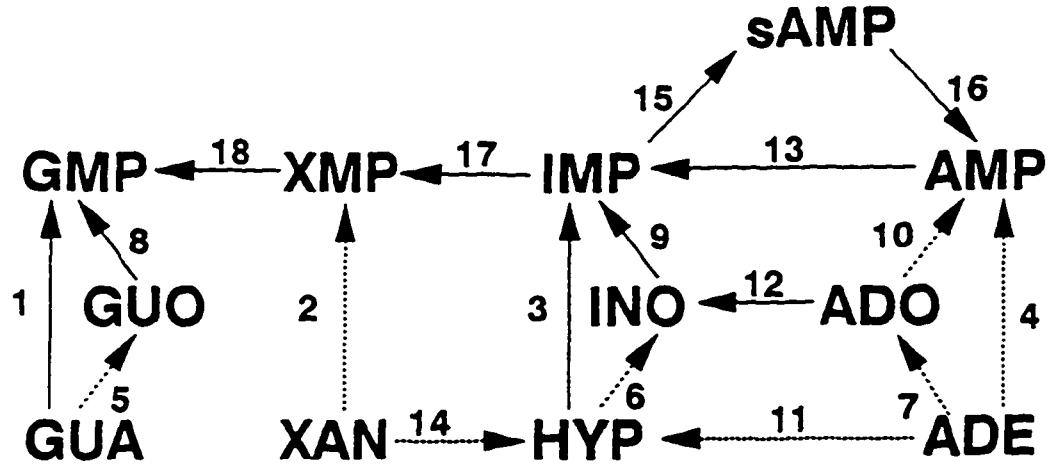

FIG. 1. Proposed pathway for purine interconversion in *M. thermoautotrophicum* Marburg. Symbols: ⟶, activities greater than 1.0 nmol/mg of protein per min; ⇢, activities greater than 0.01 but less than or equal to 0.20 nmol/mg of protein per min. Reactions 1, 2, 3, and 4 represent guanine (GUA), xanthine (XAN), hypoxanthine (HYP), and adenine (ADE) phosphoribosyltransferases, respectively; 5, 6, and 7 represent guanosine (GUO), inosine (INO), and adenosine (ADO) nucleoside phosphorylases, respectively; 8, 9, and 10 represent GUO, INO, and ADO kinases, respectively; 11, 12, and 13 represent ADE, ADO, and AMP deaminases, respectively; 14 represents XAN oxidase; 15 represents succinyl-AMP (sAMP) synthetase; 16 represents sAMP lyase; 17 represents IMP dehydrogenase; and 18 represents GMP synthetase.

Reprinted from Worrel and Nagle (1990)

Purine salvage pathways have been documented in some methanogens, including *M. thermoautotrophicum* (Worrell and Nagle, 1990) and *Methanococcus voltae* (Bowen *et al.*, 1996). These methanogens contain pathways and enzymes necessary for purine salvage. An example of one of these pathways is shown in Figure 1 (Worrell and Nagle, 1990). In other studies, DeMoll and Auffenberg (1993) reported the potential of *Methanoccocus vannielli* to use some purines as a source of nitrogen.

The thermophilic nature of the enzyme under study here raises some questions in the context of phylogeny. According to the universal tree proposed by Woese (1994), the archaea may be closer to the universal common ancestor than are the eubacteria or the eucaryotes. However, Sogin (1989) argues that eucaryotes could be as primitive as archaebacteria and eubacteria. The studies of Stetter (1996) suggest that thermophily may have been primitive, especially with his observation that thermophily is a property common to organisms close to the proposed common ancestor. The knowledge that the surface of the early Earth was unusually hot (near 100°C) (Stetter, 1986) raises the possibility that the primitive common ancestor may have been a hyperthermophile (Stetter, 1996). Thermophily is a property common to the archaeal kingdom *Euryarchaeota* (comprising methanogens and halophiles) which is closely branched from the *Crenarchaeota* lineage.

The relationship of Archaea with the domains Eubacteria and Eucarya, and the nature of a universal common ancestor to all three domains remain unresolved. The sequencing of eubacterial genomes of *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Mycoplasma genitalium* (Fraser *et al.*, 1995) has been completed. In Eucarya, at least five of the sixteen chromosomes from yeast have been sequenced (Bussey *et al.*, 1995). Recently, sequencing of the entire genome from *Methanococcus jannaschii*

was completed (Bult *et al.*, 1996). The study showed that only 44% of *M. jannaschii*'s genes could be matched with those found in known databases of eubacterial and eucaryotic genomes. It is believed that the availability of *M. jannaschii* genomic sequence data will allow detailed comparisons of genes and biochemical pathways among the three domains. Furthermore, it is expected that genome sequences of *M. thermoautotrophicum* and *Pyrococcus furiosus* could be available in near future (Morell, 1996).

This dissertation is a report of studies on purine metabolism in *M. thermoautotrophicum* Marburg using genetics, physiology, and biochemistry. Chapter 1 is a study of purine analog-resistant strains. Chapter 2 is a study of thermal properties of the purine salvage enzyme(s) hypoxanthine- and guanine phosphoribosyltransferase (HGPRTase) from *M. thermoautotrophicum*, Brewers' yeast, and *Escherichia coli*. Chapter 3 describes results of purification and properties of HGPRTase from *M. thermoautotrophicum*. Finally, Chapter 4 is a study of starvation-survival of *M. thermoautotrophicum* and an intriguing unknown role that adenosine plays in survival.

In the text of this dissertation, citations were made by author and year. In other respects, Chapters 1 and 2 were prepared after the format of the *Journal of Bacteriology*, and Chapter 3 was prepared after the format of the *Journal of Biological Chemistry*. Chapter 4 was prepared after the format of *FEMS Microbiology Letters*.

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CHAPTER 1

Isolation and biochemical characterization of *Methanobacterium thermoautotrophicum* mutants resistant to purine analogs

ABSTRACT

Methanobacterium thermoautotrophicum mutants defective in purine metabolism were isolated by selecting for resistance to purine analogs. Two mutants (MP-2 and MP-3) resistant to 8-azaguanine, a guanine analog, and one (MP-5) resistant to 6-mercaptopurine, a hypoxanthine analog, were found to be resistant to many of the purine analogs tested. All three purine analog-resistant mutant strains appeared to be defective in hypoxanthine and guanine accumulation during growth as well as in hypoxanthine-guanine phosphoribosyltransferase activity (HGPRTase; E.C. 2.4.2.8). All the mutants retained between 6-10% of base incorporation found for the wild type, whereas 0-5% of HPRTase and GPRTase activities remained. When strain MP-3 was serially transfered several times in medium without purine analog, resistance to 8-azaguanine was retained and there was no change in the levels of hypoxanthine-guanine phosphoribosyltransferase activity in extracts, indicating the resistance mutation was a stable property. Addition of bases hypoxanthine and guanine to the medium did not affect the specific activities of hypoxanthine-guanine phosphoribosyltransferase in wild type or in MP-3. Finally, crude extract of MP-3 did not contain inhibitors of HGPRTase activity when wild type and MP-3 extracts were mixed, and no effect on HGPRTase activity in wild type extract was seen.

INTRODUCTION

Purine ribonucleotides are precursors in the biosynthesis of nucleic acids, histidine, and cofactors (e.g. NAD, FAD, folic acid, thiamine, and CoA). Purine ribonucleotides can be synthesized in two ways; via *de novo* synthesis from small precursors and via salvage pathways (utilization of preformed purine bases and nucleosides). Purine salvage is less costly to the cells than *de novo* synthesis because it prevents loss of valuable energy and precursors. Diffusable purine nucleosides and bases originating from intracellular turnover of nucleic acids, and catabolic products of nucleic acid and nucleotides in decaying cells are salvaged (Nygaard, 1983).

Carl Woese (1994) proposed that the living world can be divided into three domains: Eubacteria, Archaea, and Eucarya. Archaeobacteria (here considered synonymous with archaea), although procaryotic, and like eubacteria in many ways, also share many biological properties with eucaryotes. Studies aimed at comparing biological properties and metabolic processes within these groups have provided an insight into how these three domains may have evolved. With rooting of phylogenetic trees, the new view that emerged suggests that Archaea and Eucarya lie on the same branch of the tree, and therefore may have shared a common ancestral gene pool (Woese, 1994). Salvage pathways have been documented in eucaryotic, eubacterial, and archaeal domains. Although the *de novo* purine nucleotide synthesis pathway seems to be identical in the various organisms studied, salvage pathways seem far more diverse (Nygaard, 1983). Purine phosphoribosyltransferases (PRTases) catalyze the synthesis of purine ribonucleotides from purine bases and phosphoribosyl pyrophosphate (Neuhard and Nygaard, 1987). One of the key enzymes, hypoxanthine-

guanine phosphoribosyltransferase (HGPRTase; E.C. 2.4.2.8) is involved in salvage of preformed purine bases, namely hypoxanthine and guanine (Nygaard, 1983). Eucaryotic organisms and the majority of eubacteria contain a single protein having HGPRTase activity (Olsen and Milman 1977; Saxild and Nygaard, 1987; and Nilsson and Lauridsen, 1992). However, enteric eubacteria like *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio* sp. contain separate proteins having HPRTase and GPRTase activity (Chou and Martin, 1972; Neuhard and Nygaard, 1987; Showalter and Silverman, 1990).

Studies suggest that uptake of exogenous purine bases is tightly coupled to the activation reaction catalyzed by HGPRTase (Neuhard and Nygaard, 1987). However, other studies suggest that transport of bases may involve specific transport systems energized by proton motive force (Burton, 1977 and 1983).

Purine salvage pathways have been studied in eucaryotes (Nygaard, 1983) and eubacteria (Neuhard and Nygaard, 1987), but less is known about the archaea. It is intriguing that purine bases adenine and guanine were believed to have formed readily under conditions that existed on the Earth prior to the appearance of living things (Nygaard, 1983). The knowledge of utilization of preformed purines via salvage reactions by thermophilic archaeobacteria may lead to insight into one of the more primitive biological reactions.

Purine salvage studies are of particular interest in *Methanobacterium thermoautotrophicum* strain Marburg. This organism is a strictly anaerobic, autotrophic, methane-forming, thermophilic archaeon (Zinder, 1993). Much of methanogen biochemistry has been worked out through studies in this organism (Ferry, 1993). It has been used for genetic experiments (Worrell *et*

al., 1988), contains a small plasmid (Meile *et al.*, 1983), can be cultured easily on small and large scale, and it transports many small molecules. A purine salvage pathway has been documented in this organism, and enzymes capable of interconversion of preformed nucleobases and nucleosides to AMP, IMP, XMP, and GMP have been detected in extracts (Worrell and Nagle, 1990).

Few studies have been done on purine metabolism and salvage in archaeobacteria. Knox and Harris (1988) reported isolation and studies of spontaneous mutants of mesophilic methanogens of diverse genera (*Methanobacterium* sp. strain FR-2, *M. bryantii*, *Methanobrevibacter smithii* strain PS, and *Methanosarcina mazei* strain S-6) resistant to purine analogs. A subset of methanogens, the marine methanococci, were shown to metabolize purines. *Methanococcus vannielli* is capable of degrading purines hypoxanthine, guanine, or xanthine as the sole source of nitrogen (Demoll and Tsai, 1986a and 1986b; Demoll and Auffenberg, 1993). A related methanococcus, *Mc. voltae* was shown to transport and convert purine bases to nucleotides (Bowen and Whitman, 1987). The thermophilic *M. thermoautotrophicum* also transports and converts purine bases to nucleotides (Worrell and Nagle, 1990). This study describes the isolation and characterization of purine-analog resistant mutants of *M. thermoautotrophicum* Marburg.

MATERIALS AND METHODS

Organism and growth conditions: *M. thermoautotrophicum* Marburg (DSM 2133) was grown in a liquid mineral medium (Table 1.1; Worrell and Nagle, 1990). Solid mineral medium contained, in addition to the ingredients used for making liquid mineral medium, $CaCl_2$ (2 mL of 5% w/v) , $MgSO_4$ (0.4 mL of 25% w/v) and Gelrite gellan gum (0.8 g, Kelco, San Diego, Calif.) per 100 mL of medium. The tubes were pressurized to 205 kPa under an atmosphere of H_2-CO_2 (80:20). The plates were incubated in a pressure vessel under an atmosphere of H_2-CO_2-H_2S (19:80:1, vol/vol/vol) at 300 kPa. The cultures were grown in tubes with shaking or on solid medium in pressure vessels at 60°C.

Chemicals and isotopes: The anoxic stock solutions were filter-sterilized and stored in the dark at 5°C. 8-azaguanine (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in water with the aid of 1 N NaOH. The pH of the solution was 11.3. 8-azahypoxanthine (Sigma Chemical Co., St. Louis, MO) was dissolved in water with the aid of 3 N NaOH. The pH of the solution was 9.5. Guanine (United States Biochemical Corp., Cleveland, Ohio) was dissolved in water with the aid of 1 M KOH and the final pH was 11. Hypoxanthine (Sigma Chemical Co., St. Louis, MO) was dissolved in water with the aid of 1 N NaOH, and the final pH was 10.5. Radiochemicals {[8-^{14}C]hypoxanthine (52 mCi/mmol) and [8-^{14}C]guanine (56 mCi/mmol)} were purchased from ICN Biochemicals Inc. (Irvine, Calif.).

TABLE 1.1. The composition of liquid mineral medium

Component	Amount/liter
Balch Minerals[a]	25 mL
Trace metals[b]	10 mL
$Fe(NH_4)_2(SO_4)_2.6H_2O$ (0.2% w/v)	5 mL
L-Cysteine	0.6 g
Resazurin (0.1% w/v)	1 mL
$NiCl_2.6H_2O$ (10 mM)	0.5 mL
NH_4Cl (10% w/v)	12.5 mL
$NaHCO_3$	7.5 g
$Na_2S.9H_2O$	2 mL

[a] For composition of Balch's minerals, see Table 1.2.

[b] For composition of trace metals, see Table 1.3.

TABLE 1.2. Composition of Balch's minerals

Component	Amount (g)/250 mL
KH_2PO_4	1.5
$(NH_4)_2SO_4$	1.5
NaCl	3.0
$MgSO_4.7H_2O$	0.65
$CaCl_2.2H_2O$	0.04

TABLE 1.3. Composition of trace metals

Component	Amount (g)/L
Nitrilotriacetic acid[a]	1.5
$MgSO_4.7H_2O$	3.0
NaCl	1.0
$FeSO_4.7H_2O$	0.1
$CoCl_2$	0.1
$CaCl_2.2H_2O$	0.1
$ZnSO_4$	0.1
$CuSO_4.5H_2O$	0.01
$AlK(SO_4)_2$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01
$MnSO_4.H_2O$	0.4

[a] Nitrilotriacetate was dissolved with water and 1M KOH to a pH of 6.5 and then other metals were added to the solution. The solution was then stored at 4°C.

Table 1.4. Strains of *M. thermoautotrophicum* used in this study

Strain	Proposed Genotype[a]	Phenotype selected	Original strain, source or reference[b]
Marburg	Wild type	None	DSM 2133
MP-2	*Par*	Azaguanine resistant	This work
MP-3	*Par*	Azaguanine resistant	This work
MP-5	*Par*	Mercaptopurine resistant	This work

[a] *Par*, Purine analog resistant

[b] All mutant strains were derived from strain Marburg

Isolation of *M. thermoautotrophicum* mutants resistant to base analogs: About 4 X 10^8 wild type cells (3.6 X 10^8 CFU/mL) were plated on Gelrite solid mineral medium containing 100 µg/mL of 8-azaguanine or 300 µg/mL of 6-mercaptopurine. After 7 days, several (>100) colonies grew on these plates and two colonies were picked from 8-azaguanine plates and transferred with sterile loop to liquid mineral medium containing 100 µg/mL of 8-azaguanine. Similarly, one colony from a 6-mercaptopurine plate containing 4 colonies was picked and transferred to liquid mineral medium containing 300 µg/mL of 6-mercaptopurine. All resistant mutant strains were maintained in liquid mineral medium containing the appropriate purine analog. The rate of spontaneous mutation for 8-azaguanine and 6-mercaptopurine was detrmined to be 4.4 X 10^{-7} and 2.8 X 10^{-9} (Worrell and Nagle, 1990).

Physiological characterization of wild type and mutant strains: To study inhibitory effects of various purine analogs, cultures were inoculated and grown in duplicate liquid mineral medium tubes containing appropriate analog at final concentration of 300 µg/mL. Purine analog additions did not change the pH of the medium. Growth was monitored at 660 nm (18 mm path length) with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Tubes without any analogs served as a control. Tubes were flushed and repressurized with H_2:CO_2 (80:20) after 24 h.

Incorporation of ^{14}C-labeled purine bases: The incorporation of radiolabeled bases by the wild-type and mutant strains was studied (Bowen and Whitman, 1987). The wild-type and mutant strains (MP-2, MP-3, MP-5) were grown in liquid mineral medium containing 0.1 mM hypoxanthine or guanine and 0.05 µCi of the ^{14}C-radiolabeled purine base per mL. After 48 h, 1 mL of culture

was centrifuged in a microfuge tube at 14,000 rpm/min for 2 min. The supernatant was discarded and the pellet was washed twice with liquid mineral medium (not containing sulfide and resazurin) and suspended in 0.15 mL of liquid mineral medium. Also, 0.15 mL of whole culture was kept aside in a microfuge tube from each growth tube. To pellet and whole culture, 0.1 mL of 10 mM NaOH was added, and tubes were heated in a heat block at 95°C for 10 min. 0.25 mL of heat-treated samples were added to 2 mL of Scintiverse E (Fisher Scientific Co., Pittsburgh, Pennsylvania) and radioactivity was determined using a Liquid Scintillation Counter (LS 1701; Beckman Instruments, Inc., Fullerton, Calif.).

Preparation of crude extracts and HGPRTase enzyme assay: MP-2 and MP-3 were grown in 500 mL liquid mineral medium in 8-azaguanine (100 µg/mL). Similarly, MP-5 was grown in 100 µg/mL of 6-mercaptopurine. The cultures were grown for 48 h in H_2-CO_2 (80:20) at 140 kPa and were flushed and repressurized with H_2-CO_2 (80:20) every 6 h. After growth, cells were harvested by centrifugation at 17,000 X *g* for 20 min at 4°C. The pellet was sonicated in buffer 1 (50 mM Tris-HCl, pH 7.8, 50 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 7 mM 2-mercaptoethanol) and centrifuged in a microfuge for 5 min. The supernatant, also called crude extract, was used to measure HPRTase and GPRTase activities as described below.

For HGPRTase assay, 15 µL assay mixture contained 10 mM Tris-HCl (pH 8.4 for HPRTase or pH 7.9 for GPRTase), 0.2 mM base (hypoxanthine or guanine), 0.012 µCi of ^{14}C-base (hypoxanthine or guanine; ICN Biochemicals Inc., Irvine, Calif.), 4 mM $PRPP_i$ (Sigma Chemical Co., St. Louis, MO) and 5 mM $MgCl_2$. The reaction assay minus $PRPP_i$ served as a control. One unit of activity was equal to 1 nmol of product formed per min.

The reaction was initiated by addition of enzyme and incubation at 55°C. The radioactive products (IMP or GMP) were analyzed by spotting 2 μL assay mixture onto a PEI-cellulose thin-layer chromatography plate (EM Science, Cherry Hill, N.J.) pre-spotted with 20 nmol of a base and a nucleotide. The TLC plates were developed in 2.5 M $LiCl_2$ and allowed to dry. The R_f values for hypoxanthine, guanine, IMP, and GMP were 0.41, 0.18, 0.82, and 0.48, respectively. The separated compounds were localized under ultra-violet light and bands were cut out, placed in a scintillation vial containing 2 mL of Scintiverse E (Fisher Scientific Co., Pittsburgh, Penn.) and 0.2 mL water. The radioactivity in the vials was quantitated using a scintillation counter (Model LS 1701; Beckman Instruments, Inc., Fullerton, Calif.). Recovery of ^{14}C radioactivity spotted on TLC plates was in the range of 80-90%. Tube with no added enzyme was used as a control. The precision of replicate assays was indicated by standard deviations of 0 to 11%. Protein concentration was determined by the method of Bradford (Bradford, 1976), with bovine serum albumin as the standard.

Growth in the presence of purine bases: The wild type and mutant cells were grown in liquid mineral medium in the presence of appropriate purine bases and growth was compared to cells grown without added base. The bases, hypoxanthine and guanine, were present at a final concentration of 1 mM. The tubes were pressurized to 205 kPa with H_2:CO_2 (80:20) and growth was monitored over the period of 24 h.

RESULTS AND DISCUSSION

Growth of *M. thermoautotrophicum* strains in presence of purine analogs: When wild type cells were treated with various purine analogs, growth was inhibited to a significant extent. Table 1.5 shows the response of wild type cultures when treated with hypoxanthine, guanine, and adenine analogs. The small amount of growth observed may have been due to the use of a dense inoculum, as these compounds are known to be inhibitory to this organism (Worrell and Nagle, 1990 and unpublished results). The inhibition of wild type cultures was used as a selection to isolate *M. thermoautotrophicum* mutants resistant to 8-azaguanine (strains MP-2 and MP-3), and 6-mercaptopurine (MP-5). Characterization of these mutant strains required that they be tested for several possibilities: that they were resistant to other purine analogs, were impaired in their ability to accumulate purines, and (based on previous experience (Worrell and Nagle, 1990)) may have had altered H- or GPRTase activities. As shown in Table 1.5, MP-2 and MP-3, 8-azaguanine-resistant, grew to the same extent as untreated cultures in presence of 8-azahypoxanthine and 6-mercaptopurine. Similarly, MP-5, 6-mercaptopurine-resistant, grew to the same extent as untreated cultures in presence of 8-azaguanine and 6-thioguanine. These results clearly show that MP-2 and MP-3 mutants were cross-resistant to hypoxanthine purine base analogs, whereas the MP-5 mutant was cross-resistant to guanine purine base analogs. Similar results of cross-resistance to purine analogs with *M. thermoautotrophicum* (Worrell and Nagle, 1990) and *M. voltae* (Bowen and Whitman, 1987) mutant strains were observed. All three mutant strains isolated in this study were mildly inhibited by 8-aza-2,6-diaminopurine, an adenine analog. In contrast, Worrell and Nagle(1990) observed a higher

Table 1.5. Growth of *M. thermoautotrophicum* Marburg wild type and mutants in presence of purine analogs[a]

Strain (Phenotype selected)	Addition				
	8-AzaG	6-ThioG	8-AzaHyx	6-MP	8-Aza-2,6-DAP
	Growth (% control)				
Wild type	38	25	61	22	17
MP-2 (8-AzaG^r)	105	107	102	102	70
MP-3 (8-AzaG^r)	102	90	87	83	69
MP-5 (6-MP^r)	107	98	101	109	79

[a] Growth after 19 h, expressed as a % of that in untreated control culture (A_{660}, 0.46 to 0.52). Values are the averages of two determinations, and standard errors were in the range 0-0.03%. All analogs were present at 300 μg/mL.

degree of inhibition (18-51% growth that of control culture) of mutant strains by 8-aza-2,6-diaminopurine.

Purine incorporation by *M. thermoautotrophicum* mutant strains: The ability of purine salvage mutants to incorporate hypoxanthine and guanine during growth was also examined. The incorporation of bases in eubacteria and eucaryotes is tightly coupled to the metabolic processes that convert bases to nucleotides by phosphoribosylation (Neuhard and Nygaard, 1987). The data in Table 1.6 show that radiolabelled purine incorporation was significantly less in the mutant strains than in the wild type cultures. In the three mutants, hypoxanthine accumulation was about 6% that of wild type and guanine was about 10% that of wild type. Similar observations were made in studies with more diverse group of methanogens and eubacteria. In *M. voltae*, 8-azaguanine and 8-azahypoxanthine-resistant mutants lacked the ability to incorporate hypoxanthine and guanine (Bowen *et al.*, 1996). Additionally, 6-mercaptopurine-resistant *Methanobacterium* and *Methanobrevibacter* species, and 8-azaguanine-resistant *Methanosarcina mazei* were shown to retain reduced incorporation of hypoxanthine and guanine, respectively (Knox and Harris, 1988). Similarly 8-azaguanine-resistant *Bacillus subtilis* lacked hypoxanthine and guanine incorporation (Saxild and Nygaard, 1987), where an 8-azaguanine-resistant *S. typhimurium* (Gots *et al.*, 1972) and a mutant of *E. coli* lacking xanthine-guanine PRTase (Holden *et al.*, 1976) had reduced incorporation of guanine. Our values for purine incorporation by *M. thermoautotrophicum* wild type were somewhat lower than observed by Worrell and Nagle (1990) but comparable to values obtained for *M. voltae* (Bowen and Whitman, 1987).

Table 1.6. Incorporation of ^{14}C-hypoxanthine and ^{14}C-guanine by *M. thermoautotrophicum* Marburg wild type and mutant strains

Strain	Hypoxanthine	Guanine
Wild type	7.51[a]	4.38
MP-2	0.49	0.43
MP-3	0.46	0.49
MP-5	0.39	0.47

[a] Amount (nmol) of base incorporated/10^8 cells. Cultures were grown for 48 h in mineral medium containing 0.05 μCi of ^{14}C-radiolabeled purine base/mL. Data represents value from the single determination.

Enzyme activities in *M. thermoautotrophicum* mutant strains: When levels of HPRTase and GPRTase in extracts of the three mutant strains were tested, they were found to be markedly lower than in wild type cells. Table 1.7 summarizes the results of these assays. Wild type cells had levels of GPRTase similar to those reported previously, whereas HPRTase activity was 2-fold greater than those reported which may reflect small differences in assay conditions. Extracts of all three analog-resistant strains had measurable HPRTase, at the level of 4 to 6% of wild type activities. GPRTase activity was undetectable in two instances and *ca.* 8% of wild type level in the third strain. The results of Worrell and Nagle (1990) indicated that (with one exception, discussed below), purine analog-resistant strains retained 1.2-2.5% of wild type levels of H-and GPRTase. Since all guanine or hypoxanthine analog-resistant mutants of *M. thermoautotrophicum* (in this and previous work) were cross-resistant to analogs of the other base, markedly deficient in the accumulation of either guanine or hypoxanthine during growth, and for the most part deficient in HGPRTase, it supports the hypothesis that a single enzyme may be responsible for incorporation of both of these purines.

The exception, strain VW-106, was morphologically different from wild-type in liquid culture and did not take up labelled hypoxanthine or guanine. The strain's extracts contained wild-type levels of HPRTase and essentially no GPRTase, which could be used to suggest that both activities are not associated with a single protein. The authors suggested that a change in membrane structure may have resulted in a loss of hypoxanthine uptake but retention of HPRTase activity.

It is also possible that retention of HPRTase activity in VW-106 may have been due to the modification of the substrate specificity of enzyme activities caused by a second mutation. The strain had been maintained in the

Table 1.7. H- and GPRTase activities of *M. thermoautotrophicum* Marburg wild type and mutant strains

Strain	Enzyme activity (U/mg)[a]	
	HPRTase	GPRTase
Wild type	3.89 (0.31)	3.75 (0.18)
MP-2	0.14 (0)	0.0 (0)
MP-3	0.23 (0.29)	0.29 (0.1)
MP-5	0.22 (0.09)	0.0 (0)

[a] One unit is defined as formation of one nmol product per min. The data represent the average (± standard error) of two determinations.

Abbreviations: HPRTase, hypoxanthine phosphoribosyltransferase; GPRTase, guanine phosphoribosyltransferase.

presence of the analog for some time and could easily have undergone another mutation. Similar observations were made in a *S. typhimurium gpt*⁻ mutant (lacking GPRTase), where secondary mutation modified substrate specificity of HPRTase by allowing it to utilize guanine and hypoxanthine (Benson and Gots, 1975). Strain VW-106 is no longer available and we were not able to isolate similar mutants in our studies.

Various examples of resistance to purine analogs accompanied by a lack of the corresponding PRTase activity, and incorporation of purine bases have been reported. A 6-mercaptopurine-resistant *E. coli* (Nygaard, 1983) and 8-azaguanine-resistant *S. typhimurium* (Gots *et al.*, 1972) lacked HPRTase and XGPRTase activity, respectively. 8-azahypoxanthine-resistant *Schizosaccharomyces pombe* (Groodt *et al.*, 1971), 8-thioguanine-resistant *B. subtilis* (Endo *et al.*, 1983) lacked HGPRTase activities. Interestingly, an 8-azaguanine-resistant *B. subtilis* was shown to lack transport but retain HGPRTase activities (Saxild and Nygaard, 1987). Thus, the majority of data suggests that low HPRTase and/or GPRTase activities in these organisms resulted in reduced uptake of hypoxanthine and/or guanine. Our results are in good agreement with *M. voltae* results reported by Bowen and Whitman (1993) and Bowen *et al.* (1996). 8-azaguanine and 8-azahypoxanthine-resistant mutants of *M. voltae* lacked both H- and GPRTase activities, and were found to lack ability to incorporate hypoxanthine and guanine. It seems that deficiency of H- and GPRTase in *M. thermoautotrophicum* mutants could be sufficient to account for reduced incorporation of purine bases.

Mutant strains are normally grown and maintained in liquid mineral medium containing appropriate purine analog. MP-3 was similarly maintained in liquid mineral medium containing 8-azaguanine. An experiment was designed to see if levels of H- and GPRTase were

derepressed when MP-3 was grown without its analog, 8-azaguanine. Results in Table 1.8 show that after seven transfers without analog, levels of H- and GPRTase in MP-3 were unaltered. Addition of purine bases (hypoxanthine plus guanine) to the growth medium did not induce higher H- or GPRTase specific activities in MP-3; the strain's phenotype was stable. In similar experiments with wild type grown in the presence of exogenous purines, no change in specific activities of H- and GPRTase was seen (shown in Chapter 3). Cell extracts of MP-3 were also tested for the presence of potential inhibitor(s) of PRTases by mixing its extract with the wild type extract. As shown in Table 1.9, no effect was seen on HPRTase and GPRTase activities in wild type extract in presence of MP-3 extract. Under the experimental conditions, no inhibitors of HPRTase and GPRTase activities were detected in MP-3 extract.

We also examined effects of exogenous purines on growth of wild type and mutant strains. The presence of hypoxanthine or guanine or both in the medium had no significant effect on growth of wild type and mutant strains (Table 1.10). It can be concluded that deficiency in H- and GPRTase activities in mutant strains or presence of these activities in wild type does not have any effect on growth of these cultures.

The isolation and characterization of *M. thermoautotrophicum* mutants will contribute to the development of a genetic system in this organism. Archaea, including *M. thermoautotrophicum*, are insensitive to most antibiotics commonly used for eubacteria (Knox and Harris, 1988). However, it seems likely that *hgprt* can be used as a selectable marker in HGPRTase$^-$ *M. thermoautotrophicum* cells. The idea is to transform *M. thermoautotrophicum* HGPRTase$^-$ cells with the sequence, homologous to the endogenous target sequence to be replaced, disrupted with the *hgprt* gene.

Table 1.8. H- and GPRTase activities in *M. thermoautotrophicum* MP-3 mutant strain grown either without purine analog or with purine bases

Growth Condition	Enzyme activity (U/mg)[a, b]	
	HPRTase	GPRTase
Mineral medium[c]	0.75 (0.1)	0.37 (0)
Mineral medium + hypoxanthine (100 µg/ml) + guanine (100 µg/ml)	0.74 (0.25)	0.43 (0.2)

[a] One unit is defined as formation of one nmol product per min. The data represent the average (± standard error) of two determinations.
Abbreviations: HPRTase, hypoxanthine phosphoribosyltransferase;
GPRTase, guanine phosphoribosyltransferase.

[b] Wild type strain did not show any change in specific activity of HGPRTase when grown with purines (see Chapter 3, Table 3.2).

[c] Culture was serially transferred more than seven times in mineral medium without 8-azaguanine analog.

Table 1.9. Effect of *M. thermoautotrophicum* MP-3 crude extract on H- and GPRTase activities in wild type extract

Assay condition	HPRTase (U/mg)[a]	GPRTase (U/mg)
Wild type	6.43	7.2
MP-3	0.14	0.21
Wild type + MP-3[b]	7.06	7.3
Change from predicted sum of specific activity	7.4%	-1.5%

[a] One unit is defined as formation of nmol product per min.

[b] Wild type extract containing 41 μg total protein and MP-3 extract containing 53 μg total protein was mixed and kept on ice until the assay.

Table 1.10. Growth of *M. thermoautotrophicum* Marburg wild type and mutant strains in presence of purine bases[a]

Base (mM)	Growth (% of control)		
	Wild type	MP-3	MP-5
None	100	100	100
Hypoxanthine (4.5)	109	91	96
Guanine (0.76)	111	103	ND[b]
Hypoxanthine (4.5) + Guanine (0.76)	112	101	91

[a] Growth after 24 h, and expressed as a % of that in untreated control culture. Control cultures reached A_{660} of 0.45 to 0.58. Values are the averages of at least two determinations.

[b] ND, Not determined.

M. thermoautotrophicum HGPRTase$^-$ cells containing *hgprt* and the disrupted target sequence can be selected by inhibiting *de novo* purine nucleotide synthesis with an appropriate compound, and inclusion of purine bases hypoxanthine and guanine in the selective medium. Such selection strategies are currently used in mammalian cells for gene targeting purposes (Spring *et al.*, 1994). Purine-analog-resistant mutants (MP-2, MP-3, and MP-5) can be used for studying purine biosynthesis, for understanding purine transport, and to study biochemistry and physiology of purine salvage pathways.

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CHAPTER 2

Thermostable and thermoactive hypoxanthine and guanine phosphoribosyltransferase activities in Archaea, Eucaryota, and Eubacteria

ABSTRACT

Hypoxanthine and guanine phosphoribosyltransferase (H- and GPRTase) activities from *Methanobacterium thermoautotrophicum* Marburg, converting hypoxanthine or guanine to the respective nucleotides, IMP or GMP, were studied for temperature optima and thermal stability. The maximum H- and GPRTase activities were observed between 71 and 79°C. About 95% of maximum H- and GPRTase activities remained at 88°C. The apparent energies of activation were determined to be 12800 and 11900 cal/mol below 60°C, and 3320 and 1100 cal/mol above 60°C with hypoxanthine and guanine, respectively. In thermal stability studies, both activities from *M. thermoautotrophicum* were stable at 80°C for 10 min without 5-phosphoribosyl 1-pyrophosphate ($PRPP_i$). At 92°C, the HPRTase and GPRTase activities decayed exponentially with $t_{1/2} = 4$ min (losing 50% of both activities in 4 min). Addition of substrate $PRPP_i$ led to increased stability, with thermal half-life of the enzyme increasing to greater than 23 min. In each thermal stability study, there was a parallel loss of activity for hypoxanthine and guanine.

HGPRTase from the eucaryote Bakers' yeast (*Saccharomyces cerevisiae*), and HPRTase and GPRTase activities from the eubacterium *Escherichia coli* were also found to be highly active near or above 90°C. The yeast enzyme exhibited maximum HPRTase activity at 79°C, and retained 89% of maximum HPRTase activity at 92°C. Compared to their

activity at 37°C, *E. coli* HPRTase and GPRTase retained 96% and 105% of activities at 92°C, respectively. Our results suggest that thermal stability and thermal activity may be a common feature of HGPRTase activities from *M. thermoautotrophicum*, *E. coli*, and *S. cerevisiae*.

INTRODUCTION

Hypoxanthine- and guanine phosphoribosyltransferase (HGPRTase) activities are involved in the purine salvage pathway converting preformed nucleobases to IMP and GMP, respectively (Nygaard, 1983). This reaction requires 5-phosphorylribose-1-pyrophosphate ($PRPP_i$) as the phosphopentose donor. In eucaryotes and some eubacteria, the two activities are catalyzed by a single enzyme. However, in enteric bacteria, H- and GPRTase activities are catalyzed by two distinct enzymes (Nygaard, 1983). Studies show H- and GPRTase activities from bacterial and eucaryotic systems are unusually stable at high temperatures (Liu and Milman, 1983; Veres *et al.*, 1985).

The living world is divided into three domains: Bacteria, Archaea, and Eucarya (Woese, 1994). The structure and thermostability of H- and GPRTase in the third domain of organisms, the Archaea, is not yet clear. Purine salvage pathways have been studied in methanogenic archaea (Worrell and Nagle, 1990; Bowen *et al.*, 1996), and H- and GPRTase activities are present in the thermophilic archaeobacterium *Methanobacterium thermoautotrophicum* (Worrell and Nagle, 1990). In this report, we studied the effect of temperature on activity and stability of partially-purified H- and GPRTase activities in wild-type *M. thermoautotrophicum* Marburg. The results with these thermostable activities were suggestive about the bifunctional nature of the enzyme. There have been few reports on the effect of elevated temperature on HGPRTase activities from mesophilic organisms (Miller and Bieber, 1968; Miller *et al.*, 1972). Hence, we investigated effect of higher temperature on HGPRTase activities from Bakers' yeast *Saccharomyces cerevisiae* and *Escherichia coli*.

MATERIALS AND METHODS

Organisms: *M. thermoautotrophicum* strain Marburg was grown as described in Chapter 1. *E. coli* was grown overnight at 37°C on a rotary shaker in TSB medium containing 16 g of Bacto Tryptone, 16 g of Bacto Yeast Extract, 5 g of NaCl, 2.5 g of K_2HPO_4, and 2 g of glucose per liter. Cells were collected by centrifuging at 13,800 X *g* for 30 min and the pellet obtained was stored at -20°C until used.

Preparation of enzyme extracts: *M. thermoautotrophicum* HGPRTase was purified 100-fold on a Reactive Red Agarose column as described in Chapter 3. *E. coli* cell-free extracts or 50% ammonium sulfate pellet fraction were used to assay HPRTase and GPRTase activities. Cell-free extracts were prepared by passing cells through a chilled French pressure cell twice at 16,000 p.s.i.g. followed by centrifugation at 28,000 X *g* for 20 min at 4°C. The supernatant obtained was used to assay enzyme activities. First, streptomycin sulfate was added to 10% w/v final concentration and stirred for 20 min at 4°C. To prepare ammonium sulfate-treated extract, the extract was centrifuged at 12,100 X *g* for 20 min and to the supernatant, the appropriate amount of ammonium sulfate was added to achieve 50% saturation. The extract was stirred for 60 min at 4°C followed by centrifugation. The pellet was stored at -70°C. Before assay, pellet was dissolved in 20 mM Tris-HCl (pH 7.6) and dialyzed at 4°C for 15 h against 10 mM Tris-HCl, pH 8. Protein concentration was determined by the method of Bradford (Bradford, 1976), with bovine serum albumin as the standard. Purified yeast HGPRTase was purchased from Sigma Chemical Co. (St. Louis, MO), and assay values obtained agreed with the manufacturer's reported activity.

Enzyme assay: For HGPRTase assay, 15 μL assay mixture contained 10 mM Tris-HCl (pH 8.4 for HPRTase or pH 7.9 for GPRTase), 0.2 mM base (hypoxanthine or guanine), 0.012 μCi of ^{14}C-base (hypoxanthine or guanine; ICN Biochemicals Inc., Irvine, Calif.), 4 mM $PRPP_i$ (Sigma Chemical Co., St. Louis, MO) and 5 mM $MgCl_2$. The reaction assay minus $PRPP_i$ served as a control. One unit of activity was equal to 1 nmol of product formed per min. The reaction was initiated by addition of enzyme and incubation at appropriate temperature (the standard assay temperature for *E. coli* and yeast was 37°C, and for *M. thermoautotrophicum* was 55°C). The radioactive products (IMP or GMP) were analyzed by spotting 2 μL assay mixture onto a PEI-cellulose thin-layer chromatography plate (EM Science, Cherry Hill, N.J.) pre-spotted with 20 nmol of a base and a nucleotide. The TLC plates were developed in 2.5 M $LiCl_2$ and allowed to dry. The R_f values for hypoxanthine, guanine, IMP, and GMP were 0.41, 0.18, 0.82, and 0.48, respectively. The separated compounds were localized under ultra-violet light and bands were cut out, placed in a scintillation vial containing 2 mL of Scintiverse E (Fisher Scientific Co., Pittsburgh, Penn.) and 0.2 mL water. The radioactivity in the vials was quantitated using a scintillation counter (Model LS 1701; Beckman Instruments, Inc., Fullerton, Calif.). In assays of temperature optima of activities, about 30-55% of nucleobase substrate was utilized at the end of assay for *M. thermoautotrophicum*, yeast, and *E. coli*. Recovery of ^{14}C radioactivity spotted on TLC plates was in the range of 80-90%. Temperature stability of $PRPP_i$ under assay conditions was tested by pre-incubating a reaction mixture at 90°C for 2 min followed by addition of an enzyme and 2 h incubation at 37°C. Compared to an assay mixture in which no pre-incubation at 90°C took place, about 85% of substrate was utilized in pre-incubated assay suggesting that about 15% of $PRPP_i$ may have

been lost at 90°C in 2 min. Tubes with boiled enzyme or no added enzyme were used as controls. At high temperature (92°C) in the absence of enzyme, no IMP or GMP formation was observed. Assays at high temperature (up to 92°C) were done without covering with oil. After 2 min assay period at 92°C, about 96% of reaction assay mixture was recovered, as determined by weighing, indicating that there was no significant loss of assay mixture at high temperature. The precision of replicate assays was indicated by standard deviations of 0 to 11%.

Thermal studies: For temperature-optima studies, reaction assays were incubated at various temperatures (30 to 90°C). For *M. thermoautotrophicum* HGPRTase temperature stability studies, the enzyme was incubated at 92°C for various periods of time then assayed for both activities at standard temperature of 55°C for 2 min. To study the effect of $PRPP_i$ on stability of *M. thermoautotrophicum* HGPRTase, the enzyme fraction was incubated at 92°C in presence of 2 mM $PRPP_i$ and then sampled at specified times for assay of both H- and GPRTase at 55°C. The control experiments did not contain $PRPP_i$ during incubation at 92°C. Occasionally, TCA-precipitation was done at the end of assay to remove protein that might interfere with separation of nucleotide product.

RESULTS AND DISCUSSION

Effects of temperature on stability of *M. thermoautotrophicum* HGPRTase: When the crude extract of *M. thermoautotrophicum* was heated to 80°C for 10 min and then assayed at 55°C for H- and GPRTase activities, neither activity was lost (data not shown), although with time, precipitation of proteins in the crude extract was evident.

To study effects of temperature on stability of *M. thermoautotrophicum* H- and GPRTase, the 100-fold-purified Reactive Red Agarose enzyme preparations were subjected to heat denaturation at 89°C. The results showed that HGPRTase lost 50% of both initial activities in 6 min ($t_{1/2}$= 6 min) at 89°C, and only 33% of activities remained after 8 min (Figure 2.1). When subjected to 92°C, 50% of both initial activities remained after 4 min ($t_{1/2}$= 4 min) (Figure 2.2). However, in the presence of 2 mM $PRPP_i$, the half-life of HGPRTase denaturation was greater than 23 min. These results suggested that $PRPP_i$ protected *M. thermoautotrophicum* HGPRTase from thermal denaturation at 92°C. In another study, about 88% of enzyme activities remained when HGPRTase was heated in the presence of 2 mM $PRPP_i$ at 85°C for 25 min (data not shown). With time, some precipitation of proteins and possibly $PRPP_i$ was seen. This experiment did not include a test without $PRPP_i$; however, the appreciable stability at 85°C of both activities for at least 25 min makes it possible to consider heat treatment as one of the steps in the purification of HGPRTase from *M. thermoautotrophicum*.

Most HGPRTases from various sources have been reported to be protected by $PRPP_i$ against heat denaturation. As summarized in Table 2.1, diverse HGPRTase activities survive heat treatment with $PRPP_i$. The most heat stable enzyme, chicken brain HGPRTase, had a half-life of 30 min at

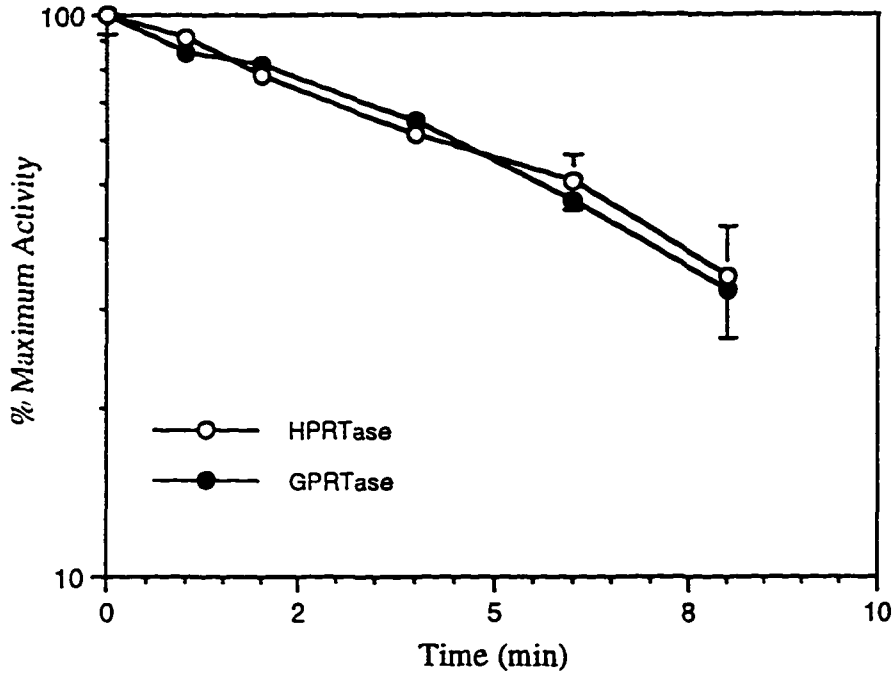

Fig. 2.1. Time course of heat inactivation at 89 °C of Reactive Red Agarose-purified HGPRTase activities from *M. thermoautotrophicum* . 4 µg protein (in 10 mM Tris-HCl, pH 7.8, 1 M NaCl and 7 mM ß-mercaptoethanol) was incubated at 89°C for time indicated and then transferred to assay vials for standard assay at 55°C. Each data point represents the mean± standard error of two determinations. Activities without preincubation were designated as 100%, representing 0.8 units (HPRTase) and 0.8 units (GPRTase).

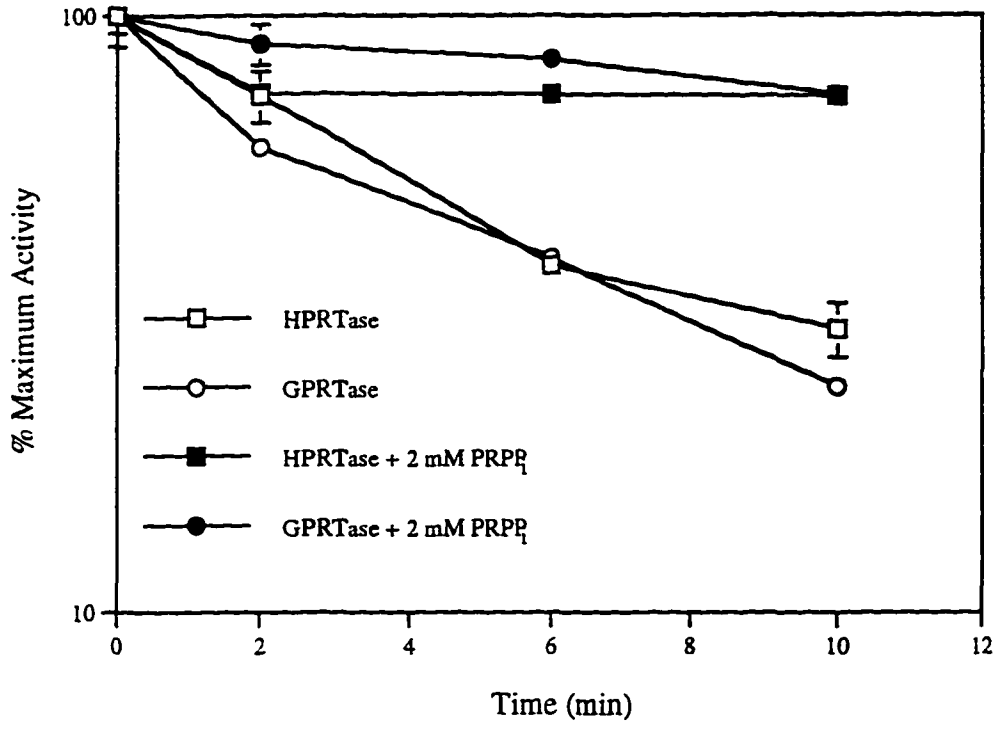

Fig. 2.2. Thermal inactivation of *M. thermoautotrophicum* HGPRTase at 92 °C. Reactive Red Agarose-purified enzyme fractions (4 μg protein) were incubated at 92 °C in the presence or absence of 2 mM $PRPP_i$. At specified times, enzyme fractions were assayed for HGPRTase activities at 55 °C. Each data point represents the mean± standard error of two determinations. Activities without preincubation were designated as 100%, representing 0.8 units (HPRTase) and 0.8 units (GPRTase).

TABLE 2.1. Thermal stability of HGPRTases

Source	Preincubation without $PRPP_i$[a]		Preincubation with $PRPP_i$[b]	
	Condition T, °C; t, min	Activity remaining (%)	Condition T, °C; t, min	Activity remaining (%)
Human erythrocyte[1]	ND[c]		65°; 10 min	70
	80°; 8 min	25	ND	
	ND		85°; 10 min	85
Chicken brain[2]	ND		85°; 30 min	50
Chinese hamster[3]	85°; 10 min	19	85°; 10 min	80
Trematode[4]	ND		85°; 10 min	90
Yeast[5]	50°; 29 min	45	ND	
	70°; 15 min	64	ND	
Crithidia HPRTase[6]	ND		55°; 10 min	70
Crithidia GPRTase[6]	ND		55°; 10 min	100
Eimeria[7]	37°; 30 min	25	ND	
Plasmodium[8]	50°; 30 s	40	50°; 30 min	90
Tritrichomonas[9]	ND		37°; 5 min	0
E. coli HPRTase[10]	60°; 10 min	20	ND	
E. coli XGPRTase[11]	65°; 2 min	50	65°; 90 min	50
M. thermoautotrophicum[12]	**80°; 10 min**	**100**	**85°; 25 min**	**88**
	92°; 4 min	**50**	**92°; 23 min**	**50**

[a] Enzyme preparations were preincubated without $PRPP_i$ at specified temperatures and time, and assayed at standard assay temperatures

[b] Enzyme preparations were preincubated with $PRPP_i$ at specified temperature and time, and assayed at standard assay temperatures

[c] Not determined.

1 Kelley et al. (1967); Muensch and Yoshida (1977); Olsen and Milman (1977)
2 Veres *et al.* (1985)
3 Olsen and Milman (1974)
4 Dovey *et al.* (1986)
5 Miller and Bieber (1968); Schmidt *et al.* (1979)
6 Kidder *et al.* (1979)
7 Wang and Simashkevich (1981)
8 Queen *et al.* (1988)
9 Beck and Wang (1993)
10 Krenitsky *et al.* (1970)
11 Liu and Milman (1983)
12 This study

85°C when $PRPP_i$ was present (Veres *et al.*, 1985). The exception, *Tritrichomonas*, had HGPRTase activities which were completely inactivated after 5 min at 37°C with $PRPP_i$ (Beck and Wang, 1993). *M. thermoautotrophicum* Marburg HGPRTase appears to be the most thermostable HGPRTase studied to date.

The parallel denaturation kinetics for *M. thermoautotrophicum* H- and GPRTase activities with or without $PRPP_i$ suggest that both enzyme activities came from the same protein. Such parallel denaturation kinetics for HGPRTase have been observed in *Schistosoma mansoni* (Dovey *et al.*, 1986), *Plasmodium falciparum* (Queen *et al.*, 1988), *Tritrichomonas foetus* (Beck and Wang, 1993) and *Lactobacillus casei* (Krenitsky *et al.*, 1970), each of which produce a single enzyme possessing both activities. On the contrary, in those organisms that produce biochemically distinct enzymes, thermal stabilities differ. *E. coli* HPRTase denatured more rapidly than its XGPRTase (xanthine-guanine phosphoribosyltransferase) (Krenitsky *et al.*, 1970). Similar observations were made with *Crithidia fasciculata*, a eucaryote that produces distinct enzymes for hypoxanthine and guanine (Kidder *et al.*, 1979). Our data show that both activities from *M. thermoautotrophicum* decreased at a similar rate with heat inactivation treatment. Additional lines of evidence also suggest that H- and GPRTase activities from *M. thermoautotrophicum* may reside on the same protein. Both activities from *M. thermoautotrophicum* were co-purified (Chapter 3). With one exception, spontaneously-occuring purine analog-resistant mutants were deficient in both H- and GPRTase activities. In Chapter 1, three such strains were isolated, and by Worrell and Nagle (1990) four such strains were characterized. An observation that can not yet be explained in the context of a bifunctional HGPRTase in *M. thermoautotrophicum* is strain VW-106,

whose extracts were deficient in GPRTase but not in HPRTase (see discussion in chapter 1; Worrell and Nagle, 1990). In the distantly related marine methanogen *Methanococcus voltae*, Bowen *et al.* (1996) suggest that more than one enzyme with overlapping substrate specificities (analogous to some eubacterial systems) might be present.

Effects of temperature on HGPRTase activities: The effects of temperature on the activity of Reactive Red Agarose-purified HGPRTase of *M. thermoautotrophicum* were investigated. The temperature/activity profile in Figure 2.3 demonstrates broad temperature optima for H- and GPRTases over the range 71-79°C. At 88°C, well above T_{opt}, 95% of maximum H- and GPRTase activities remained, whereas at 30°C, 14-17% of maximum H- and GPRTase activities were detected.

An Arrhenius plot [log V_{max} versus 1/T (°K)] was used to determine apparent energies of activation for H- and GPRTase of *M. thermoautotrophicum*. As shown in Figure 2.4, a biphasic profile was obtained with a change in slope at about 60°C. Over the range 30-55°C, the apparent energy of activation (E_a) was 12.8 and 11.9 Kcal mol^{-1} with hypoxanthine and guanine as substrate, respectively. Above 58°C, the apparent respective E_a values were 3.3 and 1.1 Kcal mol^{-1}. These values are in line with those reported in other systems. Similarly, heat-treated Reactive Red fraction retained near 100% of maximum H- and GPRTase activities at 92°C (data not shown). *M. thermoautotrophicum* is a moderately thermophilic methanogen that has a T_{opt} for growth of 65°C (Brandis *et al.*, 1981), and appears to have a thermoactive HGPRTase with 95% of maximum activities remaining at 88°C. Temperature effects on HGPRTase activities

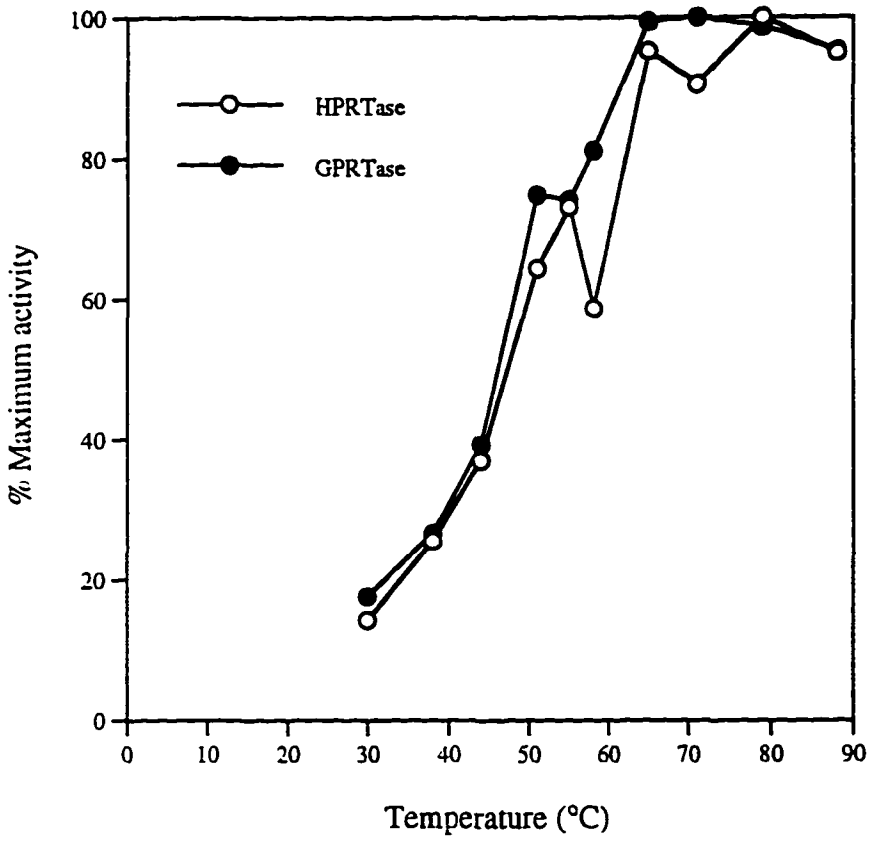

Fig. 2.3. Temperature dependence of the rate of IMP and GMP formation by Reactive Red Agarose-purified HGPRTase from *M. thermoautotrophicum* . Each assay contained 0.84 μg of protein. Maximum activity was 1.1 μmoles/min/mg for IMP formation and 1.3 μmoles/min/mg for GMP formation.

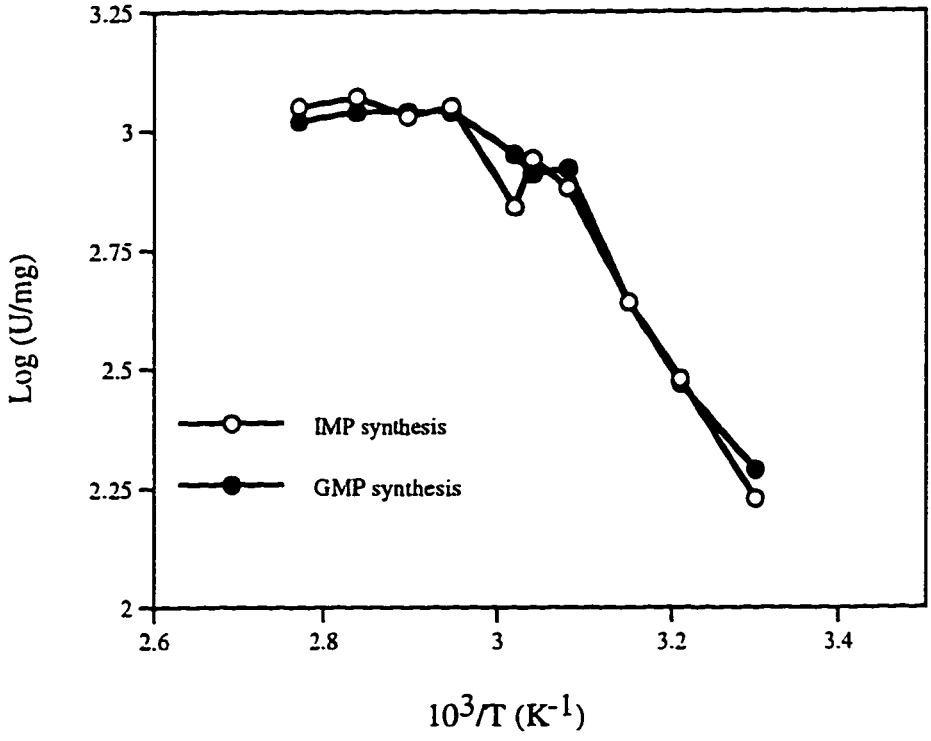

Fig. 2.4. Arrhenius plot for the effect of temperature on rate of IMP and GMP formation by Reactive Red Agarose-purified HGPRTase from *M. thermoautotrophicum* . Data points represent specific activities (U/mg) and temperature values from fig. 2.3.

from mesophilic organisms have been studied at temperatures up to 50°C. The bifunctional Brewers yeast HGPRTase was assayed by Miller and Bieber (1968 and 1969) from 0°C to 50°C. An Arrhenius plot of HPRTase data yielded a single E_a of 11.6 Kcal mol^{-1}, whereas GPRTase activity was biphasic with E_a of 5.7 Kcal mol^{-1} below 19°C and 11.3 Kcal mol^{-1} between 19°C and 50°C. In Ehrlich ascites cell extracts, above 23°C the E_a for GPRTase was 13 Kcal mol^{-1} (below 23°C, competing activities interfered with the Arrhenius plot analysis) (Murray, 1967). In *E. coli*, the substrate specific GPRTase had an apparent E_a below 23°C of 12.8 Kcal mol^{-1}, and between 23°C and 50°C the E_a value was 3.37 Kcal mol^{-1} (Miller *et al.*, 1972). The occurrence of a biphasic Arrhenius plot with guanine as a substrate was common in reported data with *E. coli*, Brewers yeast, and Ehrlich ascites cells. We observed a biphasic Arrhenius plot for *M. thermoautotrophicum* HGPRTase with break with change in slope at 55°C. The different energies of activation below and above 55°C may indicate a conformational change in the *M. thermoautotrophicum* HGPRTase protein near this temperature. The absence of a sudden drop in the Arrhenius plot, similar to Bakers' yeast HGPRTase (this study; discussed below), also suggest that methanogen enzyme had not become inactivated between 58°C and 88°C.

Our results indicating that *M. thermoautotrophicum* HGPRTase was active at temperatures far above its growth optimum, and the apparent complete retention of activity up to 50°C by H- and GPRTases from other organisms led us to test the enzymes from two mesophilic sources at significantly elevated temperatures (up to 90°C). HPRTase activity of the bifunctional HGPRTase from Bakers yeast (*S. cerevisiae*) was more active at 69°C and 79°C than it was at 37°C, being stimulated 1.6-fold at 79°C (Fig.

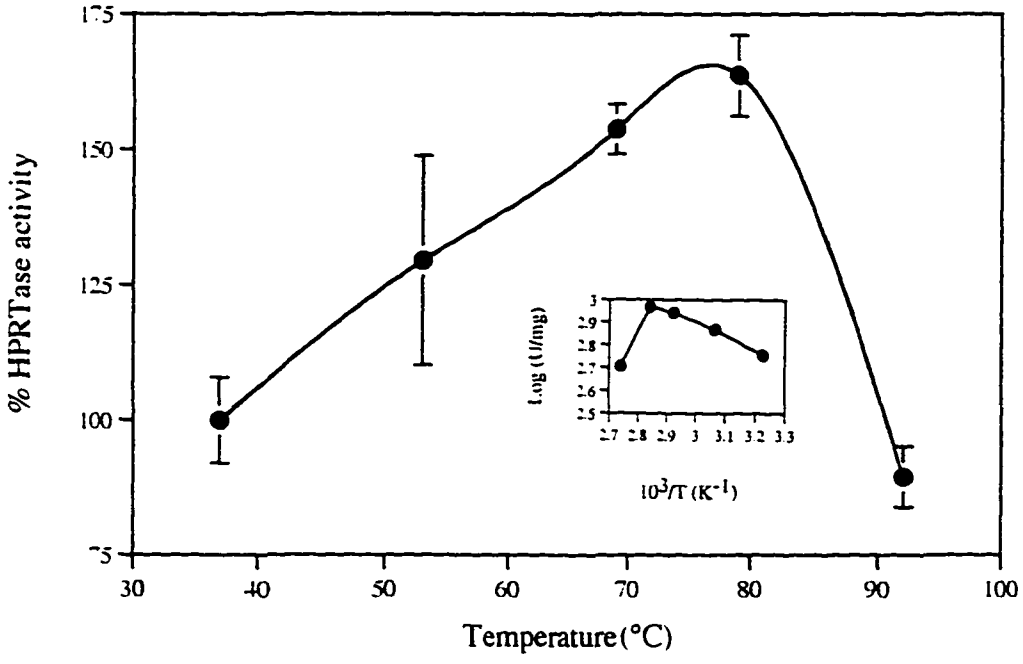

Fig. 2.5. Temperature dependence of the rate of IMP formation by HGPRTase from Baker's Yeast. Each assay contained 0.96 μg of protein. 100% activity was 0.5 nmoles/min for IMP formation. Each data point represents the mean $\pm$ standard error of two determinations. Inset, Arrhenius plot of the same data.

Table 2.2. Comparison of HGPRTase activities at elevated temperatures

Organism (Domain)	T_{opt} growth (°C)	Assay temperature (°C)		Activity at elevated temperature[a] (% of that at normal temperature)	
		normal	elevated	HPRTase	GPRTase
M. thermoautotrophicum (Archaea)	65	55	88	120	120
S. cerevisiae (Eucarya)	30	37	79	164	ND[b]
			92	89	ND
E. coli (Eubacteria)	37	37	92	136	105

[a] Activities at normal temperature for enzyme preparations from the three organisms: *M. thermoautotrophicum* H- and GPRTase activities were 0.7 and 0.9 nmol/min, respectively; *S. cerevisiae* HPRTase activity was 0.7 nmol/min; and *E. coli* H- and GPRTase activities were 1 and 0.5 nmol/min, respectively.

[b] Not determined.

2.5 and Table 2.2). Figure 2.5 (inset) is an Arrhenius plot of temperature/activity profile of HPRTase from Bakers' yeast. The Arrhenius plot was biphasic, and the apparent E_a values were 2.6 Kcal mol^{-1} between 37°C and 79°C, and -11.8 Kcal mol^{-1} between 79°C and 92°C. The drop in the plot at 79°C may indicate denaturation of Bakers' yeast HGPRTase. In our studies, we did not determine effects of temperature on Bakers' yeast GPRTase activity.

For the mesophilic eubacterium *E. coli*, which has monofunctional H- and GPRTases, crude extracts were tested for both activities. A complete temperature/activity profile could not be carried out in these extracts at moderate temperatures (50°C to 80°C), since phosphatase(s) removed nucleotide product as it was formed in this temperature region (data not shown). At highly elevated temperatures, phosphatase activities did not interfere, and as shown in Table 2.2, at 92°C *E. coli* H- and GPRTase were stimulated 1.36 and 1.05-fold over their activities at 37°C. The result with *E. coli* HPRTase was confirmed with an ammonium sulfate-precipitated fraction; at 93°C the preparation retained 96% of the activity assayed at 37°C. The results with Bakers' yeast and *E. coli* are unexpected since the T_{opt} for growth of Bakers' yeast and *E. coli* is 30°C and 37°C, respectively.

Thermal activity of some enzymes from mesophilic organisms has been reported. Recent examples include β-galactosidase from *Sterigmatomyces elviae* (Onishi and Tanaka, 1995), amidase from *Klebsiella pneumoniae* (Nawaz *et al.*, 1996), and NADP-isocitrate dehydrogenase from *Cephalosporium acremonium* (Olano *et al.*, 1995). HGPRTase has long been known to be thermostable (Table 2.1), and as reported here, enzymes from mesophiles are highly thermoactive. The results suggest that thermal stability and activity at elevated temperatures of HGPRTases may be an

intrinsic property of these enzymes regardless of the source. It is noteworthy that hyperthermophiles are represented phylogenetically in the deepest branches of the lineages of *Bacteria*, *Eucarya*, and *Archaea* (Stetter, 1996), and thermal properties of HGPRTases may have been conserved in these organisms.

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CHAPTER 3

Purification and properties of hypoxanthine-guanine phosphoribosyltransferase enzyme from *Methanobacterium thermoautotrophicum*

ABSTRACT

The H- and GPRTase activities (HGPRTase; E.C. 2.4.2.8) from a moderately thermophilic, methanogenic archaeon *Methanobacterium thermoautotrophicum* Marburg have been purified using ammonium sulfate precipitation, hydroxylapatite chromatography, QAE-Sephadex A-50 chromatography, Reactive-Red Agarose-120 chromatography, and heat step (85°C for 30 min). The specific activities of the purified H- and GPRTase fraction were 1200-fold greater than those in the crude extract. H- and GPRTase activities coeluted in all chromatography steps and resisted heat inactivation to the same extent, suggesting that they are associated with the same enzyme protein. On Sephadex G-150 chromatography, 100-fold-purified HGPRTase eluted over a broad molecular weight range (27,000 to more than 100,000 Da), suggesting that the enzyme was dissociating into subunits. The molecular mass of the subunit estimated from sodium dodecyl sulfate polyacrylamide gel electrophoresis was 28,000. The specific activities of the PRTases were not affected by the growth in medium containing nucleobases, however acetate led to a two-fold increase in specific activities with both hypoxanthine and guanine.

The pH activity profile of HGPRTase exhibited a broad pH range for activity with pH optimum of 9.9. The enzyme followed a Michaelis-Menten kinetics with substrates hypoxanthine, guanine, and $PRPP_i$. The apparent K_m values were 6.1 μM (hypoxanthine), 38.7 μM (guanine), and 86.3 μM ($PRPP_i$

in presence of hypoxanthine). Both activities were stimulated by Mg^{2+}, Mn^{2+}, and Zn^{2+}. Guanine inhibited HPRTase activity at low hypoxanthine concentrations. H- and GPRTase activities were inhibited by IMP and PP_i, but unaffected by GMP, AMP, adenine, and xanthine. H- and GPRTase activities were inhibited by 8-azahypoxanthine and 6-mercaptopurine base analogs.

INTRODUCTION

Hypoxanthine- and guanine phosphoribosyltransferase (HGPRTase), a purine salvage enzyme, catalyzes the condensation of 5-phosphoribosyl-1-pyrophosphate ($PRPP_i$) with either hypoxanthine or guanine to yield the corresponding purine mononucleotide. H- and GPRTase activities are present in the archaeon *Methanobacterium thermoautotrophicum* (Worrell and Nagle, 1990). In this organism, the evidence suggests that both of these activities are found on same enzyme. First, parallel losses in H- and GPRTase activity were observed in thermal denaturation studies (Chapter 2). Second, hypoxanthine and guanine were not incorporated from the medium by purine-analog-resistant strains. Third, extracts of these spontaneously resistant strains were deficient in both H- and GPRTase activity (Chapter 1; Worrell and Nagle, 1990).

M. thermoautotrophicum is a member of the domain Archaea, which together with Eucaryota and Eubacteria makes up the three-domain organizational system of organisms proposed by Woese and colleagues (Woese, 1994). Comparisons of biological properties in different organisms can be useful in understanding the relationships among these three groups. In the case of nucleobase metabolism, most organisms possess both the *de novo* pathway and salvage pathways for the synthesis of purine nucleotides. The *de novo* pathway for synthesis of purine nucleotides appears to be the same in all organisms, whereas salvage pathways seem far more diverse in their nature and distribution (Nygaard, 1983).

Hypoxanthine and guanine salvage is an example of such diversity. Eucaryotes and many of the eubacteria contain a single enzyme for both hypoxanthine and guanine utilization, whereas enteric bacteria contain two separate enzymes. Examples of eucaryotic cells with a bifunctional enzyme

to convert hypoxanthine and guanine to their respective nucleotides include human (Holden and Kelley, 1978; Olsen and Milman, 1978), mouse (Hughes *et al.*, 1975), hamster (Olsen and Milman, 1978), trematode (Dovey *et al.*, 1986), yeast (Nagy and Ribet, 1977; Schmidt *et al.*, 1979; Nussbaum and Caskey, 1981), and parasitic protozoa (Tuttle and Krenitsky, 1980; Aldritt and Wang, 1986; Schimandle *et al.*, 1987; Queen *et al.*, 1988; Allen *et al.*, 1989; Beck and Wang, 1993). Examples of eubacteria with a bifunctional enzyme for hypoxanthine and guanine include *Bacillus subtilis* (Endo *et al.*, 1983), *Lactococcus lactis* (Nilsson and Lauridsen, 1992), and *Rhodobacter capsulatus* (Beckman and Kranz, 1991).

In contrast, enteric bacteria, notably *Escherichia coli* (Martin and Yang, 1972), *Salmonella typhimurium* (Hochstadt, 1978) and *Vibrio harveyi* (Showalter and Silverman, 1990) contain separate enzymes for the utilization of hypoxanthine and guanine, respectively. H- and GPRTase activities from *E. coli* and *S. typhimurium* were resolved into two fractions by means of chromatography, and comparison of kinetic responses confirmed the conclusion that two separate enzymes exist in these organisms.

There are some differences in substrate specificity among eucaryotic and eubacterial HGPRTase enzymes. In many protozoa, in addition to hypoxanthine and guanine, xanthine also serves as a substrate for the bifunctional HGPRTase enzyme (Queen *et al.*, 1988; Beck and Wang, 1993). In contrast, human HGPRTase is unable to recognize xanthine as a substrate (Krenitsky *et al.*, 1969; Muensch and Yoshida, 1977). In *E. coli* and *S. typhimurium*, HPRTase (hypoxanthine phosphoribosyltransferase) and XGPRTase (xanthine-guanine phosphoribosyltransferase) display cross-activity if other purine substrate is provided at high enough concentrations (Nygaard, 1983). In the enteric eubacteria, HPRTase exhibits a pronounced

kinetic preference for hypoxanthine, while XGPRTase will utilize guanine or xanthine (Nygaard, 1983). To date, however, although substrate utilization patterns may differ, H- and GPRTases from different organisms share some properties which include a requirement for magnesium ion for activity, stabilization of activity by $PRPP_i$, and thermal stability (the latter property is described in detail in Chapter 2).

The archaea represent organisms that are as distantly related to eubacteria as they are to eucaryotes (Keeling and Doolittle, 1995). In fact, numerous archaeal biological properties led to the hypothesis that the archaea are on the same line of descent that gave rise to the eucaryotic nucleus. Nucleobase salvage reactions have some appeal for the possibility of providing comparative information about the three domains. Purines were likely to exist on Earth prior to the appearance of living organisms; mononucleotide synthesis from purines may represent one of the more primitive biological reactions (Nygaard, 1983). As noted in Chapter 1, there have been several studies on purine analog susceptibility in methanogenic archaea. In one case, *Methanococcus voltae*, Bowen *et al.* (1996) reported properties of H- and GPRTase but were not able to determine if one or more than one enzyme was present in this organism. In the moderate thermophile, *M. thermoautotrophicum* strain Marburg, preliminary results given in Chapters 1 and 2 suggest that a single HGPRTase enzyme is responsible for hypoxanthine and guanine conversion to nucleotides.

In this chapter, purification and characterization of an apparently bifunctional H- and GPRTase from *M. thermoautotrophicum* is reported. This will significantly contribute to our knowledge of HGPRTase and purine salvage in this primitive thermophilic methanogenic archaeon. Studies of its structure and biochemical properties will permit comparison to known

enzymes from eucaryotes and eubacteria. The complete sequence of an archaeal genome, the hyperthermophile *Methanococcus jannaschii*, (Bult *et al.*, 1996) and ongoing sequencing of *M. thermoautotrophicum* (Morell, 1996) will provide a context for the purine salvage pathways results.

MATERIALS AND METHODS

Bacterium and growth conditions: *M. thermoautotrophicum* Marburg was grown in a 100L fermentor containing mineral medium (Chapter 1) and 5 mM sodium acetate at 60°C under an atmosphere of H_2:CO_2. About 3.9 g L^{-1} of *M. thermoautotrophicum* cells were obtained after 85 h of cultivation. After harvest, the cells were stored in 20 mM Tris-HCl buffer (pH 7.6) under an atmosphere of H_2 at -20°C.

HGPRTase assay: The H- and GPRTase activities were determined by measuring the formation of radiolabeled nucleotide from the radiolabeled base substrate and $PRPP_i$ as described before (Worrell, 1991). Unless otherwise noted, the total volume of the reaction mixture was 15 µL. The reaction mixture contained 50 mM Tris-HCl (pH 8.4 for HPRTase; pH 7.9 for GPRTase), 5 mM $MgCl_2$, 4 mM $PRPP_i$, 0.2 mM unlabeled base (hypoxanthine or guanine), and [8-^{14}C]purine base (hypoxanthine or guanine). [^{14}C]Hypoxanthine (52 mCi/mmol) and [^{14}C]guanine (56 mCi/mmol) were purchased from ICN Biomedicals, Inc. (Irvine, Calif.). The radioactive substrates were present at a final specific radioactivity of 1.3 mCi/mmol. $PRPP_i$ (Sodium salt; Sigma Chemical Co., St. Louis, MO) was prepared fresh each time in buffer 1 (Table 3.1). The assay mixture was prewarmed for 2 min at 55°C, and the reaction was initiated by addition of the enzyme. The assays were incubated at 55°C for 2-4 min, and the radiolabeled bases and nucleotide products were analyzed by spotting 2 µL of the reaction mixture on a PEI-cellulose thin-layer chromatography plates (EM Science, Cherry Hill, N.J.) prespotted with 20 nmoles of appropriate base and nucleotide. The spotted plate was developed in ascending direction in 2.5 M LiCl solvent.

The plate was allowed to dry, and separated base and mononucleotide product were visualized under ultra-violet light. The R_f values for hypoxanthine, guanine, IMP, and GMP were 0.41, 0.18, 0.82, and 0.48, respectively. The spots were cut out and placed in a scintillation vial containing 2 mL of Scintiverse E (Fisher Scientific Co., Pittsburgh, Pennsylvania) and 0.2 mL water. The radioactivity in the vials was measured using a Beckman LS scintillation counter (LS 1701; Beckman Instruments, Inc., Fullerton, Calif.). The HGPRTase enzyme assay was $PRPP_i$-dependent, and linear with time and concentration of protein. The precision of the assay was determined to be S.D. $\pm$ 0 to 0.17 U/mg for both activities. The accuracy of the assay, as determined with commercial yeast HGPRTase (Sigma Chemical Co., St. Louis, MO), was determined to be near 100% (1 U of commercial enzyme produced one nmol IMP per min).

Precipitation of H- and GPRTase activities with ammonium sulfate: A pilot scale ammonium sulfate fractionation was done to achieve maximum precipitation of H- and GPRTase activities from the streptomycin sulfate-treated crude extract of *M. thermoautotrophicum*. 15 mL of this extract was divided into three fractions. Solid, ground ammonium sulfate was added slowly at 4°C to each fraction to obtain the desired partial saturation. Once all salt dissolved, the solution was stirred for 15 min followed by centrifugation at 28,000 X *g* for 20 min. The precipitate and supernatant obtained were dialyzed against buffer 2 for 24 h and assayed for H- and GPRTase activities.

Agarose-GMP affinity chromatography: 1 mL agarose-GMP (3.7 µmoles of 5'-GMP insolubilized on beaded 4% agarose, Sigma Chemical Co., St. Louis,

Table 3.1. Buffer compositions

Buffer	Components[a]
Buffer 1	Tris-HCl (50), KCl (50), $MgCl_2$ (6), EDTA (0.1), 2-Mercaptoethanol (7), pH 7.8
Buffer 2	Tris-HCl (20), 2-Mercaptoethanol (7), pH 8.0
Buffer 3	Tris-HCl (10), $MgCl_2$ (10), EDTA (0.1), 2 Mercaptoethanol (7), pH 7.8
Buffer C	Tris-HCl (50), KCl (25), $MgCl_2$ (10), Dithioerythritol (1), pH 7.4
Gel filtration buffer	Tris-HCl (50), KCl (100), pH 7.5
SDS sample buffer	Trizma-HCl (62.5), SDS (2% w/v), 2-Mercaptoethanol (5% v/v), glycerol (10% v/v), bromophenol blue (0.001% w/v), pH 6.75
SDS tank buffer	Tris (25), glycine (192), SDS (1% w/v)
Electroblotting transfer buffer	Tris (25), glycine (192), MeOH (20% v/v),

[a] Unless otherwise noted, numbers in parentheses represent millimolar concentrations.

MO) was equilibrated with buffer C (see Table 3.1) before application of the sample. After the sample was applied, the column was washed with buffer C. To elute H- and GPRTase, the column was washed with buffer C containing 5 mM GMP as described by Schmidt *et al.* (1979). Finally, the column was washed with 2 M NaCl to remove any residual bound protein.

Purification of H- and GPRTase activities from *M. thermoautotrophicum*:
An outline of the purification scheme for *M. thermoautotrophicum* HGPRTase is shown in Figure 3.1. For buffer compositions, see Table 3.1.

Step 1: Preparation of crude extract: About 227 g of *M. thermoautotrophicum* cells were used for purification scale up. Cells were suspended in 225 mL of buffer 1. Aerobic conditions were used. For enzyme purification, the cells were passed through a chilled French Pressure Cell twice at 16,000 p.s.i.g. The broken cells were centrifuged at 28,000 X *g* for 20 min at 4°C.

Step 2: Streptomycin sulfate precipitation: The pellet from step 1 was discarded, and to the supernatant (crude extract), solid streptomycin sulfate was added to a final concentration of 20 mg/mL. After stirring for 20 min at 4°C, the crude extract was centrifuged at 28,000 X *g* for 20 min at 4°C.

Step 3: 65-100% ammonium sulfate precipitation: To the supernatant from step 2, ground ammonium sulfate was added to achieve 65% saturation, and the mixture was stirred for 30 min at 4°C. The 65%-saturated ammonium sulfate fraction was centrifuged at 28,000 X *g* for 20 min at 4°C. The pellet was discarded, and to the supernatant, more ground ammonium sulfate was

Fig 3.1. Protocol for purification of HGPRTase from *M. thermoautotrophicum*

M. thermoautotrophicum cells grown on H_2:CO_2 + acetate

↓

Harvest aerobically and pass through French Press twice @ 16,000 p.s.i.g.

↓

Cell-free extract

↓

$(NH_4)_2SO_4$ Fractionation
65% saturation

Pellet ← ↓

Supernatant

↓

100% saturation

↓

Pellet

↓

Hydroxylapatite chromatography

↓

Gradient to 0.5 M KH_2PO_4

↓

QAE-Sephadex A-50 chromatography

↓

Gradient to 0.9 M KCl

↓

Reactive-Red Agarose-120 chromatography

↓

Gradient to 1.75 M NaCl

↓

Peak fractions were collected and stored at -20°C

↓

Heat treatment at 85°C for 30 min in presence of 2 mM $PRPP_i$,

supernatant containing H- and GPRTase activity stored at -20°C

added to achieve 100% saturation. This solution was stirred for 30 min at 4°C, and centrifuged as before to collect the pellet. The pellet was dissolved in buffer 1 and stored at -70°C until use.

Step 4: Hydroxylapatite chromatography: The redissolved undialyzed 65-100% ammonium sulfate pellet from step 3 was loaded on a Bio-Gel HTP hydroxylapatite column (Bio-Rad Laboratories, Richmond, CA; 2.6 X 32 cm column with 200 mL bed volume) equilibrated with buffer 3. After application, a 600 mL gradient from 0 to 0.5 M KH_2PO_4 was started at a flow rate of 75 mL/h. Fractions of 10 mL volume were collected, and those containing the majority of H- and GPRTase activities (coeluted at 0.16 to 0.42 M KH_2PO_4) were pooled. Pooled fractions 19-32 (10 mL each) contained 140 mL total volume.

Step 5: QAE-Sephadex A-50 chromatography: The pooled H- and GPRTase fractions from the hydroxylapatite column were dialyzed against buffer 2, and 160 mL fraction was applied to a QAE-Sephadex A-50 column (2.6 X 12 cm column with 65 mL bed volume) equilibrated with buffer 3. H- and GPRTase activities were eluted with a 300 mL gradient from 0 to 1 M KCl in buffer 3 at a flow rate 60 mL/h. Fractions containing the majority of H- and GPRTase activites (coeluted at 0.55 to 0.86 M KCl) were pooled. Pooled fractions 13-26 (10 mL each) contained 140 mL total volume.

Step 6: Reactive Red Agarose-120 chromatography: The pooled H- and GPRTase fractions from the QAE-Sephadex A-50 column were dialyzed against buffer 2, and 50 mL concentrated fraction applied to a Reactive Red Agarose-120 column (Sigma Chemical Co., St. Louis, MO; 1 X 43 cm

column with 33 mL bed volume) equilibrated with buffer 3. H- and GPRTase activities were eluted with a 200 mL gradient from 0.2 to 1.5 M NaCl in buffer 3 at a flow rate 30 mL/h. Fractions containing the majority of H- and GPRTase activities (coeleuting between 0.9 to 1.2 M NaCl) were pooled. Pooled fractions 9-15 (5 mL each) contained 35 mL total volume.

Step 7: Heat step: The pooled H- and GPRTase fractions from the Reactive Red Agarose column were heated at 85°C for 30 min in the presence of 2 mM $PRPP_i$. The heated fraction was centrifuged in an airfuge (50,000x *g*; Beckman Instruments, Inc., Fullerton, Calif.) at high speed for 30 min. After centrifugation, the pellet obtained was discarded and supernatant containing H- and GPRTase activities was stored at -20°C without glycerol.

Activity stain of HGPRTase: An activity stain for *M. thermoautotrophicum* HGPRTase was attempted as described by Vasquez and Bieber (1978). For activity detection of H- and GPRTase activities of *M. thermoautotrophicum*, 7.5% acrylamide gels were run and equilibrated in 0.2 M Tris-HCl (pH 7.4) for 15 min. After equilibration, slab gel or lanes were immersed in reaction mixture in a petri plate containing 50 mM Tris-HCl (pH 7.4), 0.15 mM hypoxanthine or guanine, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.45 mM $PRPP_i$. After incubation for 1 h at 55°C, gels were transferred to precipitation solution (0.01 M $MnCl_2$ in 0.02 M succinate, pH 6) and observed for appearance of precipitation bands. Controls included no enzyme in the lane or no base or no $PRPP_i$ in the reaction mixture.

In an alternate method for detection of activity in gels, gel lanes containing *M. thermoautotrophicum* H- and GPRTase were cut into 2-mm slices, transferred into microfuge tubes containing the 150 µL of appropriate

assay mixture (same as described for HGPRTase assay except [8-^{14}C]purine base was at a final specific radioactivity of 0.07 mCi/mmol). The HPRTase and GPRTase activity was determined after 18 h of incubation at 55°C by spotting 2 µL of the assay mixture on PEI-cellulose TLC plate followed by separation and detection of radioactive nucleotides as described above.

Molecular Sieve Chromatography: To determine whether H- and GPRTase activities in *M. thermoautotrophicum* are associated with a single protein, and to determine the molecular mass, gel filtration was performed using Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, CA; 5000-100000 Dalton fractionation range; 1.4 X 62 cm column). The column was equilibrated with gel filtration buffer at room temperature, and the flow rate was 8.5 mL/h. The molecular weight standards (Sigma Chemical Co., St. Louis, MO) used to calibrate the column were as follows: cytochrome *c* (M_r 12,384), and Vitamin B_{12} (M_r 1,350). The elution of Blue Dextran 2000, standard proteins (V_e), and vitamin B_{12} were followed by absorbance readings at 280 nm. The void volume (V_0) determined by the elution of Blue Dextran 2000 was 21.6 mL. Freshly prepared crude extract (2 mL) of *M. thermoautotrophicum* containing 5% (v/v) glycerol was applied to the column. 1.2-mL fractions were collected using a microfractionator (Gilson), and the elution volume for H- and GPRTase was determined by assaying enzyme activities as before. A standard curve of logarithm of known molecular weights of standard proteins versus V_e/V_0 was used to calculate molecular mass of H- and GPRTase in crude extract of *M. thermoautotrophicum*.

In another experiment, 120 µg of highly purified enzyme fraction containing 5% (v/v) glycerol from Reactive Red column was chromatographed on Sephadex G-150 (Pharmacia Fine Chemicals Inc.,

Piscataway, NJ; 5000-300000 Dalton fractionation range; 1.5 X 26 cm column) equilibrated with gel filtration buffer at room temperature. The column was calibrated with standards β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000), cytochrome *c* (M_r 12,500), and vitamin B_{12} (M_r 1,350). The flow rate was 20 mL/h and 0.5-1.0 mL fractions were collected for enzyme assay as before.

Polyacrylamide gel electrophoresis: Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to determine the subunit molecular weight and purity of the H- and GPRTase. Electrophoresis was run on slab gels [10% w/v polyacrylamide (made from 19:1 w/w acrylamide/bisacrylamide) with stacking gel (5% polyacrylamide)]. Both stacking and resolving gel for SDS and native gel were prepared in 125 mM Tris-HCl (pH 6.8) and 380 mM Tris-HCl (pH 8.8). Samples were boiled for 1-2 min in SDS sample buffer and electrophoresed at 10-15 mA using SDS tank buffer. Carbonic anhydrase (M_r 29,000), egg albumin (M_r 45,000), bovine albumin (M_r 66,000), phosphorylase *b* (M_r 97,400), β-galactosidase (M_r 116,000), and myosin (M_r 205,000) were used as standards. Proteins on the gel were stained with Coomassie brilliant blue R-250. The molecular weights were determined from a standard curve for the six proteins run on the same slab gel.

Native gel electrophoresis was performed with a stacking gel (5% polyacrylamide) and resolving gel (10% polyacrylamide) with bromphenol blue as the tracking dye. The proteins were electrophoresed using Tris-glycine (pH 8.5). The gels were run at 10-15 mA for 2 h and stained with Coomassie brilliant blue R-250 for proteins.

Kinetic studies: Enzyme kinetics were performed with the Reactive Red Agarose-purified enzyme preparation in a buffer of 50 mM Tris-HCl, 5 mM $MgCl_2$, 4 mM $PRPP_i$, at pH 8.4, with hypoxanthine or pH 7.9 with guanine. Radiolabeled [^{14}C]nucleobase and increasing concentrations of the non-radiolabeled nucleobase substrate were present. For determination of K_m values for the purines, hypoxanthine and guanine were varied over the range 13.3 to 320 μM. When determining the apparent K_m of $PRPP_i$, this substrate was present at concentrations over the range 6.25 to 400 μM. The concentration of hypoxanthine in the latter experiment was 0.2 mM.

pH optimum: Enzymatic activity was monitored by the production of IMP from [^{14}C]hypoxanthine and GMP from [^{14}C]guanine. The buffer mixture consisted of 16 mM each of Bis-Tris Propane, CAPS (3[cyclohexylamino]-1-propanesulfonic acid), and TES (*N*-tris-[hydroxymethyl]-methyl-2-aminoethane sulfonic acid) over the pH range of 6.3-10.6. Buffer pH was adjusted at room temperature and reported for room temperature.

Inhibition of HPRTase activity by guanine, IMP and PP_i: Inhibition studies were performed with Reactive Red Agarose-purified H- and GPRTase fraction. Hypoxanthine was the substrate, and guanine, IMP or PP_i was putative inhibitor. For guanine inhibition, radiolabelled hypoxanthine concentrations of 100, 200, or 300 μM were challenged with 0, 130, 270, 400, and 830 μM guanine and IMP formation was measured. For IMP (2 mM) or PP_i (2 mM) inhibition studies, the assay mixture contained 0.2 mM hypoxanthine and $PRPP_i$ concentrations were varied over the range 60-300 μM.

NH_2-terminal sequence determination: For NH_2-terminal sequence of *M. thermoautotrophicum* HGPRTase, protein was separated using an SDS-PAGE minigel (9.5 cm X 6.5 cm X 0.5 mm) as described before. After electrophoresis, proteins were transferred onto a PVDF-plus membrane (Micron Separations Inc., Westborough, MA) in a electroblotting transfer buffer at 70 mA (5V) for 5 h. After electroblotting, the membrane was stained with Coomassie brilliant blue R-250 (0.1% Coomassie brilliant blue R-250 in 1% (v/v) acetic acid and 40% (v/v) methanol) for 10-15 min. The membrane was then destained with 50% methanol-10% acetic acid for 15 min. The membrane was allowed to air dry and stored at -20°C before sequencing. The NH_2-terminal sequencing was performed by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center. Analyses of amino acid sequences were performed on a VAX using OUGCG and BLAST database program and PileUp program.

Protein and conductivity measurements: Protein concentrations were measured by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Protein concentrations in the heat-treated fractions were estimated by visual comparison with protein standards on gels. Conductivity was measured using an analyzer (Markson Science Inc., CA).

RESULTS AND DISCUSSION

We have studied effects of purines and acetate on levels of HGPRTase in *M. thermoautotrophicum* by growing it in the presence of exogenous purines in combination or acetate (Table 3.2). The H- or GPRTase activities were the same in extracts prepared from cells grown in mineral medium or with purine supplements. These results are similar to observations made in *E. coli*, *S. typhimurium* and *M. voltae*. Studies in the former two organisms indicated that addition of purines to the medium did not increase the intracellular levels of the purine PRTases (Nygaard, 1983). Similarly, H- and GPRTase activities in the archaeon *M. voltae* were not induced in the presence of exogenous purines, either singly or in combination (Bowen and Whitman, 1993; Bowen *et al.*, 1996). However, it was found that extracts from acetate-supplemented *M. thermoautotrophicum* cells had significant increases in HPRTase and GPRTase (2.1-fold and 2.3-fold, respectively). It is not clear why acetate would increase the levels of H- and GPRTase in *M. thermoautotrophicum*, although the sparing of early biosynthetic pathways by addition of the two carbon precursor could lead to shifts in need for down stream reactions. This is the first observation of HGPRTase activities being influenced by a substrate like acetate.

HGPRTase precipitation with ammonium sulfate: Our previous results showed that *M. thermoautotrophicum* H- and GPRTase activities remained soluble in 70% ammonium sulfate supernatant (data not shown; Worrell 1991). Attempts were made to achieve maximum precipitation of both activities. The results of a pilot experiment are shown in Table 3.3 where it can be seen that most of the HPRTase activity was recovered in the

TABLE 3.2. Effects of exogenous supplements on the levels of HGPRTase activities in *M. thermoautotrophicum* Marburg[a]

Addition to the medium (mM)	Enzyme activities (U/mg)[b]	
	HPRTase	GPRTase
None	3.89	3.75
Hypoxanthine (4.5) + Guanine (0.33)	3.30	2.15
Acetate (5)	8.33	8.57

[a] Cells were grown for 28 h at 60°C in 500 mL of mineral medium containing appropriate supplement. Cells were harvested, followed by preparation of extracts and assayed for H- and GPRTase activities.

[b] One unit is defined as the amount of activity leading to formation of 1 nmol product per min.

Table 3.3. Ammonium sulfate precipitation of H- and GPRTase activities from *M. thermoautotrophicum*

Fraction (% Am. sulfate)	Total protein (mg)	Total activity (Units)		Sp. Act. (U/mg)		Fold purification		Yield (%)	
		HPRTase	GPRTase	HPRTase	GPRTase	HPRTase	GPRTase	HPRTase	GPRTase
Crude extract	80.6	284	492	3.5	6.1	1	1	100	100
Streptomycin sulfate supernatant	59.3	492	730	8.3	12.3	2.4	2	173	148
0-65% pellet	45	9.4	31	0.2	0.6	0.06	0.1	3.3	0.06
0-65% sup	5.5	305	509	55.4	92	15.8	15.1	107	103
65-80% pellet	2.8	112	280	40	100	11.4	16.3	39	57
65-80% sup	8.1	148	180	18.3	22.2	5.2	3.6	52	36
65-90% pellet	6.7	234	199	34.9	29.7	10	4.9	82	40
65-90% sup	0.6	150	2.5	230.7	3.84	65.9	0.6	53	0.01
65-100% pellet	8.9	279	235	31.3	26.4	8.9	4.3	98	48
65-100% sup	0.3	95	12.1	316.6	40.3	90.4	6.6	33	0.02

65-100% ammonium sulfate pellet resulting in a 6-fold purification. The low recovery of GPRTase activity in 65-90 and 65-100% pellet of this pilot scale separation may represent an underestimate, since supernatant from the same fractions did not contain significant GPRTase activity. Our assumption that maximum H- and GPRTase activities precipitate in 65-100% ammonium sulfate fraction was later confirmed when purification of these activities was scaled up (Table 3.4). This procedure allowed concentration of both activities before loading onto a hydroxylapatite column without dialysis.

Purification of H- and GPRTase from *M. thermoautotrophicum*: To purify HGPRTase activities requires reasonable yields at each step and a large purification factor. Based on purification protocols in the literature (see Table 3.14) and the work of Worrell (1991), various combinations of pilot chromatography steps were tried, including affinity (agarose-GMP), dye-ligand (Reactive Red Agarose), ion-exchange (DEAE-cellulose, Cellex E, and QAE-Sephadex A-50), and hydrophobic (hydroxylapatite) chromatography. Based on the results, a purification protocol was set up, described below, and shown in Table 3.4.

In the process of developing the protocol, extensive attempts to exploit an agarose-GMP column at different stages in the purification scheme were made. Activity in ammonium sulfate fractions did not bind to the column. In one attempt, a dialyzed, 70% ammonium sulfate supernatant was loaded onto the column, permitted to equilibrate with the matrix for a few minutes, and then the column was washed with buffer. Briefly, 67% of loaded protein, 78% of HPRTase activity, and 44% of GPRTase were recovered in the break-through fraction. In the first wash (buffer C) an additional 28% of HPRTase and 31% of GPRTase were recovered; less than 4% of the

activities were recovered in the buffer plus GMP (5 mM) wash. It was possible that it might require a more purified enzyme fraction for this affinity method to function, if column capacity were overcome by competing enzymes. Therefore activity that had been purified >10-fold from QAE-Sephadex A-50 or hydroxylapatite columns was applied to the agarose-GMP column. Again, in both cases, the majority of H- and GPRTase activities eluted in the pass-through fraction (data not shown).

Use of GMP-affinity chromatography has been very succesful in purifying HGPRTase from yeast (Schmidt *et al.*, 1979; Nussbaum and Caskey, 1981), *G. lamblia* (Aldritt and Wang, 1986), and mammalian sources (Holden and Kelley, 1978). The failure of GMP-agarose chromatography in purification of *M. thermoautotrophicum* HGPRTase may be due to a lack of affinity of the enzyme for GMP. As discussed later in the chapter, IMP and not GMP inhibited HGPRTase activity. An IMP-affinity column may perform better for purification of *M. thermoautotrophicum* HGPRTase.

Enzyme was purified 800-1100-fold when a pilot scale purification was carried out as follows. After ammonium sulfate precipitation and dialysis, QAE-Sephadex, hydroxylapatite, and Reactive Red Agarose columns were run sequentially; the specific activity of the Reactive Red eluate was 3900 U/mg for HPRTase and 6200 U/mg for GPRTase.

The most successful large-scale purification scheme is presented in Table 3.4. H- and GPRTase activities were purified 1200 to 1500-fold from crude extract after ammonium sulfate precipitation, chromatography on hydroxylapatite, QAE-Sephadex A-50, and Reactive Red Agarose, and a heat step at 85°C. Specifics of these purification steps follow.

As indicated in the table, the 66 to 100% ammonium sulfate pellet contained about half of the initial activities, purified about 1.6 to 1.8-fold.

TABLE 3.4. Purification of HGPRTase from *M. thermoautotrophicum* Marburg

Fraction	Protein (mg)	Sp. Activity (U/mg) HPRT	GPRT	Total Activity(U) HPRT	GPRT	Fold Purification HPRT	GPRT	Yield (%) HPRT	GPRT	Ratio
Crude extract[a]	7000	8.4	8.8	58600	61600	1	1	100	100	0.95
Streptomycin SO^{4-}	7700	6.4	5.3	49300	40800	0.8	0.6	84	66	1.2
Ammonium sulfate	2070	13.8	15.8	28600	32700	1.6	1.8	49	53	0.87
Hydroxylapatite	175	123	140	21500	24500	14.6	15.9	37	40	0.9
QAE-Sephadex A-50	72	217	217	15600	15600	26	25	27	25	1
Reactive-Red Agarose	1.4	846	1105	1190	1550	101	125	2	2.5	0.8
Heat step[b] (85°C; 30 min)	0.14	8500	11000	1200	1500	1000	1250	2	2.5	0.8

[a] 227 g of cells were subjected to this purification protocol.

[b] Recovery normalized for use of part of the preparation at this step.

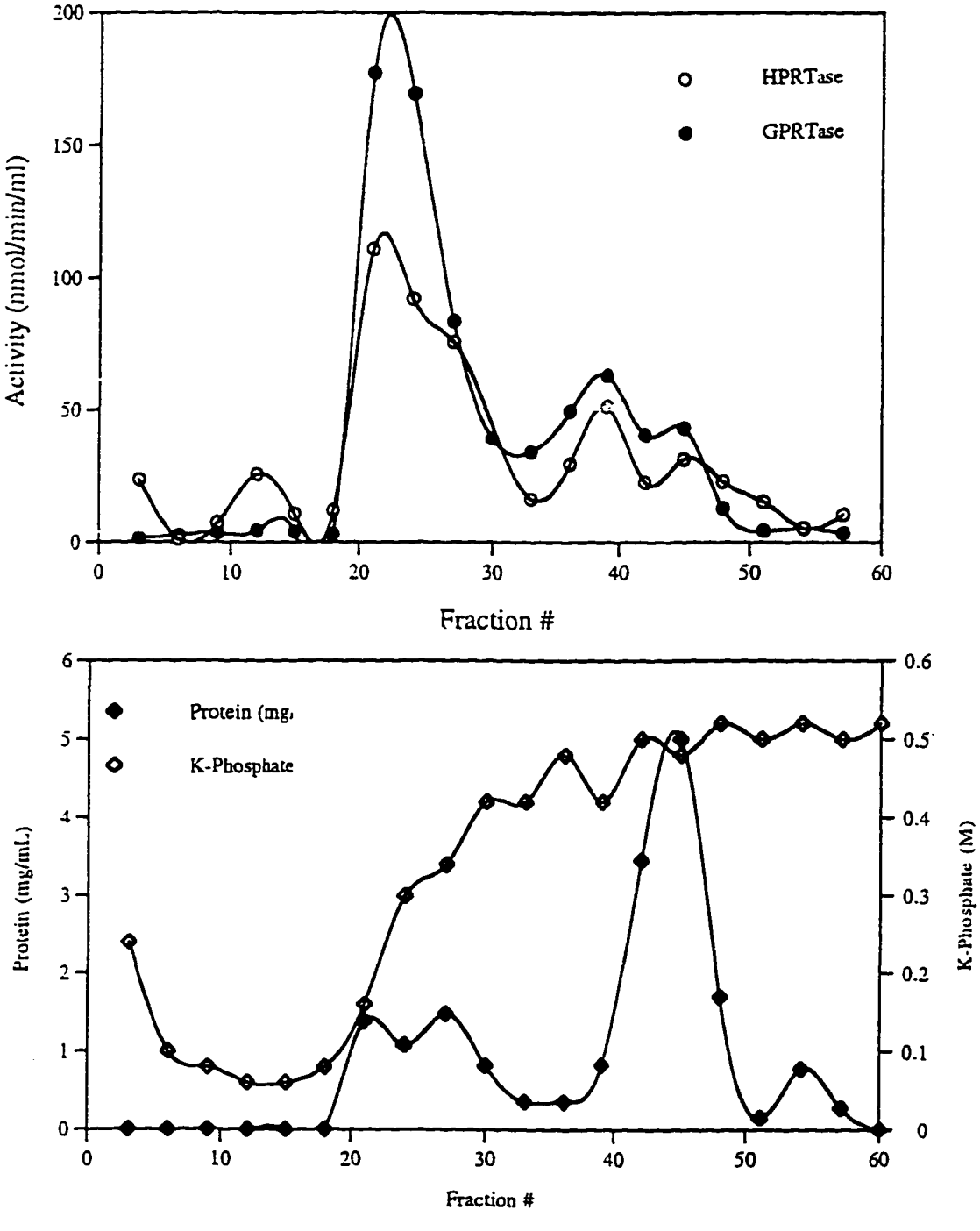

Figure 3.2. Elution profile of hydroxylapatite chromatography of H- and GPRTase activities from *M. thermoautotrophicum*. PRTase activities in the fractions with substrates hypoxanthine and guanine were measured as described under Materials and Methods. Column buffers and flow rates are described in the text.

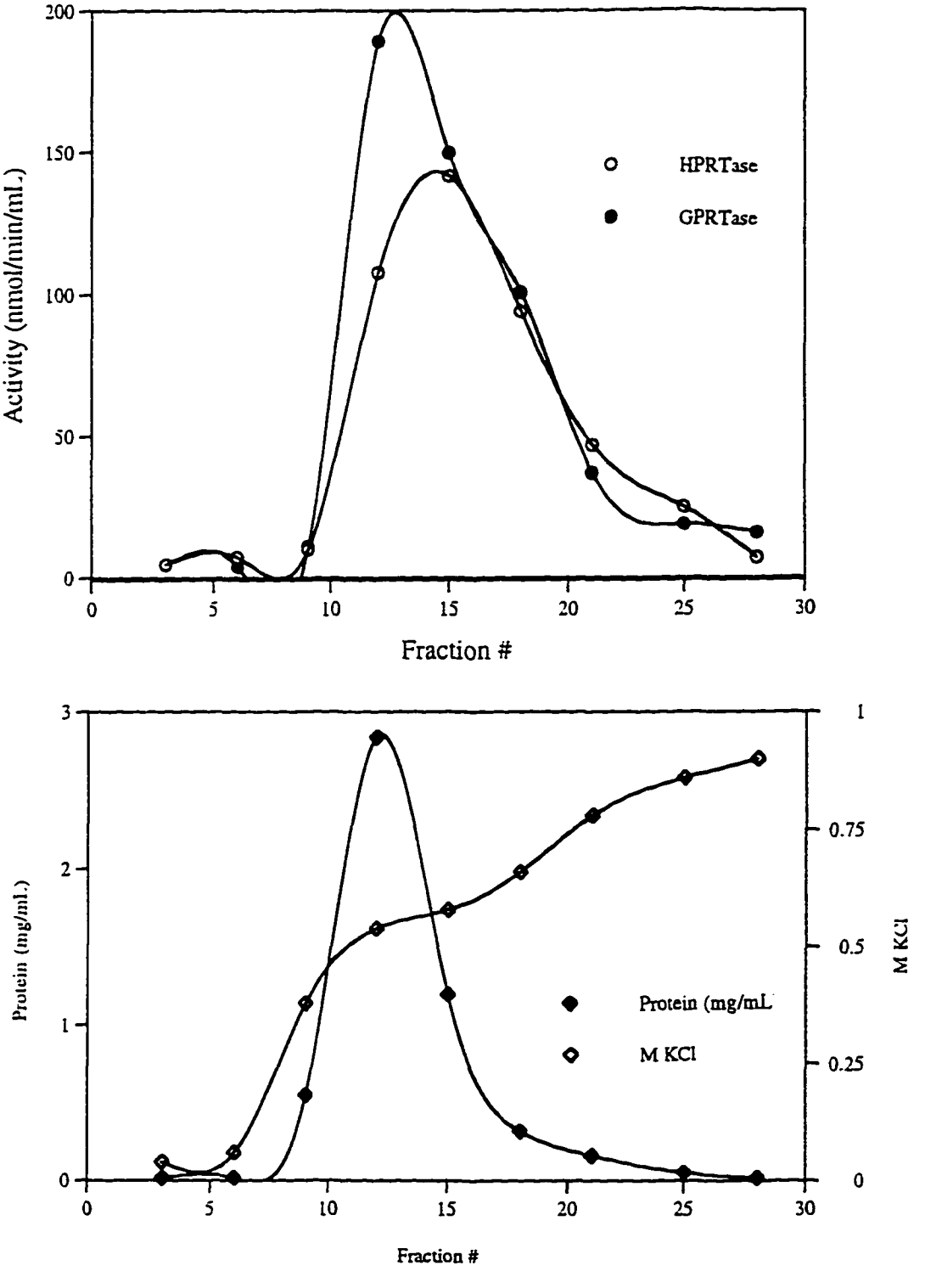

Figure 3.3. Elution profile of QAE-Sephadex A-50 chromatography of H- and GPRTase activities from *M. thermoautotrophicum*. PRTase activities in the fractions with substrates hypoxanthine and guanine were measured as described under Materials and Methods. Column buffers and flow rates are described in the text.

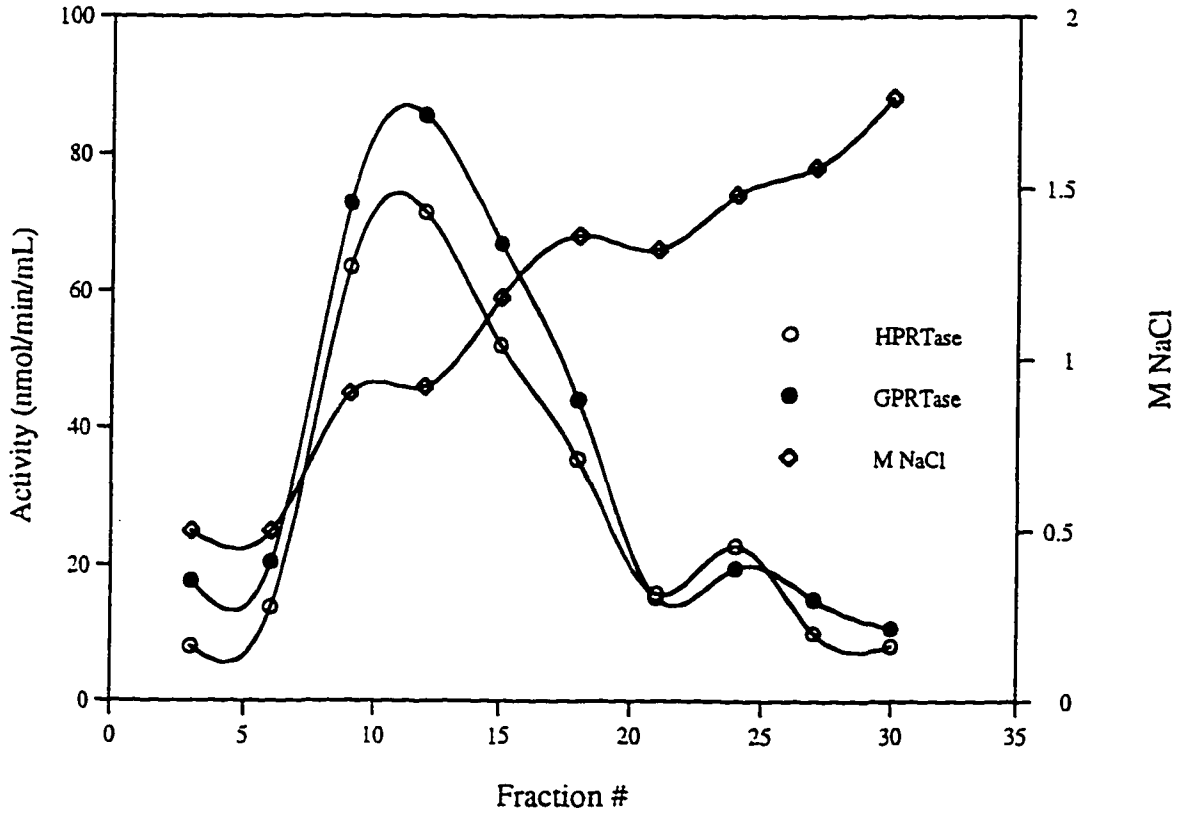

Figure 3.4. Elution profile of Reactive Red Agarose chromatography of H- and GPRTase activities from *M. thermoautotrophicum*. PRTase activities in the fractions with substrates hypoxanthine and guanine were measured as described under Materials and Methods. Column buffers and flow rates are described in the text.

The 66% pellet contained about 27% of HPRTase and 39% of GPRTase activity, respectively, significantly more than had been observed in that fraction previously (Table 3.3). The temperature at which fractionations were carried out may influence the recovery, since all previous pilot ammonium sulfate cuts were done at 4°C, whereas the scaled-up cut was done at room temperature in an anaerobic chamber. This procedure, done to permit separation of formate dehyrogenase for other purposes, did not purify the enzyme as much as did pilot steps. This may have negatively influenced later purification steps. As much as 9% of GPRTase activity remained in the 100% ammonium sulfate supernatant fraction, but HPRTase was not detected there.

The undialyzed ammonium sulfate fraction was applied to a hydroxylapatite column. In the profile shown in Figure 3.2, H- and GPRTase activities coeluted from the hydroxylapatite column at 0.16 to 0.42 M K-phosphate. Fractions 19 to 32, containing the majority of H and GPRTase activity (*ca.* 75%) and about 8% of the protein were pooled and stored at -20°C. Overall recovery of protein from this column was 100%.

Pooled hydroxylapatite fractions were diluted with buffer 3 to lower the conductivity prior to loading onto a QAE-Sephadex A-50 column whose elution profile is shown in Figure 3.3. Fractions 13 to 26 (0.55 to 0.86 mM KCl) were pooled and frozen, containing 73% of the HPRTase activity, 62% of the GPRTase activity, and 41% of the applied protein.

The A-50 fractions were thawed, diluted with buffer 3, and applied to a Reactive Red Agarose-120 column (Figure 3.4). Much of the activity broke through this column and did not bind. About 29 to 45% of HGPRTase was recovered in the pass-through fraction. The activities which bound and were recovered in the eluate between 0.9 to 1.2 M NaCl (fractions 9 to 15)

represented only 7 to 10% of the HGPRTase loaded on the column. About 44 to 63% of total HGPRTase activities applied was not accounted for.

This particular column run was much less successful than pilot columns which produced specific activities ten-fold higher than shown in Table 3.4. Comparison of gel electrophoresis patterns (below) prompted an additional purification step. A portion of the Reactive Red Agarose fraction was heated at 85°C for 30 min in the presence of 2 mM $PRPP_i$. The supernatant from this heat step contained as much as 96 to 100% of treated H- and GPRTase activities, with specific activities of 8,500 and 11,000 Units/mg of protein, respectively.

HGPRTase enzyme appears to be resistant to heat denaturation particularly in presence of $PRPP_i$ (see Chapter 2). A heat step was sucessfully used as one of the steps in purification of HGPRTase from eucaryotic organisms. Human enzyme (Olsen and Milman, 1974), Chinese hamster brain enzyme (Olsen and Milman, 1978), and chicken brain enzyme (Veres *et al.*, 1985) were subjected to treatment at 85°C for 10-15 min with or without $PRPP_i$. For enzyme purification from beef brain (Paulus *et al.*, 1980), rat brain (Gutensohn and Guroff, 1972), mouse liver (Huges *et al.*, 1975), *Schistosoma* (Yuan *et al.*, 1990), and Yeast (Schmidt *et al.*, 1979), the enzyme fractions were heat denatured in 65-80°C range without $PRPP_i$.

In a pilot scale purification, the ammonium sulfate precipitation yielded similar results (1.2 to 1.9-fold purification of HGPRTase) to a scaled up preparation. However, in sequential steps QAE-Sephadex A-50, hydroxylapatite and Reactive Red Agarose chromatography resulted in 35-fold, 110-fold, and >800-fold purification of HGPRTase, respectively. In all of the purification steps starting from crude extract to the Reactive Red Agarose, the majority of H- and GPRTase were co-purified and the ratio of

specific activity of HPRTase to GPRTase varied in the range of 0.7 to 1.3. These data strongly suggest that a single enzyme represents the majority, if not all, of H and GPRTase in *M. thermoautotrophicum*. For scaled-up purification, the order of QAE-Sephadex A-50 and hydroxylapatite was reversed to minimize purification time by eliminating a dialysis step before loading onto the hydroxylapatite column.

Native and SDS polyacrylamide gels: Native gel electrophoresis on 10% polyacrylamide of constant activity from different fractions that were collected during purification is shown in Figure 3.5a. The ammonium sulfate fraction contained fewer protein bands than crude extract. The hydroxylapatite and QAE-Sephadex fractions show similar amounts of one protein and the loss of some high molecular weight proteins. The Reactive Red Agarose fraction showed one major band and one less intense band of protein. Figure 3.5b shows native gel electrophoresis of the heat-treated Reactive Red fraction, and there appears to be a single polypeptide. Although these gels were run at different times, the major band from Reactive Red is not clearly visible, suggesting that one of the other bands has been enriched in the fraction and its intensity may be similar to a less intense band in the Reactive Red fraction. The molecular size was not determined using native gel electrophoresis.

The polypeptide compositions of fractions at different stages of purification (except the heat step) are illustrated in the SDS-polyacrylamide gel in Figure 3.6a, which contains a set of lanes with constant activity and a set with constant protein. Lanes with constant activity contained a band of approximately equal intensity at each step, migrating at M_r of 36,600 ($\pm$1300, six determinations). A less intense band of M_r 28,000 ($\pm$ 1100, four

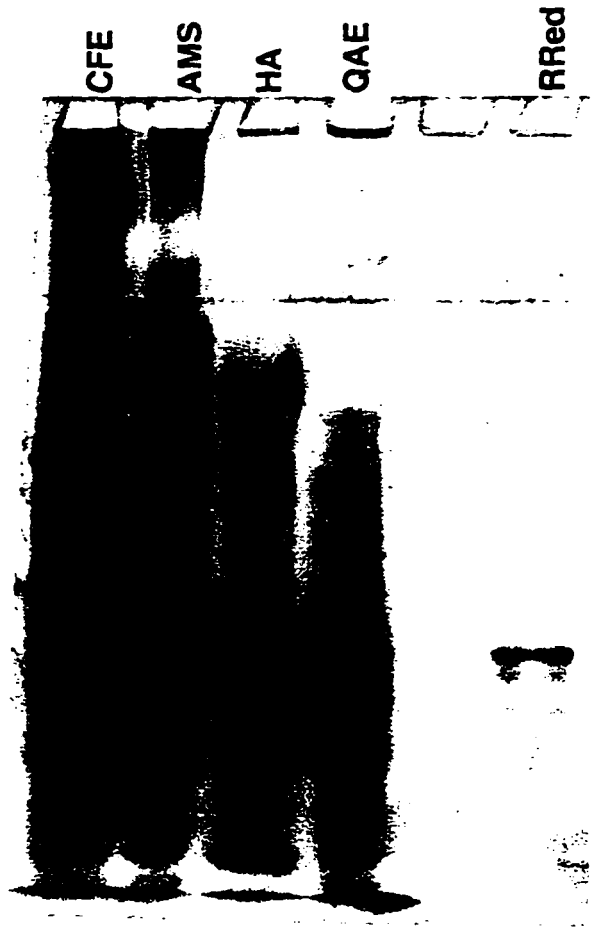

Figure 3.5a. Native gel electrophoresis in 10% polyacrylamide. Enzyme samples from successive steps in the purification procedure. Lanes contained 5.9 units of HPRTase activity. CFE; Crude extract. AMS; 65-100% ammonium sulfate pellet fraction. HA; hydroxylapatite fraction. QAE; QAE-Sephadex A-50 fraction. RRed; Reactive Red fraction.

Figure 3.5b. Native gel electrophoresis in 10% polyacrylamide. Single lane from native gel containing 5.9 units of HPRTase activity from heat-treated Reactive Red fraction.

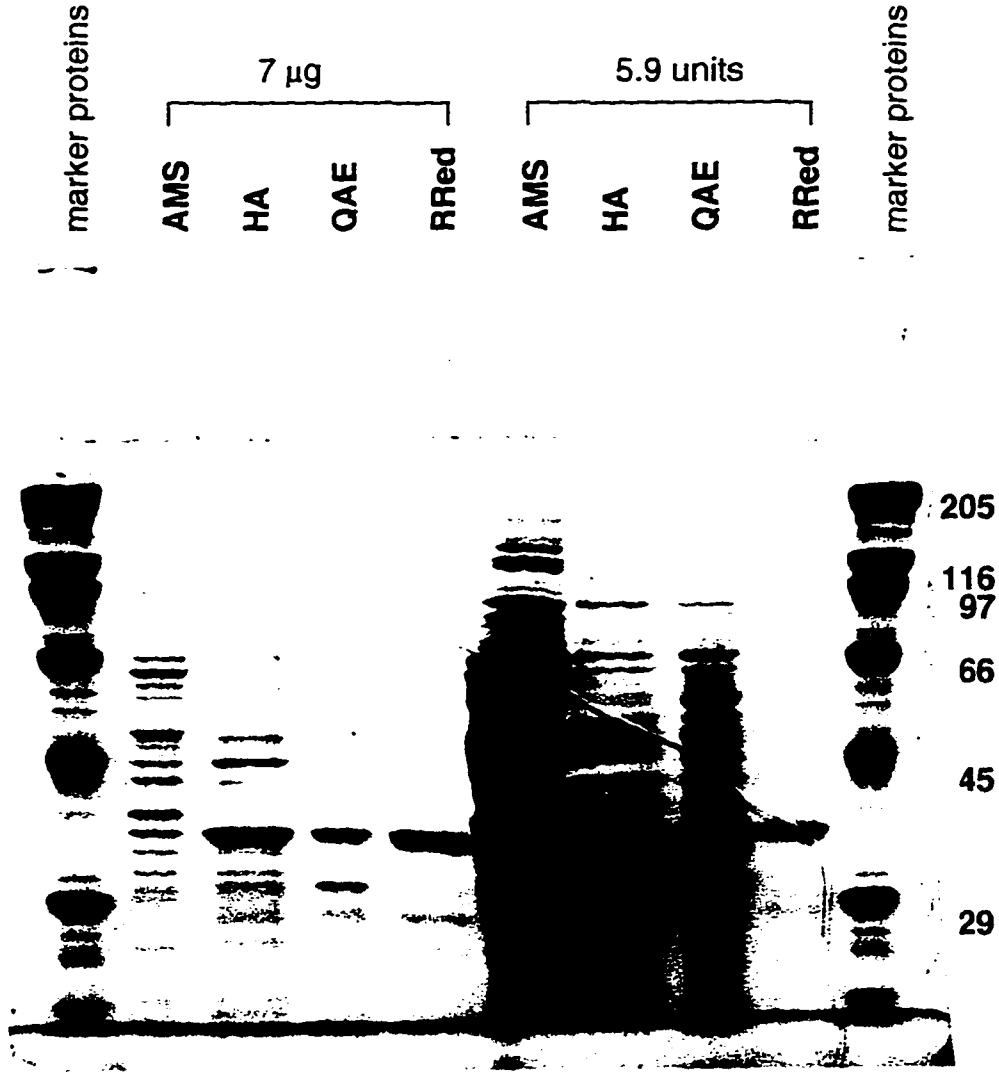

Figure 3.6a. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide. Lanes at both ends contained same molecular weight markers as indicated. Enzyme samples from successive steps in the purification procedure. Lanes contained 7 µg of protein or 5.9 units of HPRTase activity. AMS; 65-100% ammonium sulfate pellet fraction. HA; hydroxylapatite fraction. QAE; QAE-Sephadex A-50 fraction. RRed; Reactive Red fraction.

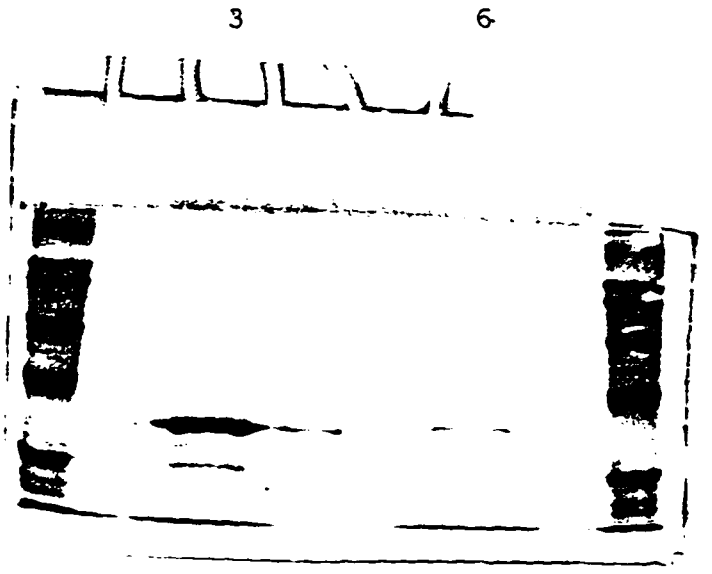

Figure 3.6b. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide. Lanes contain 5.9 units HPRTase activity. Lane 3; Reactive Red fraction. Lane 6; Heat-treated Reactive Red fraction.

determinations) was also visible in later stages of the purification. Gel patterns from pilot preparations suggested that the protein band of 36.6 kDa was associated with HGPRTase. It was essentially homogeneous in 100-fold purified enzyme fractions on Coomassie blue-stained gel lanes containing about 3 μg protein (figure not shown).

However, evidence correlating the 28,000 molecular weight protein band with HGPRTase was obtained by heating experiments. A sample of the Reactive Red Agarose-purified enzyme was heated 30 min at 85°C in 2 mM $PRPP_i$, centrifuged, with complete recovery of enzyme activities in the supernatant. The heated Reactive Red Agarose-purified enzyme was also run on an SDS-polyacrylamide gel. As shown in Figure 3.6b, the majority of the 37 kDa band was lost, while the one at 28 kDa did not change in intensity. Use of Silver stain, a more sensitive technique than Coomassie blue, will also rule out the possibility of HGPRTase being present in undetectable and trace amount in heated Reactive Red fraction.

These data suggested that 28 kDa and not 37 kDa subunit belonged to HGPRTase of *M. thermoautotrophicum*. NH_2-terminal amino acid sequences were obtained from blots of denaturing gels of the 36.6 kDa protein and the 28 kDa protein. The first 16 NH_2-terminal amino acids of the 36.6 kDa protein were Met-Lys-Phe-Gly-Ile-Glu-Phe-Val-Pro-Asn-Glu-Pro-Ile-Glu-Lys-Ile. A search for this sequence in data bases showed that it was identical to the sequence reported for Coenzyme F420-dependent N_5,N_{10}-methylenetetrahydromethanopterin reductase from *M. thermoautotrophicum* Marburg and ΔH strains (Nolling *et al.*, 1995). This enzyme from strain Marburg was reported to have a subunit size of 35 kDa. Its co-purification with HGPRTase is not yet explained, but this reductase represents a major catabolic activity and would be expected to comprise a significant portion of

cell protein. Blue Sepharose CL-6B® column might be applied to remove this contaminant protein according to Ferry (1992).

The 28 kDa protein band was assigned to HGPRTase because of its consistent appearance in all highly purified fractions. Strong evidence in support of this conclusion is based on the NH_2-terminal sequence of this protein, as described below and indicated in Tables 3.12 and 3.13, and from the gel filtration data, below.

Activity stain of HGPRTase: Detection of H- and GPRTase activities *in situ* was attempted for *M. thermoautotrophicum* H- and GPRTase. Several methods have been reported for the detection of HGPRTase activity after gel electrophoresis. These include pyrophosphate precipitation with $MnCl_2$ (Vasquez and Bieber, 1978), precipitation of nucleotide products with lanthanum chloride and localization by autoradiography (Bakay *et al.*, 1969, Tischfield *et al.*, 1973), cellogel & DEAE-cellulose electrophoresis of enzyme followed by localization by autoradiography (Shin *et al.*, 1971), and incubating cut gel slices in a reaction mixture followed by quantitation of nucleotide product.

The availability of a working HGPRTase activity stain in *M. thermoautotrophicum* should serve two purposes. First, it would be possible to properly assign individual protein bands detected thereby with specific catalytic activities. Second, it would also show whether both activities, H- and GPRTase, are catalyzed by a single protein or separate proteins. For these reasons, significant effort was spent on attempts to develop activity stains for H- and GPRTases from *M. thermoautotrophicum*. Certain precautions were taken to maximize the probability of locating enzyme activity. Polymerized gels were left at room temperature for at least 24 h

since ammonium persulfate was used to initiate polymerization and may be detrimental to the enzyme(s). Gels were pre-electrophoresed with running buffer to help eliminate any residual initiator.

Initial runs on methanobacterial HGPRTase preparations, using the principle of precipitation of pyrophosphate product with $MnCl_2$ were unsuccessful; precipitation bands associated with the gel were not observed (data not shown). For the most part, crude extract was used for attempts to detect HGPRTase activities. About 10 units of HGPRTase activities were loaded. In fact, the entire reaction mixture became turbid during the incubation, which might hinder observation of an enzyme-related manganese pyrophosphate precipate. It was thought possible that PRPPi was not stable during the prolonged incubation at elevated temperature. According to the manufacturer (Sigma, Inc.), $PRPP_i$ stability is temperature- and concentration dependent. The background turbidity was $PRPP_i$-dependent and time-dependent; no turbidity was seen at time zero or when it was omitted from gel-stain mixtures. Lower temperatures were tested to slow down the background precipitation reaction, and H-and GPRTase activities in crude extract assayed at 42 and 49°C were approximately 60% of those detected at 55°C (0.56 nmol IMP and 0.88 nmol GMP/min, respectively), and therefore were thought to be sufficiently active for detection. Although the mixture remained relatively clear, activity stains of native gels performed at 42°C were not successful. The background turbidity problem was also addressed by leaving $MnCl_2$ out of the reactions, which allowed the mixture and the gel to remain clear until the manganese solution was added. But again the mixture became turbid upon addition of $MnCl_2$, and no precipitation bands were detected with the methanogen enzyme.

After these activity staining attempts failed, about 3 units of commercial baker's yeast HGPRTase, provided in a form lyophylized with $MgCl_2$, were electrophoresed for 30 min and subjected to the $MnCl_2$ staining protocol (gels are not shown). A lane stained for protein with Coomassie blue dye lane revealed three bands: the dye front; a high molecular weight band, probably from non-proteinaceous substances in the protein sample or buffers (since it was never observed with any other gels) and a protein band which had migrated 3.1 cm from the origin. In lanes incubated in H- or GPRTase mixtures, including $MnCl_2$, white precipitation bands formed exactly 3.1 cm from the origin. Thus the commercial enzyme remained active during the brief electrophoresis period and could be detected by the published activity stain procedure.

For the reasons given above, it was deemed worthwhile to continue attempts to detect HGPRTase activities from *M. thermoautotrophicum* on gels. After native gel electrophoresis of crude extract preparation containing 15 units per lane, gel slices (2 mm thick) were cut and incubated in standard radiochemical assay mixtures at 55°C for 18 h. No significant formation of radioactive nucleotide products was detected (not shown). Assay mixtures after incubation contained unutilized radioactive substrate (hypoxanthine or guanine), suggesting that H- and GPRTase enzyme(s) were probably inactivated even before the gel slices were transferred into the assays.

Finally, to determine if H- and GPRTase remained active at all in the polyacrylamide gel after electrophoresis, crude extract of *M. thermoautotrophicum* was electrophoresed on a native gel. Then entire lanes were incubated in reaction mixtures containing ^{14}C-hypoxanthine or ^{14}C-guanine. After 40 h at 55°C, 0.04 nmol IMP and 0.14 nmol GMP were

recovered, a minute fraction of that expected for the amount of activity applied.

It seemed clear that *M. thermoautotrophicum* H- and GPRTase activity was sensitive to native gel electrophoresis, although traces were recovered in entire gel. A final attempt was made in which gels were photopolymerized with riboflavin instead of ammonium persulfate, and preelectrophoresed before sample was loaded. Activity was not detected with $MnCl_2$ staining. Activity stain using other matrix such as starch or an agarose gel was not attempted.

Sizing column chromatography: This experiment was done to see if H- and GPRTase activities from *M. thermoautotrophicum* could be separated from each other, and to determine the molecular mass of each activity. A Bio-Gel® P-100 sizing column was calibrated with cytochrome *c* and Vitamin B_{12}. Bovine serum albumin and alcohol dehydrogenase were not detected at 280 nm after repeated attempts. Figure 3.7a shows the results of chromatography of *M. thermoautotrophicum* crude extract through Bio-Gel® P-100. A major peak of H- and GPRTase activities at V_e/V_0= 1.3 (fraction 31.2 mL) was assigned a molecular weight of roughly 100,000. This was based on the elution volumes of cytochrome *c* and vitamin B_{12}. The sample applied contained 37.2 mg of protein. The specific activities of H- and GPRTase loaded were 4.9 and 5.8 U/mg, respectively. In the most active fraction (31.2 mL), the specific activities of H- and GPRTase were 31.3 and 171.6 U/mg, respectively. This fraction contained 4 and 19% of H- and GPRTase activities, respectively. The change in ratio of HPRTase/GPRTase in the peak fraction is most likely not due to separation of a major amount of HPRTase activity. It is more likely explained by the assay conditions, where

column buffer (pH 7.5) was present (23.5 μL enzyme/30 μL assay) and probably neutralized the pH 8.4 HPRTase assay buffer, yielding a pH of about 7.9. As seen in Figure 3.11, this would move HPRTase even further down the pH activity profile, leading to an underestimate of its activity. About half-maximal activity was seen at a pH of approximately 7.8 for HPRTase. Although major H- and GPRTase activities coeluted, there was about 20% IMP-forming activity that eluted later. Between fractions 42 and 60, traces of IMP-forming activity were detected. Although these assays were barely above the detection limit (< 1X background radioactivity), it can't be ruled out that a small % of HPRTase was separated. The overall recoveries of H- and GPRTase activity were 80 and 69%, respectively.

Gel filtration chromatography was also performed on Sephadex G-150 with Reactive Red Agarose-purified fraction to determine the molecular size of H- and GPRTase activities. The column profile shown in Figure 3.7b shows that the HPRTase activity eluted in a broad envelope (fractions 18 to 30 mL) over a molecular weight range of about 27 to 149 kDa. Approximately 26.5% of total HPRTase activity applied was recovered in assayed fractions. Several of these individual fractions were concentrated using Amicon microconcentrators (10K-cutoff) and loaded onto the SDS gel (Figure 3.7c). It can be seen that all lanes contained an intense band at 37 kDa position (identified as Coenzyme F_{420}-dependent N_5,N_{10}-methylenetetrahydromethanopterin reductase) and a faint band at 28 kDa position. The intensity of 28 kDa band in each fraction from G-150 column was visually proportional to the amount of enzyme activity in that fraction. These data, therefore, substantiate the hypothesis that the protein that migrated on SDS gels with molecular weight of 28 kDa corresponds to *M. thermoautotrophicum* HGPRTase activities.

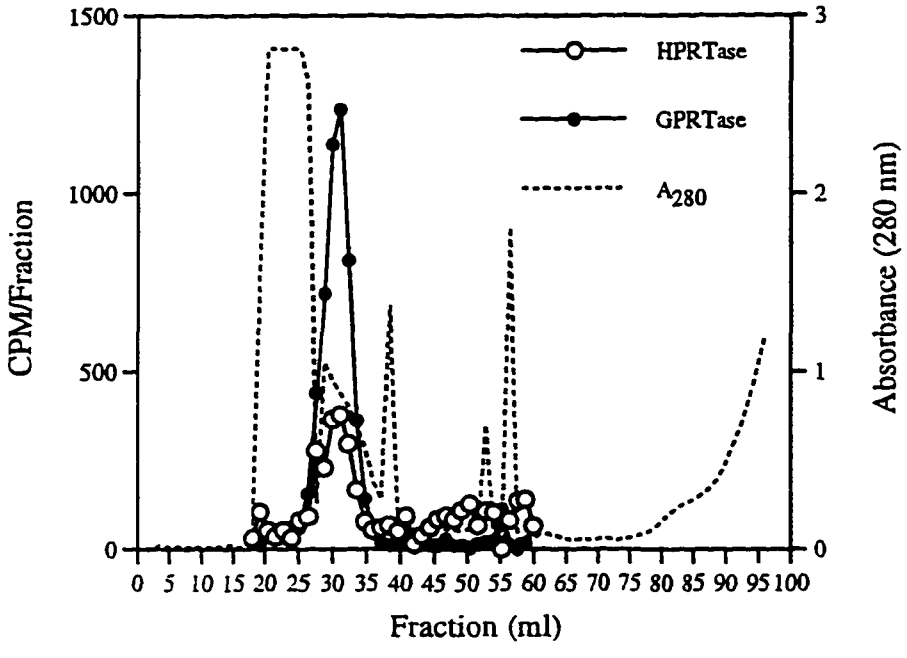

Figure 3.7a. Bio-Gel® P-100 chromatography of the H- and GPRTase activities of *M. thermoautotrophicum* crude extract. The void volume (V_0) was 21.6 mL. For additional details, see the Materials and Methods section.

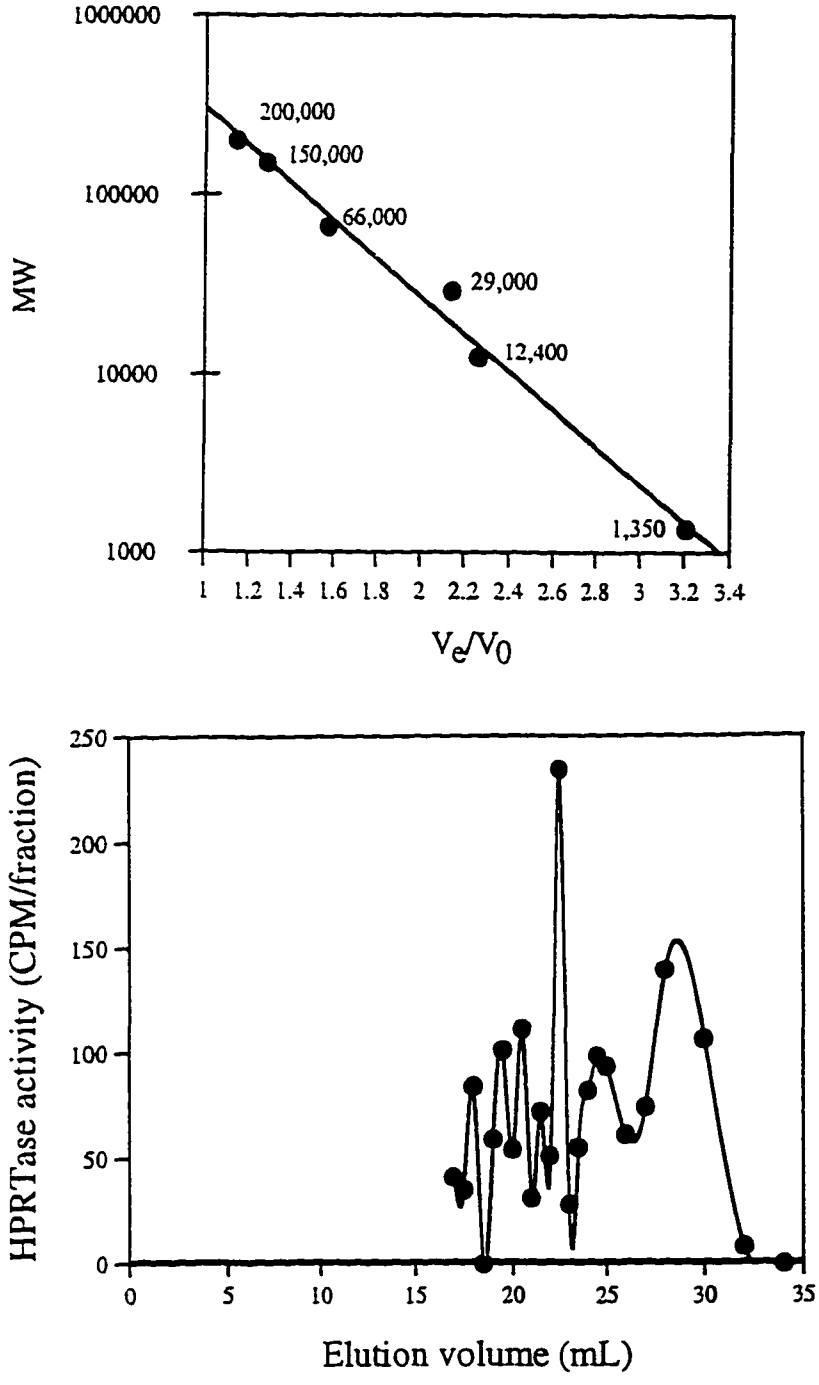

Figure 3.7b. Sephadex G-150 chromatography of Reactive Red Agarose-purified *M. thermoautotrophicum* HGPRTase. The top figure shows calibration by molecular weight markers β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome *c* (12,900), and vit. B_{12} (1,350). The bottom figure shows elution profile of HPRTase on the column. The void volume (V_0) was 14 mL.

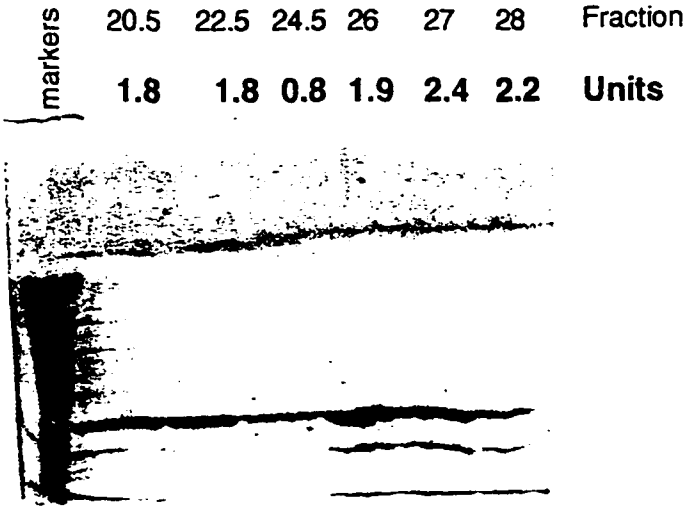

Figure 3.7c. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide. The figure shows correlation of HGPRTase protein amount (28,000 Da, lower band) with HPRTase activity eluted in fractions 20.5, 22.5, 24.5, 26, 27, and 28 mL. The total number of units loaded are also indicated.

When Reactive Red Agarose-purified *M. thermoautotrophicum* HGPRTase was loaded onto an FPLC Sepharose-12 column, a third kind of sizing column, two peaks of protein eluted with native molecular mass of 98,200 and 13,000 (data not shown). These fractions were concentrated, and were assayed for H- and GPRTase activities. The fraction containing 98,200 Da protein contained both H- and GPRTase activities, but fraction with 13,000 Da protein did not have any activity for either base. On an SDS polyacrylamide gel, the fraction with enzyme activities showed an intense band at 37 kDa (the methylenetetrahydromethanopterin reductase) and very faint band at the 28 kDa position (data not shown). These observations are analogous to our observations with G-150 column, and support our hypothesis regarding identity of 28 kDa protein being *M. thermoautotrophicum* HGPRTase.

It is possible that *M. thermoautotrophicum* H- and GPRTase activities exist as a tetramer of 28 kDa in a native state, as shown by gel-filtration of crude extract. However, the gel-filtration of Reactive Red Agarose-purified fraction suggests that enzyme activities may dissociate into smaller forms. Therefore, stabilization of elution conditions (suitable pH or ionic strength) or a chemical cross-linking study of this enzyme may be necessary to confirm a such hypothesis (Holden and Kelley, 1978). Any possible association of methylenetetrahydromethanopterin reductase and HGPRTase is not addressed in these discussions.

There has been a precedent for difficulty in determining native structures of HGPRTases from other organisms. For example, in studies of the native weight of human enzyme, Strauss (1975) found multiple peaks of HGPRTase activities on gel filtration. Similar results were seen when enzyme was subjected to SDS electrophoresis followed by renaturation in the

presence of $PRPP_i$. For the human enzyme, it was later shown that $PRPP_i$ (Strauss *et al.*, 1978), ionic strength (Johnson *et al.*, 1979), and pH affected aggregation of the enzyme. Strauss *et al.* (1978) observed occurence of a tetrameric form of human and Chinese hamster enzyme in presence of $PRPP_i$, but the same enzymes existed in dimeric and trimeric forms in absence of $PRPP_i$. Under conditions of low ionic strength, the enzyme existed as a dimer, but was converted to a tetramer form under high ionic strength (Johnson *et al.*, 1979). In contrast, cross-linking studies with highly purified human enzyme suggested that the enzyme may exist a tetramer in the native state (Holden and Kelley, 1978). Other examples include *L. donovani*, where gel-filtration studies provided molecular weight of HGPRTase 110,000 and 60,000 in absence and presence of $PRPP_i$, respectively (Tuttle and Krenitsky, 1980). $PRPP_i$ was also shown to affect the state of aggregation of HGPRTase in *T. cruzi*. This enzyme behaved as a dimer in presence of $PRPP_i$, but dissociated to a monomeric form in absence of $PRPP_i$. In the archaeon *M. voltae*, a single peak containing H- and GPRTase activities eluted on a gel filtration column at an estimated molecular mass of 90,000 (Bowen *et al.*, 1996). However, chromatography of this active fraction on an ion-exchange column resulted in multiple peaks containing both activities.

Kinetic studies: Michaelis-Menten kinetic constants were determined with Reactive Red Agarose-purified enzyme preparation. Standard assay conditions [pH 8.4 for hypoxanthine, pH 7.9 for guanine, 5 mM $MgCl_2$, excess $PRPP_i$ (4 mM) and 55°C] were utilized. The apparent K_m value for $PRPP_i$ was determined in the presence of excess hypoxanthine (0.2 mM, a concentration 33 times its apparent K_m value). *M. thermoautotrophicum* H- and GPRTase displayed Michaelis-Menten kinetics when one of the

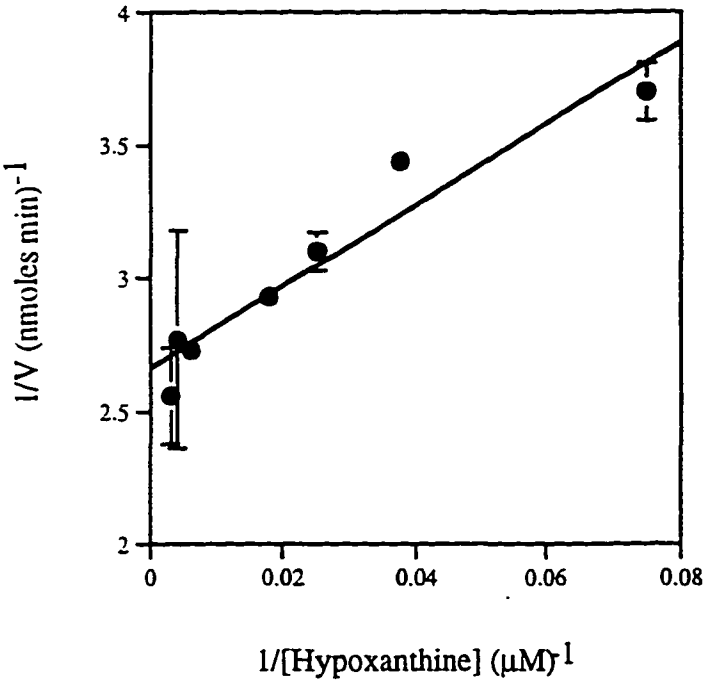

Fig. 3.8. Lineweaver-Burk analysis of the effect of hypoxanthine concentrations on HPRTase activity from *M. thermoautotrophicum* . The ability of the Reactive Red Agarose-purified *M. thermoautotrophicum* HGPRTase to phosphoribosylate hypoxanthine was determined at a number of substrate concentrations (13.3-320 μM) in presence of 4 mM $PRPP_1$ as described in Materials and Methods. Each data point represents the mean± standard error of two determinations.

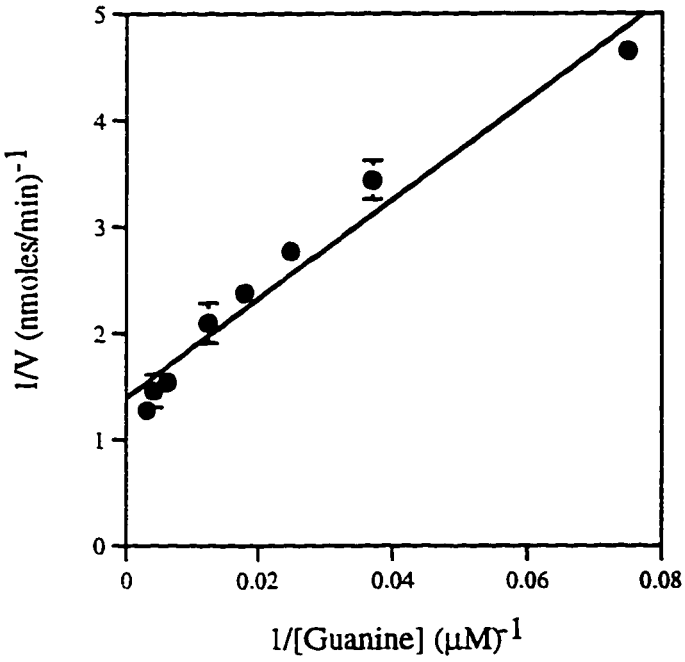

Fig. 3.9. Lineweaver-Burk analysis of the effect of guanine concentrations on GPRTase activity from *M. thermoautotrophicum* . The ability of the Reactive Red Agarose-purified *M. thermoautotrophicum* HGPRTase to phosphoribosylate guanine was determined at a number of substrate concentrations (13.3-320 μM) in presence of 4 mM $PRPP_i$ as described in Materials and Methods. Each data point represents the mean± standard error of two determinations.

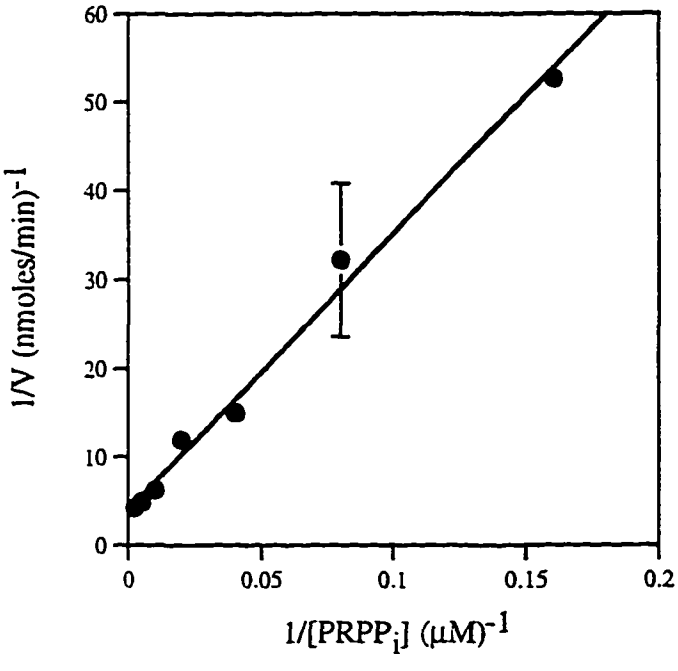

Fig. 3.10. Lineweaver-Burk analysis of the effect of PRPP $_i$ concentrations on HPRTase activity from *M. thermoautotrophicum* . The ability of the Reactive Red Agarose-purified *M. thermoautotrophicum* HGPRTase to phoshoribosylate hypoxanthine was detrmined at a number of PRPP$_i$ concentrations (6.25-400 μM) in presence of 0.2 mM hypoxanthine as described in Materials and Methods. Each data point represents the mean ± standard error of two determinations.

substrates was in limiting amounts (hypoxanthine or guanine) and the other ($PRPP_i$) was in excess. Lineweaver-Burk plots of $(velocity)^{-1}$ *versus* $(substrate\ concentration)^{-1}$ for hypoxanthine (Figure 3.8), guanine (Figure 3.9), and $PRPP_i$ (Figure 3.10) were used to determine the K_m values for hypoxanthine, guanine, and $PRPP_i$ to be 6.3, 35.7, and 81.5 μM, respectively. The correlation coefficients for kinetic plots for hypoxanthine, guanine, and $PRPP_i$ were 0.96, 0.98, and 0.99, respectively. K_m values from Eddie-Hofstee plot were determined to be 6, 41.7, and 91.1 μM for hypoxanthine, guanine, and $PRPP_i$, respectively (figures not shown). The correlation coefficients for these kinetic plots for hypoxanthine, guanine, and $PRPP_i$ were 0.93, 0.94, and 0.94, respectively. The average and range of K_m possible from the two plots is 6.1 (± 0.2), 38.7 (± 4.2), and 86.3 (± 6.8) μM for hypoxanthine, guanine, and $PRPP_i$, respectively. A similar K_m value (81.5 μM) for $PRPP_i$ in presence of excess hypoxanthine was obtained in an independent experiment (Figure 3.12). The lower K_m value for hypoxanthine than for guanine suggest hypoxanthine is a better substrate than guanine for the *M. thermoautotrophicum* HGPRTase.

The *M. thermoautotrophicum* K_m values are compared to K_m values of the enzymes from other organisms in Table 3.14. Except *G. lamblia* enzyme, HGPRTases in other organisms have typically low K_ms for purine bases (in the range 0.25-28 μM) suggesting bases to be effective substrates for this class of enzyme. High apparent K_m for $PRPP_i$ (in the range 2.9-450 μM; 10-20 times higher than for the base) also seems to be a common feature of HGPRTases from different organisms.

Effects of pH on HGPRTase activities: Figure 3.11 shows the pH activity profile of *M. thermoautotrophicum* HGPRTase. The effect of pH on both

PRTase activities was studied using a mixed buffer system over a pH range of 6.3-10.5. The enzyme activities showed identical pH dependence over a broad range between pH 6.3 and pH 9.9 with both hypoxanthine and guanine. HPRTase and GPRTase activities had a single pH optimum at 9.9. Half-maximal activities were at a pH of approximately 7.8 for both PRTase activities. Beyond pH 9.9, HPRTase activity, but not GPRTase activity, fell off sharply. In many of the experiments reported througout this dissertation, standard assay conditions (pH 8.4 and 7.9 for H- and GPRTase, respectively) were used. These had been reported as optimal in other systems. Even though these conditions were later determined not to be optimum for the methanogen enzyme, they were used for consistency.

There are some differences in pH requirements between eubacterial and eucaryotic enzymes. Compared to eubacterial enzymes, the enzymes from eucaryotes seem to have higher pH optima (Table 3.14). Few examples of pH dependence of enzymes from enteric bacteria are known. The HPRTase and XGPRTase from *E. coli* have pH optima at 8.4 and 7.9, respectively. This was the source used to develop the initial H- and GPRTase assays in *M. thermoautotrophicum* (Worrell, 1991). However, another group found *E. coli* XGPRTase activity to be maximal at pH 9.5 (Liu and Milman, 1983).

Eucaryotic enzymes from beef brain, Chinese hamster brain and chicken brain are known to exhibit maximum activity at pH 10. Enzymes from Chinese hamster brain and chicken brain are also active over a broad pH range. Beef brain enzyme was active from pH 5.5-10.0, whereas Chinese hamster enzyme was active from pH 5.5-11.0.

Additionally, broad pH optima or biphasic pH response of enzyme activity is common in some organisms. HGPRTase from *P. lophurae* and *T.*

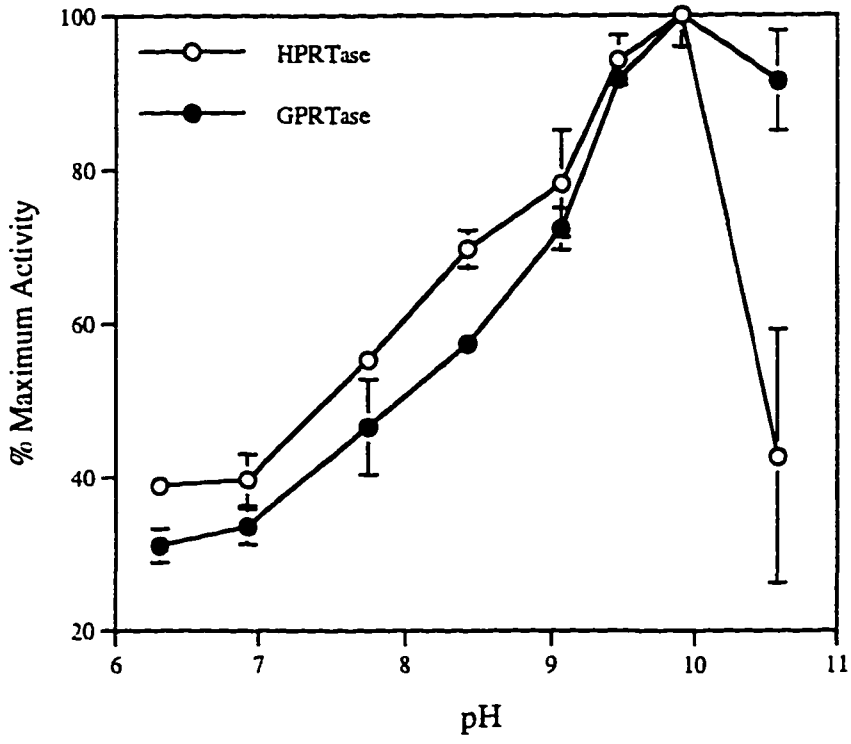

Fig. 3.11. pH activity profile of *M. thermoautotrophicum* HGPRTase. The rate of IMP and GMP formation were determined from pH 6.3-10.6 in a buffer system containing 16 mM each of Bis-Tris Propane, CAPS and TES. The pH values were measured at room temperature. Each asay contained 0.84 µg of protein. Maximum activity was 1.4 µmoles/(min.mg) for IMP formation and 1.5 µmoles/(min.mg) for GMP formation. Each data point represents the mean ± standard error of two determinations.

foetus has broad pH optimum for activity. Maximal activity for *P. lophurae* H- and GPRTase occurs between pH 6-10 and pH 7.5-9.5, respectively. The HGPRTases of *Artemia* sp., *S. pombe*, and *P. falciparum* have a biphasic pH responses. In *Artemia* sp., two apparent buffer-independent pH optima, at 7.0 and 9.5 were seen. *S. pombe* HGPRTase exerts maximum activity at pH 8.0 and 9.5.

Another notable feature of bifunctional HGPRTases in some protozoans include non-identical pH profiles with hypoxanthine and guanine. HGPRTases from *L. donovani*, *G. lamblia*, and *P. lophurae* had different pH profiles for hypoxanthine and guanine. *L. donovani* HGPRTase had maximal activity at pH 6.9 with hypoxanthine as substrate, but maximal activity for guanine occured over a broad pH range from 7.4 to 9.1. In the case of *P. lophurae* HGPRTase, maximal HPRTase and GPRTase activities were observed from pH 6-10 and pH 7.5-9.5, respectively.

pH optima and pH range of *M. thermoautotrophicum* HGPRTase activities were analogous to beef brain and and Chinese hamster brain enzyme. *M. thermoautotrophicum* enzyme exhibited maximal activity with hypoxanthine and guanine at pH 9.9, and both activities occured over a broad pH range. The only other report of a pH optimum for HGPRTase from an archaeon is in *M. voltae*, where H- and GPRTases activities were maximal at pH 9 (Bowen *et al.*, 1996). In our studies, pH values were measured at room temperature. This means that *M. thermoautotrophicum* HGPRTase had a pH optimum of 9.4. These methanogen enzymes appear to have unusually high pH optima since the internal pH of 6.7 has been reported for both *M. thermoautotrophicum* (Jarrell and Sprott, 1981) and *M. voltae* (Jarrell and Sprott, 1985).

TABLE 3.5. Effect of metal ions on *M. thermoautotrophicum* HGPRTase activities

Metal ion (5 mM)[a]	% Activity[b]	
	HPRTase	GPRTase
None	100	100
Ba^{2+}	82	97
Ca^{2+}	106	74
Co^{2+}	119	131
Cs^{2+}	96	169
Cu^{2+}	96	34
Fe^{2+}	82	96
Hg^{2+}	96	98
Mg^{2+}	152	128
Mn^{2+}	517	545
Na^{+}	112	117
Ni^{2+}	138	115
Zn^{2+}	180	138

[a] Chloride salts were used.

[b] The effects of cations (5 mM) on the rates of IMP and GMP formation were determined in duplicate using Reactive Red Agarose-purified HGPRTase. In control reactions, 0.14 nmol/min for IMP formation and 0.19 nmol/min for GMP formation was observed.

Effects of metals: Effects of various metals were studied using dialyzed Reactive Red Agarose-purified HGPRTase from *M. thermoautotrophicum* (Table 3.5). Ba^{2+}, Fe^{2+}, Hg^{2+}, and Na^{+} had little or no effect on either PRTase activity. Both activities were stimulated by Mg^{2+}, Mn^{2+}, and Zn^{2+}, with about 5-fold stimulation was seen for HGPRTase activities in presence of Mn^{2+}. Ni^{2+} somewhat stimulated HPRTase activity. For GPRTase activity, Co^{2+} and Cs^{2+} were stimulatory, whereas Ca^{2+} and Cu^{2+} were inhibitory. This result is not explained. We were not able to show an absolute requirement for Mg^{2+} with purified enzyme. Similar observations were made in experiments with dialyzed cell-free extracts (data not shown). The rates of IMP and GMP formation remained unaffected in dialyzed cell extracts in absence of Mg^{2+} (data not shown). It is possible that a required metal may have been tightly bound to *M. thermoautotrophicum* HGPRTase, or trace metal contamination may have been enough to allow for the enzyme activities. In numerous cases in other organisms, an absolute requirement for Mg^{2+} for HGPRTase activities has been reported. These are summarized in Table 3.14; examples include *Artemia* sp. (Montero and Llorente, 1991), *S. pombe* (Nagy and Ribet, 1977), *S. cerevisiae* (Schmidt *et al.*, 1979), *T. cruzi* (Gutteridge and Davies, 1982), *T. foetus* (Beck and Wang, 1993), and enteric bacteria (Hochstadt, 1978). It appears that Mg^{2+} cation is generally used as cofactor for PRTase reactions. However, Table 3.14 does show variation in the response of HGPRTases to activation by cations. Other divalent cations such as Mn^{2+}, Zn^{2+}, Fe^{2+} and Ca^{2+} have been shown to increase enzyme activities from different sources. Mn^{2+} was more effective than Mg^{2+} for HGPRTase activities from *Artemia* sp. Ca^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, and Ba^{2+} were inhibitory for enzyme activities from other sources. From the metal effector study, it can be concluded that *M. thermoautotrophicum* HGPRTase

TABLE 3.6. Inhibitory effects of chemical reagents on *M. thermoautotrophicum* HGPRTase activities[a]

Chemical reagent (5 mM)	% Activity	
	HPRTase	GPRTase
None	100	100
Dithiothreitol	119	99
2-Mercaptoethanol	102	103
PMSF	60	88
Guanidine-HCl	72	83
Urea	74	92
SDS	39	49
EDTA	101	65

[a] The effects of various chemical reagents (5 mM) on IMP and GMP formation were carried out using Reactive Red Agarose-purified HGPRTase. Each enzyme assay contained 0.84 μg of protein. Maximum activity in control reactions was 0.9 μmol min^{-1} mg^{-1} for IMP formation and 0.95 μmol min^{-1} mg^{-1} for GMP formation.

is similar to enzymes from lower organisms which are activated by Mg^{2+}, Mn^{2+}, and Zn^{2+}.

Effects of chemical reagents: The effects of various chemical reagents on Reactive-Red Agarose-purified HGPRTase from *M. thermoautotrophicum* were examined (Table 3.6). 2-Mercaptoethanol had no effect on either PRTase activity. PMSF, Guanidine-HCl, and urea showed slight inhibition of both PRTase activities. Addition of 5 mM SDS produced a marked reduction of *M. thermoautotrophicum* HGPRTase activities. Dithiothreitol showed slight stimulation of HPRTase activity but had no effect on GPRTase activity.

Dithiothreitol had been shown to activate HPRTase activity from *P. falciparum*, rat liver, and human erythrocytes. About 2.5-fold increase in activity of malarial enzyme was seen, whereas human enzyme showed 2-fold increase in enzyme activity only when exposed to dithiothreitol for a longer time (65 h at 3°C). HGPRTase from rat liver was strongly inactivated by *p*-chloromercuribenzoate and 5,5'-dithiobis(2-nitrobenzoic acid) (Natsumeda *et al.*, 1977). HPRTase activity from *Salmonella* sp., *P. falciparum*, and human erythrocytes was similarly inhibited by sulfhydryl reagents *p*-chloromercuribenzoate and *N*-ethylmaleimide. In other enzymes, the characteristic sensitivity to sulfhydryl reagents and activation by thiols may imply that one or more cysteine residues may be involved in the overall catalytic process in these cases. The role of cysteine residues in *M. thermoautotrophicum* HGPRTase remains to be determined.

Regulation of *M. thermoautotrophicum* HGPRTase activities by reaction products and other purine compounds: The possibility of inhibition by the purine nucleotide products and by a number of compounds including other

TABLE 3.7. Inhibition of *M. thermoautotrophicum* HGPRTase by various purine compounds[a]

Compound present (2 mM)	% Activity[b]	
	HPRTase	GPRTase
None	100 (1.8)	100 (0.3)
Adenine	112 (11.1)	82 (3.8)
Xanthine	112 (5.5)	150 (3.2)
AMP	103 (8.3)	100 (10.4)
ATP	91 (1.2)	82 (10.4)
IMP	57 (12.1)	41 (0)
GMP	95 (1.3)	88 (8.0)
GTP	86 (3.5)	87 (0.8)
Phosphate	111 (5.0)	94 (1.0)
PP_i	59 (3.8)	43 (0)

[a] Tests of the effects of purine compounds (2 mM) on the rates of IMP and GMP formation were carried out using Reactive Red Agarose-purified HGPRTase. Each assay contained 0.84 μg of protein. Maximum activity in control reactions was 0.8 μmoles min^{-1} mg^{-1} for IMP formation and 0.96 μmol min^{-1} mg^{-1} for GMP formation.

[b] The data represent the average (± standard error) of two determinations.

inhibitors of the HGPRTase from *M. thermoautotrophicum* but both activities were inhibited by IMP and PP_i. IMP inhibited HPRTase and GPRTase activities by 57 and 41%, respectively. Similarly, PP_i inhibited HPRTase and GPRTase activities by 59 and 43%, respectively. IMP and PP_i are end products of HGPRTase-catalyzed reaction. Interestingly, GMP the other product of this enzyme did not significantly inhibit both activities. Whether the IMP and PP_i end-product inhibition observed with *M. thermoautotrophicum* HGPRTase is physiologically relevant would be dependent upon intracellular substrate and product concentrations.

Inhibition of HGPRTase activities from other sources by purine compounds is well known. GMP was a more effective inhibitor than IMP for enzymes from rat brain, *S. pombe*, *L. donovani*, and *E. coli*. The 0.5 mM GMP inhibited about 90% of yeast GPRTase activity, whereas only 32% inhibition was seen with 0.5 mM IMP. Similarly, 1 mM GMP and 1 mM IMP inhibited 80% and 40% of *E. coli* XGPRTase activity, respectively. HGPRTase from *Artemia* sp. was inhibited by GMP but not by IMP. Bowen *et al.* (1996) reported that *M. voltae* GPRTase activity was inhibited by GMP, whereas IMP was ineffective. By contrast, GMP and IMP stimulated HPRTase activity in *M. voltae*. Additionally, PP_i, a common reaction product, was shown to inhibit HPRTase activity in human and *L. donovani*, and GPRTase activity in *S. pombe*.

Other purine compounds such as AMP, adenine, and xanthine did not inhibit *M. thermoauotrophicum* HGPRTase. In inhibition studies with HGPRTases from other sources, except for enzymes from mouse liver and rat brain, AMP does not seem to have any effect on enzyme from Chinese hamster brain, *E. coli*, and *M. voltae*. Adenine or xanthine did not affect protozoal HPRTase activity confirming xanthine phosphoribosyltransferase

(XPRTase) to be biochemically distinct from HGPRTase (Allen *et al.*, 1989; Allen and Ullman, 1993). Similarly, no inhibition of *M. voltae* HGPRTase by AMP, adenine or xanthine was seen (Bowen *et al.*, 1996). Our inhibition data and those of Bowen *et al.* (1996) may indicate substrate specificities of H- and GPRTase activities in methanogens. Since adenine did not inhibit either activity in either methanogen, these data suggest that methanogen HGPRTase may be biochemically distinguishable from adenine phosphoribosyltransferase (APRTase), the same as in other organisms. These suggestion is further supported by enzyme activity measurements in purine-analog-resistant mutant strains by Worrell and Nagle (1990). These strains lacked H- and GPRTase activities but contained normal levels of APRTase activity in the extracts. Lack of inhibition of either activity by xanthine also suggests that methanogenic HGPRTase may differ from XGPRTase of enteric organisms, which can also utilize xanthine as substrate. These data again indicate that HPRTase and GPRTase activities belong to a single protein in methanogens.

Inhibition of HPRTase activity by guanine, IMP, and PPi: Preliminary studies on the competitive inhibition of HPRTase by the alternative substrate guanine were carried out. If both enzyme activities (H- and GPRTase) had the same active site, then inhibition of either activity would be expected by the opposite base. 150-fold purified H- and GPRTase from a pilot Reactive Red column (data for this preparation not shown) was used to study inhibition of HPRTase activity by guanine.

The data in Table 3.8 show that guanine inhibited reactions at two different concentrations of hypoxanthine. At low concentrations of hypoxanthine (17 to 33 times its K_m), high concentrations of guanine (4 to

Table 3.8. Inhibition of *M. thermoautotrophicum* HPRTase activity by guanine

Hypoxanthine present (mM)	Guanine added (mM)				
	0[a]	0.13	0.27	0.40	0.83
	Activity, % control				
0.1	100[b]	45	36	45	2
0.2	100[b]	41	29	28	39

[a] 150-fold purified Reactive-Red Agarose fraction was used. HPRTase activity in absence of guanine at indicated hypoxanthine concentration was used as 100% control.

[b] HPRTase activity at 0.1 and 0.2 mM hypoxanthine was 0.1 and 0.22 nmol/min, respectively.

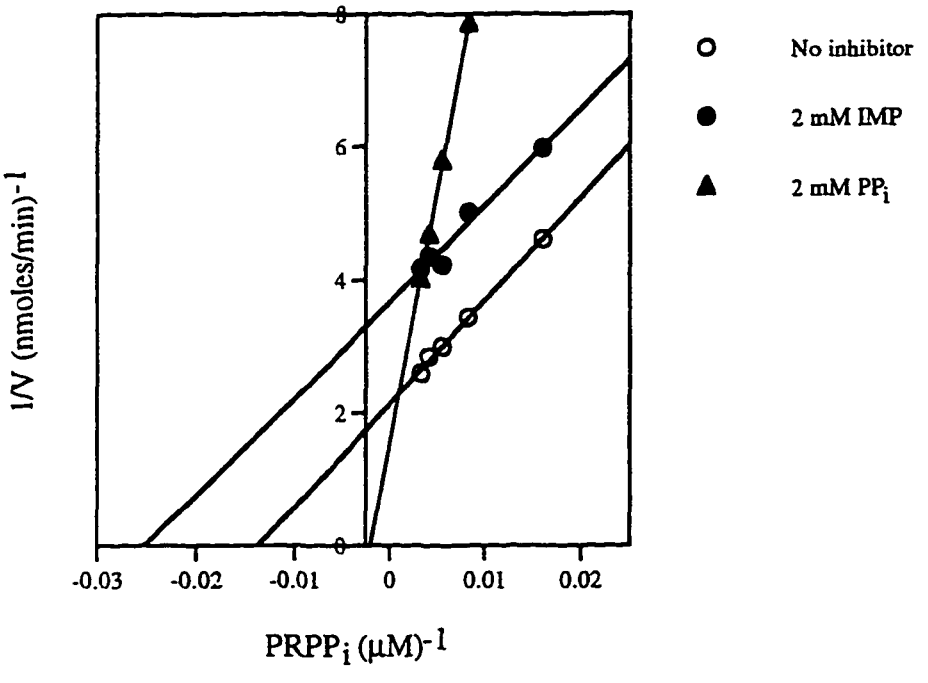

Fig. 3.12. Inhibition of *M. thermoautotrophicum* HPRTase activity with PRPP $_i$ by IMP and PP $_i$.
The standard assay was utilized, with hypoxanthine held constant at 0.2 mM and $PRPP_i$ was varied over the 60-300 μM range. The IMP and PP_i concentrations were 2 mM, respectively. Values are the averages of two determinations.

23 times its K_m) were slightly inhibitory (<50% inhibition) for HPRTase activity. The data were not sufficient to determine if guanine competitively inhibited HPRTase activity. In *M. voltae*, hypoxanthine and guanine were shown to inhibit GPRTase and HPRTase activity, respectively, and again, the inhibition pattern was inconclusive. However, each purine base was shown to competitively inhibit utilization of the other base for nucleotide synthesis in bifunctional HGPRTases from human (Jadhav *et al.*, 1979), yeast (Nussbaum and Caskey, 1981), and *B. subtilis* (Endo *et al.*, 1983).

Product inhibition studies were carried out to learn more about the HGPRTase reaction. When the product IMP was included in initial velocity assays in which $PRPP_i$ was varied (from 0.7 X K_m to 3.5 X K_m), the double reciprocal plot was parallel to that for reactions with no added IMP (Figure 3.12). This result suggests that IMP inhibition was uncompetitive with respect to $PRPP_i$ (Plowman, 1972).

With product PP_i (2 mM) included in similar assays, both the slope and intercept changed compared to the plot for reactions with no added product (Figure 3.12). The initial interpretation of such a plot would be that PP_i is competitive with respect to $PRPP_i$. However, the negative Y intercept of the PP_i curve is an unusual finding. Perhaps the most reasonable interpretation of this curve is that inhibition by PP_i is complex. PP_i is a product, a substrate analog (being a portion of $PRPP_i$), and a magnesium chelator. For the enzyme from human cells, in reports by Krenitsky and Papaioannou (1969) and Craft *et al.* (1970), inhibition by PP_i was a function of the level of magnesium in the reaction mixture; PP_i inhibition was more effective at lower concentrations of magnesium. In our assays, PP_i was at 2 mM concentration and Mg^{2+} was at 5 mM concentration. Variations in free Mg^{2+} might be expected to complicate kinetic interpretations.

Kinetic analysis of H- and GPRTase in general has yielded diverse and complicated inhibition patterns. At face value, our results with respect to $PRPP_i$ (IMP uncompetitive and PP_i competitive) are in contrast to the case of enzymes described from other sources. IMP has been reported to be competitive with $PRPP_i$ for human HPRTase activity (Henderson *et al.*, 1968; Krenitsky and Papaioannu, 1969); whereas for rodent brain enzyme and $PRPP_i$ it was neither strictly competitive nor strictly non-competitive (Chinese hamster brain enzyme, Olsen and Milman, 1974) or it exerted strong and complicated inhibition (rat brain enzyme, Gutensohn and Guroff, 1972). Thus, there is not one common inhibition pattern for HGPRTases.

For GMP with respect to $PRPP_i$, the human GPRTase enzyme (Henderson *et al.*, 1968; Krenitsky and Papaioannu, 1969) and the rat brain enzyme were inhibited competitively; the fission yeast (*S. pombe*) enzyme was inhibited in a non-linear competitive fashion (Nagy and Ribet, 1977). The human enzyme was non-competitively inhibited by GMP with respect to guanine.

The product PP_i yielded an unusual inhibition pattern by competitively inhibiting methanogen HPRTase with respect to $PRPP_i$ (Figure 3.12). This is in contrast to the results reported for the human GPRTase in which PP_i was a non-competitive with respect to both $PRPP_i$ and guanine.

We have not examined the kinetic mechanism for the HGPRTase reaction in *M. thermoautotrophicum*. These mechanisms for the HGPRTase-catalyzed reactions have been studied in human (Krenitsky and Papaioannou, 1969), yeast (Ali and Sloan, 1982), and *Schistosoma* sp. (Yuan *et al.*, 1992) with different conclusions. In the case of *Schistosoma* sp., substrates bind to the enzyme in a defined order (first $PRPP_i$ followed by the purine bases) followed by the ordered release of the products (first PP_i followed by purine

nucleotides). Yeast HGPRTase follows sequential ordered or random binding of substrates or release of products. Previously, a ping-pong kinetic mechanism was proposed for the human HGPRTase (Henderson *et al.*, 1968). Recently, careful studies suggested sequential ordered bi rapid equilibrium random bi mechanism for the human enzyme (Yuan *et al.*, 1992), where $PRPP_i$ binds to the enzyme first followed by the purine base, and the products (PP_i and the purine nucleotide) are released in a random fashion.

Effects of purine analogs on *M. thermoautotrophicum* HGPRTase activities: Various purine analogs were found to inhibit growth of *M. thermoautotrophicum* (Chapter 1 and Worrell and Nagle, 1990). The purine analogs may serve as a substrate for HGPRTase, and by its action, together with further phosphorylation and other modifications (eg., incorporation into RNA), begin to become toxic to the cell. We studied effects of some of the purine-analogs on HGPRTase activities from *M. thermoautotrophicum* in the standard assay (Table 3.9). 8-aza-2,6-diaminopurine, 2-aminopurine, 8-azaguanine, 6-thioguanine, and 6-chloropurine did not inhibit HPRTase activity, but showed marked inhibition of GPRTase activity. Hypoxanthine-analogs, 8-azahypoxanthine and 6-mercaptopurine inhibited HPRTase activity by about 30%. However, these analogs inhibited GPRTase activity by about 70%. It appears that GPRTase activity was sensitive to inhibition by all analogs tested, whereas HPRTase was inhibited by only two analogs. We did not determine whether these purine analogs were acting as substrates or inhibitors of HGPRTase activities. It should be noted that effects of purine-analogs on *M. thermoautotrophicum* HGPRTase activities were tested using standard enzyme assays containing hypoxanthine or guanine concentrations higher than their apparent K_ms.

TABLE 3.9. Effect of various purine analogs on HGPRTase activities in *M. thermoautotrophicum*[a]

Purine analog (1 mM)	% activity[b]			
	HPRTase		GPRTase	
None	100	(0.6)	100	(0.2)
8-Azahypoxanthine	63	(27)	26	(19.2)
6-Mercaptopurine	70	(3.9)	31	(6.4)
8-Aza-2,6-Diaminopurine	91	(0)	77	(0)
2-Aminopurine	101	(9.9)	63	(8.8)
8-Azaguanine	97	(3.4)	66	(3.5)
6-Thioguanine	114	(2.4)	30	(6.8)
6-Chloropurine	98	(4.2)	47	(5.8)

[a] The rates of IMP and GMP formation were determined in presence of 1 mM purine analog. Each enzyme assay contained 0.84 μg of Reactive Red Agarose purified HGPRTase. Maximum activity in control reactions was 1.3 μmoles min^{-1} mg^{-1} for IMP formation and 0.9 μmoles min^{-1} mg^{-1} for GMP formation.

[b] The data represent the average (± standard error) of two determinations.

Several examples of inhibition of HGPRTase by purine analogs have been reported. 8-azaguanine has been shown to serve as a substrate for GPRTase from *Salmonella*, and at 2 mM concentration inhibited 59% of GPRTase activity. Allopurinol was shown to inhibit *Eimeria tenella* HGPRTase, but not HGPRTase from chicken liver (Wang and Simashkevich, 1981). 6-Mercaptopurine and 6-thioguanine were potent inhibitors of chicken liver HGPRTase. 6-thioguanine was a strong inhibitor of HPRTase activity in *S. cerevisiae*. Allopurinol and 8-azaguanine inhibited HPRTase activity from *P. lophurae*. Compounds with substitution at the 6-position of the purine ring, such as 6-thioinosine and 6-chloropurine, were also effective inhibitors for HGPRTase from *P. lophurae*, *P. chabaudi*, and human. In *M. voltae*, both PRTase activities were inhibited by 8-azaguanine and 8-azahypoxanthine, whereas 2-thioxanthine inhibited only HPRTase activity. Interestingly, 8-aza-2,6-diaminopurine and 6-mercaptopurine did not inhibit either PRTase activity from *M. voltae*. Inhibition of *M. thermoautotrophicum* HGPRTase by some purine analogs was analogous to enzymes from chicken liver, yeast, and *M. voltae*.

Immunological study of *M. thermoautotrophicum* HGPRTase: A simple immunological characterization of *M. thermoautotrophicum* H- and GPRTase activities was attempted. Rabbit antiserum raised against the trypanosome *T. brucei* HGPRTase was used to see if there was a homology between the antigen determinants of the two enzymes. An ammonium-sulfate precipitated preparation of *M. thermoautotrophicum* HGPRTase was incubated with antiserum to the *T. brucei* HGPRTase. The antiserum failed to recognize *M. thermoautotrophicum* HGPRTase when immunoprecipitation followed by enzyme assay of the supernatant showed that the majority of *M.*

Table 3.10. Effect of antiserum against protozoal HGPRTase on *M. thermoautotrophicum* HGPRTase[a]

Tube	Addition (μg protein)	% Maximum activity	
		HPRTase	GPRTase
1	None (control)	100	100
2	Normal rabbit serum (1300)	96	107
3	Anti-HGPRTase crude serum raised against *T. brucei* (1350)	89	84

[a] Appropriate additions were made to 400 μg of *M. thermoautotrophicum* HGPRTase (2-fold-purified) obtained by ammonium sulfate precipitation. Rates of IMP and GMP formation in control assays were 0.5 μmol/min. Tubes were incubated in cold room for 14 h, and prior to assay, were centrifuged for 15 min at 14,000 X min^{-1}. The supernatant was assayed for HGPRTase activities.

thermoautotrophicum activities remained in the supernatant (Table 3.10). Only 11-16% of *M. thermoautotrophicum* HGPRTase activity was lost either due to inactivation by binding of or precipitation upon incubation with *T. brucei* HGPRTase antiserum after 14 h. There was total recovery of activity in tubes treated with non-immune serum. Since immune serum did not cause significant inactivation or precipitation of H- and GPRTase from *M. thermoautotrophicum*, there may be only a very limited homology between the *T. brucei* and the methanogen HGPRTase.

In other studies, mouse antiserum raised against chicken HGPRTase cross-reacted with hamster enzyme, indicating homology between the antigenic determinants of the two enzymes (Veres *et al.*, 1985). In contrast, *S. mansoni* HGPRTase (Dovey *et al.*, 1986) and *G. lamblia* (Aldritt and Wang, 1986) did not show any homology to human erythrocyte HGPRTase and *E. coli* XGPRTase. An antiserum raised against yeast HGPRTase also did not react with Chinese hamster or human HGPRTase (Nussbaum and Caskey, 1981). Also, no cross reactivity was seen between antiserum against rat brain HGPRTase with rabbit or human erythrocytes HGPRTase (Gutensohn and Guroff, 1972). The majority of studies indicate that HGPRTases from various sources may be immunochemically different from one another.

Stability of *M. thermoautotrophicum* HGPRTase: The purification of *M. thermoautotrophicum* HGPRTase was carried out aerobically at room temperature and at 4°C. Previous observations by Worrell (1991) suggested that aerobic conditions and choice of temperature had no differential effects on either activities or yields of these activities during purification. In our study, it was observed that Reactive Red Agarose-purified enzyme was stable

for at least 10 months at -20°C, and about 50% of initial activities were recovered after this period. In contrast, 65-100% ammonium sulfate pellet fraction or Reactive Red Agarose fraction heated in presence of $PRPP_i$ were highly stable at -20°C. In comparison, enzyme activities from *M. voltae* were similarly unaffected by air, but both activities were highly unstable to storage (Bowen *et al.*, 1996).

Magnesium and $PRPP_i$ are known to stabilize HGPRTase enzyme from different sources. $PRPP_i$ was shown to stabilize enzymes from *T. foetus* (Beck and Wang, 1993), *T. cruzi* (Allen and Ullman, 1994), and enteric bacteria (Hochstadt, 1978). $MgCl_2$ was also able to stabilize *T. foetus* HGPRTase. An exception was *P. lophurae* where $MgSO_4$ and not $PRPP_i$ stabilized the enzyme (Schimandle *et al.*, 1987). Some other properties of $PRPP_i$ are noteworthy. The role of $PRPP_i$ in protecting HGPRTases against heat is well known (Chapter 2). $PRPP_i$ was also able to protect rat liver HGPRTase from the inactivation by sulfhydryl agents and trypsin (Natsumeda *et al.*, 1977). Additionally, $PRPP_i$ was shown to interact with heat-denatured, inactive form of *E. coli* XGPRTase and led to its partial reactivation (Liu and Milman, 1983).

Amino-terminal sequence of HGPRTase from *M. thermoautotrophicum*: The amino-terminal sequence of the first 20 amino acids of the 28 kDa protein band assigned to *M. thermoautotrophicum* HGPRTase was determined (Table 3.11). Searches of the SWISSPROT, GenBank and PDB databases using the NH_2-terminal sequence of *M. thermoautotrophicum* HGPRTase did not reveal significant match to other proteins (Table a, Appendix). It is not unexpected since limited sequence was available to perform database search. A search of the *M. jannaschii* genomic sequence database (Bult *et al.*, 1996)

TABLE 3.11. Putative mRNA sequence for NH_2-terminal amino acid sequence of HGPRTase from *M. thermoautotrophicum*[a]

M L D K L K E S L R N S
AUG CUC GAU AAG CUC AAG GAG UCA CUC AGG AAC UCA

P V I K K G E Y
CCC GUU AUA AAG AAG GGA GAG UAC(U)

---

[a] Codons selected were based on frequency of use by *M. thermoautotrophicum* (Reeve *et al.*, 1986). Amino acids are listed above the corresponding codon.

using the putative codon sequence of the first 20 amino acids of *M. thermoautotrophicum* HGPRTase (Table 3.11; Reeve *et al.*, 1986) did not reveal any similarity. One explanation is that codon preference for certain amino acids may differ between *M. thermoautotrophicum* and *Methanococcus* sp. (Reeve *et al.*, 1986). We did not search *M. jannaschii* genomic database using degenerate code for these amino acids.

A vast amount of information is available about primary structure of HGPRTase in eucaryotes, eubacteria, and HPRTase and XGPRTase in enterics. These enzymes from different organisms appear to be composed of one type of subunit. The amino acid sequences of these enzymes are known, and three conserved regions in these amino acid sequences have been identified (Table 3.12). The stretches of amino acids corresponding to the putative purine binding site and $PRPP_i$ binding site of HGPRTase enzyme from eucaryotic organisms are found to be highly conserved (Craig *et al.*, 1988; Allen and Ullman, 1993). There is one more region found to be conserved at -COOH terminal of HGPRTase, HPRTase, and XGPRTase, believed to be involved in catalytic competence and binding of substrate (Table 3.12). Interestingly, $PRPP_i$-binding and catalytic competence regions were found to be highly conserved in eubacteria, which possessed a single enzyme for HGPRTase activities or possessed only HPRTase activity. In contrast, XGPRTases from *E. coli* and *S. typhimurium* have somewhat different amino acid sequences for these two regions that are conserved in HPRTases and HGPRTases. Such detailed analysis of primary structure of *M. thermoautotrophicum* HGPRTase will require isolation and sequencing of the gene.

Examinations of PRTase sequences after alignment also revealed a variety of amino acid deletions and insertions throughout their primary

Table 3.12. Amino acid sequences of HGPRTases, HPRTase, and XGPRTases from different organisms[a]

Human	MATRSPGVVISDDEPGYDLDLFCIPNHYAEDLERVFIPHGLIMDRTERLARDVMKEMGGHHIVALCVLKGGYKFFADLLDYIKALNRN SDRSIPMTVDFIRLKSYCNDQSTGDIKVIGGDDLSTLTGKNVLIVEDIIDTGKTMQTLLSLVRQYNPKMVKVASLLVKRTPRSVGYK PDFVGFEIPDKFVVGYALDYNEYFRDLNHVCVISETGKAKYKA
Mouse	MPTRSPSVVISDDEPGYDLDLFCIPNHYAEDLEKVFIPHGLIMDRTERLARDVMKEMGGHHIVALCVLKGGYKFFADLLDYIKALNRN SDRSIPMTVDFIRLKSYCNDQSTGDIKVIGGDDLSTLTGKNVLIVEDIIDTGKTMQTLLSLVKQYSPKMVKVASLLVKRTSRSVGYR PDFVGFEIPDKFVVGYALDYNEYFRNLNHVCVISETGKAKYKA
S. mansoni	MSSNMIKADCVVIEDSFRGFPTEYFCTSPRYDECLDYVLIPNGMIKDRLEKMSMDIVDYYEACNATSFTLMCVLKGGFKFLADLVDGL ERTVRARGIVLPMSVEFVRVKSYVNDVSHHEPILTGLGDPSEYKDKNVLVVEDIIDTGKTITKLISHLDSLSTKSVKVASLLVKRTSP RNDYRPDFVGFEVPNRFVVGYALDYNDNFRDLHHICVINEVGQKKFSVPCTSKPV
P. faciparum	MPIPNNPGAGENAFDPVFVKDDDGYDLDSFMIPAHYKKYLTKVLVPNGVIKNRIEKLAYDIKKVYNNEEFHILCLLKGSRGFFTALLKH LSRHHNYSAVEMSKPLEGEHYVRVKSYCNDQSTGTLEIVSEDLSCLKGKHVLIVEDIIDTGKTLVKFCEYLKKFEIKTVAIACLFIKR TPLWNGFKADFVGFSIPDHFVVGYSLDYNEIFRDLDHCCLVNDEGKKKYKATSL
B. subtilis	MMKHDIEKVLISEEEIQKKVKELGAELTSEYQDTFPLAIGVLKGALPFMADLIKHIDTYLEMDFMDVSSYGNSTVSSGEVKIIKDLDTSV EGRDILIIEDIIDSGLTLSYLVELFRYRKAKSIKIVTLLDKPSGRKADIKADFVGFEVPDAFVVGYGLDYAERYRNLPYIGVLKPAVY ES
R. capsulatus	MSQSGYVIDQMISAKAIAARVEALGAETTEAFKDTDRLVVVGLLRGSFVFIADLIREIGVPCEVDFLEASSYGNETTSTREVRVLKLRGD GGRDVLVVEDIIDTGITISKVMEMLRARAPRRIECCAMLDKPSRREVDVKARWTGFEIPDEFVVGYGLDYAQNHRNLPFIGTVR FTDPQ
E. coli HPRTase	MVRDMKHTVEVMIPEAEIKARIAELGRQITERYKDSGSDMVLVGLLRGSFMFMADLCREVQVSHEVDFMTASSYGSGMSTTRDVKIL KDLDEDIRGKDVLIVEDIIDSGNTLSKVREILSLREPKSLAICTLLDKPSRREVNVPVEFIGFSIPDEFVVGYGIDYAQRVRHLPVIG KVILL.
E. coli XGPRTase	MSEKYIVTWDMLQHIARKLASRLMPSEQWKGIIAVSRGGLVPGALLARELGIRHVDTVCISSYDHDNQRELKVLKRAEGDGEGFIVID DLVDTGGTAVAIREMYPKAHFVTIFAKPAGRPLVDDYVVDIPQDTWIEQPWDMGVVFVPPISGR
S. typhimurium XGPRTase	MSEKYVVTWDMLQHIARKLASRLMPSEQWKGIIAVSRGGLVPGALLARELGIRHVDTVCISSYDHDNQRELKVLKRAEGDGEGFIVI DDLVDTGGTAVAIREMYPKAHFVTIFAKPAGRPLVDDYVVDIPQNTWIEQPWDMGVVFVPPISGR

[a] The conserved regions purine binding site (underlined), $PRPP_i$ binding site (bold and underlined), and catalytic competence and substrate binding site (bold) are shown.

Table. 3.13. Reported comparison of HGPRTase amino acid sequences (% identity)[a]

	Human	Mouse	*S. mansoni*	*P. falciparum*	*T. brucei*	*V. harveyi* HPRTase	*E. coli* XGPRTase
S. mansoni	46		100				
P. falciparum	44		34	100			
T. brucei	21		23	22	100	36	
T. cruzii	24		25	21	50	36	
T. gondii	39		36	51	30	30	
T. foetus	27		25	23		36	
R. capsulatus	31	31	30	30		57	23
V. harveyi HPRTase	29		27	28		100	

[a] Adapted from Beckman and Kranz (1991); Allen and Ullman (1994); Chin and Wang (1994); and Vasanthakumar *et al.* (1994)

structures (Allen and Ullman, 1993). Amino acid regions outside the three conserved areas are highly divergent among the PRTase proteins with only a few regions of extensive homology or sequence identity among the aligned PRTase sequences. The amino acid sequence divergence among the HGPRTase proteins from phylogenetically diverse organisms suggest that conservation of primary structure is not critical for enzyme function. Table 3.13 shows % identity of sequences of H- and GPRTases from various sources. It can be seen that trematode and malarial enzymes are closely related to human enzyme (>40% identity). HGPRTase from *R. capsulatus* is closely related to *V. harveyi* HPRTase (>50% identity). Other comparisons reveal significantly less identity.

The majority of H- and GPRTase activity in *M. thermoautotrophicum* is associated with a single protein. However, it can not be ruled out that a small subset of an exclusively IMP-forming activity was detected. A summary of properties of H- and GPRTases is presented in Table 3.14, including data on the *M. thermoautotrophicum* enzyme. The temperature stability data for these enzymes are summarized in Table 2.1 in Chapter 2. Our investigations with highly-purified *M. thermoautotrophicum* HGPRTase have demonstrated that, in general, the methanogen HGPRTase resembles the eubacterial and eucaryotic enzymes with regard to its catalytic properties and subunit molecular weight. Although these enzymes in general do not show high similarity in their amino acid sequences, it is clear that subunit molecular weights, metal stimulation, and inhibition by end-products are similar to many other HGPRTases. These results suggest that purine salvage pathway may be very ancient and purine salvage reaction may have been present in the ancestor common to all organisms. It has been suggested that structural and functional homology observed in functionally related

phosphoribosyltransferases including HGPRTase indicates a common structural feature (binding of $PRPP_i$) and a common evolutionary origin (divergent evolution) (Musick, 1981). The more detailed comparison of *M. thermoautotrophicum* HGPRTase to other enzymes awaits. Isolation and sequencing of gene, which would be achieved with oligonucleotide probes based on the NH_2-terminal protein sequence, would allow such comparison. *M. thermoautotrophicum* is one of the well-studied moderately thermophilic methane-producing archaea (Ferry, 1993) and many of its genes have been cloned and sequenced (Reeve, 1992). Isolation of *hgprt* gene will also raise the possibility of using this gene as a selection marker in molecular studies (discussed in Chapter 1). Finally, the properties of *M. thermoautotrophicum* HGPRTase isolated are given in Table 3.15.

TABLE 3.14. Comparison of HGPRTases from different organisms

Organism	Structure (kDa) Subunit	Native	pH optima	K_m (μM) Hyx	Gua	$PRPP_i$	Inhibition[d]	Metal requirements
Human								
Erythrocytes[1]	34.5	α_2 (68)	8.7	17	5	250	IMP, GMP, GTP, PP_i, *p*-CMB	Mg^{2+}
	45	α_2 (85)						
	26	α_3 (81)						
	24	α_4 (100)						
Animal								
Beef brain[2]	26	α_3 (84)	9.8 [5.5–10][a]	0.99	0.42	2.9–18.6[b]		
Rodent								
Chinese ham. brain[3]	25	α_3 (78)	10 [5.5-11][a]	0.52	1.1	5.3	IMP, GMP	
Mouse liver[4]	27	α_3 (80)				200		
Rat brain[5]	26	α_3 (72)				200	GMP, GDP, GTP, UMP, CMP, AMP, ATP, IMP	
Bird								
Chicken brain[6]	26	α_4 (85)	10	5.2	1.8	20-50[b]		
Brine Shrimp								
Artemia[7]	19	α_4 (66)	7.0, 8.5-9	<1	<1	15	Ca^{2+}	Mg^{2+}, Mn^{2+}, Zn^{2+}
Trematode								
S. mansoni[8]	26	α_2 (105)		5.4	3	9-18[b]		
Yeast								
S. pombe[9]	42/48[c]	α (42/48)	7.6-8, 9.2-9.5		28	100	IMP, GMP, GTP, PP_i	Mg^{2+}
S. cerevisiae[10, 11]	51	α (51)	8.5	23	18	50		Mg^{2+}, Co^{2+}, Zn^{2+}
	29.5	α_2 (54.5)						
Protozoa								
C. fasciculata[12]								
HPRTase							GMP, GDP, GTP, IMP, ATP	Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}
GPRTase							GMP, GDP, GTP, IMP, ATP	Co^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}
L. donovani[13]	24	α_2/α_4 (60/110)	6.9, 7.4-9.1	7.6	3.8	65	GMP, GTP, IMP, PP_i	
G. lamblia[14]	29	α_2 (58)	8	514	291			
P. lophurae[15]		(79)	6-10, 7.5-9.5	3.8	2.4		6-CP, 8-AG	

P. falciparum[16]		(56)		0.46	0.3	21	Hg^{2+}, Cd^{2+}, Zn^{2+}, *p* CMB, *N*-ethylmaleimide	
T. brucei[17]	23.4			2.3	4.8		GTP, IMP, GMP, ATP, AMP	
T. cruzi[18]	29	α/α_2 (29/49)	8.2	6.4	9.9			Co^{2+}, Mn^{2+}, Mg^{2+}
T. foetus[19]	24	α (24)	7-9	4.1	3.8	74, 31[b]	Ca^{2+}, Ni^{2+}, Cd^{2+}	Mg^{2+}, Mn^{2+}, Zn^{2} Fe^{3+}, Ca^{2+}
E. tenella[20]	23		7.0-7.5	0.25	0.37			
Eubacteria								
E. coli								
HPRTase[21]		(75)	8.4	120		200	Ba^{2+}, Ca^{2+}, Zn^{2+}	Mg^{2+}, Mn^{2+}
XGPRTase[22]	18.6	α_3 (55)	7.9	167	2.6	95	GMP, XMP, IMP	Mg^{2+}, Mn^{2+}
S. typhimurium[23]								
XGPRTase				22	5	450		
Archaea								
Mc. volnae[24]		(90)	9	15	200		Guanine, GMP	
Mb. thermoautotro[25].	28		9.9 [6.3-10.5][a]	6.1	38.7	86.3	IMP, PP_i, PMSF, SDS	Mn^{2+}, Mg^{2+}, Zn^{2+}

1 Arnold and Kelley, 1971; Muensch and Yoshida, 1977; Olsen and Milman, 1977; Holden and Kelley, 1978
2 Paulus *et al.*, 1980
3 Olsen and Milman, 1974
4 Hughes *et al.*, 1975
5 Gutensohn and Guroff, 1972
6 Veres *et al.*, 1985
7 Montero and Llorente, 1991
8 Craig *et al.*, 1988; Yuan *et al.*, 1990
9 Nagy and Ribet, 1977
10 Schmidt *et al.*, 1979
11 Nussbaum and Caskey, 1981
12 Kidder *et al.*, 1979
13 Tuttle and Krenitsky, 1980
14 Aldritt and Wang, 1986
15 Schimandle *et al.*, 1987
16 Queen *et al.*, 1988
17 Allen and Ullman, 1993
18 Gutteridge and Davies, 1982; Allen and Ullman, 1994
19 Beck and Wang, 1993
20 Wang and Simashkevich, 1981
21 Hochstadt, 1978
22 Liu and Milman, 1983
23 Adye and Gots, 1965
24 Bowen *et al.*, 1996
25 This study

[a] Enzyme has a broad pH activity range
[b] Range for $PRPP_i$ K_m values with hypoxanthine and guanine
[c] Molecular weights of 42,000 and 48,000 found in the presence and absence of $MgCl_2$, respectively
[d] *Abbreviations*: *p*-CMB, *p*-chloromercuribenzoate; 6-CP, 6-chloropurine; 8-AG, 8-azaguanine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate

TABLE 3.15. Properties of *M. thermoautotrophicum* HGPRTase

Parameter	Result
Molecular mass (kDa) as determined by:	
Denaturing 10% polyacrylamide gel	28 kDa subunit
Temperature (optimum)	71-79°C
Temperature (stability)	$t_{1/2}$=4 min, 92°C, (-$PRPP_i$)
	$t_{1/2}$>23 min, 92°C, (+$PRPP_i$)
pH (optimum)	9.9 (6.3-10.5)
Metal requirements	$Mn^{2+} > Zn^{2+} \geq Mg^{2+}$
Inhibitors	IMP, PP_i
Kinetic constants (K_m):	
Hypoxanthine	6 μM
Guanine	39 μM
$PRPP_i$	86 μM

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CHAPTER 4

Effect of starvation on *Methanobacterium thermoautotrophicum*

ABSTRACT

Cells of *Methanobacterium thermoautotrophicum* were subjected to starvation by incubating them under H_2, N_2, and N_2:CO_2 headspaces and observing the changes in viability with time. In preliminary studies, it was found that *M. thermoautotrophicum* remained viable for at least 15 days when incubated under N_2:CO_2, but viability was lost much more rapidly under N_2 or H_2. The cells also lost viability when incubated under N_2:CO_2 either in presence of protein synthesis inhibitors neomycin or 5-methyl tryptophan compared to cells without compound, suggesting that protein synthesis may be involved in starvation survival. Adenine nucleobases and nucleotides had little effect on growth of *M. thermoautotrophicum*. The nucleoside adenosine, by an unknown mechanism, may have contributed to extending the viability of cells starved for H_2.

INTRODUCTION

In nature, bacteria encounter situations where essential nutrients are available in limiting amounts due to competition. In response to nutritional scarcity or starvation, many eubacteria such as *Bacillus* and *Clostridium* sp. differentiate into endospores, or *Myxobacterium* sp. into myxospores. However non-differentiating bacteria do not show any comparable morphological changes.

How do non-differentiating eubacteria maintain viability during a starvation period? Even though these organisms are at a disadvantage of not making spore-like structures, many bacteria have evolved highly sophisticated mechanisms that allow them to maintain viability during starvation and resume growth when nutrients are available (Siegele and Kolter, 1992). Dramatic physiological responses in such microorganisms are well documented. A summary of physiological changes during starvation of bacteria is shown in Table 4.1. The physiological studies have provided insights into the mechanism of survival. In *E. coli*, about 30-50 proteins were induced when cells were starved for an essential carbon source, such as glucose (Groat *et al.*, 1986). *Vibrio* sp. strain S14 was induced for synthesis of about 60 proteins during starvation for glucose, amino acids, ammonium, or phosphate (Nystrom *et al.*, 1990). Although functions of these stress proteins are not known, the importance of protein synthesis in these organisms during starvation was shown by inhibitory effects of chloramphenocol and rifampin on long-term survival, suggesting that *de novo* synthesized gene products were essential.

Among the archaea, effect of environmental conditions on survival of some species has been documented. In the methanogenic archaea, a study

Table 4.1. Various responses reported in eubacteria upon starvation[a]

1. morphological changes such as reduction in cell size, formation of periplasmic space
2. protein degradation
3. RNA degradation
4. DNA level stable or increased
5. decrease in carbohydrate level
6. changes in fatty acid profile
7. reduction in endogenous respiration rate
8. decrease in ATP level
9. decrease in adenylate energy charge
10. viability loss
11. sporulation
12. synthesis of stress proteins
13. utilization of storage polymers (PHB and polyphosphate)[b]
14. shift to higher affinity nutrient transport
15. maintenance of proton motive force

[a] Unless otherwise specified, adapted from Morgan and Dow (1986).
[b] From Dawes, 1985; Matin, 1992.

in *Methanospirillum hungatii* indicated that cells lost considerable viability under an H_2 gas phase compared to N_2, and H_2-N_2 gas-phases (Breuil and Patel, 1980). Declines in ATP, RNA, protein, carbohydrate, and amino acid pools were observed over a 30 day period of carbon starvation. In an unpublished study, McInerney (personal communication) studied loss of viability of *Methanobacterium ruminantium* strains M1, isolated from the rumen, and P1, isolated from an anaerobic sewage digester, over a 20 h period. He found that the M1 strain was much more sensitive than the P1 strain to H_2-CO_2 starvation as measured by viable counts. Another environmental stress, heat shock (exposure of cells to rapid shifts in temperature) has been studied in a mesophilic methanogen *Methanococcus voltae* (Hebert *et al.*, 1991), an extreme halophile *Halobacterium halobium* (Daniels *et al.*, 1984), and an extreme thermophile *Sulfolobus* sp. strain B12 (Trent *et al.*, 1990). These organisms induced a number of heat shock proteins (HSPs) in response to a shift in temperature (Macario and Macario, 1994).

Methanobacterium thermoautotrophicum Marburg is a methane-producing, thermophilic, autotrophic archaebacterium which grows at the expense of H_2:CO_2. Little is yet known about the effects of environmental challenges, such as energy- and carbon-starvation, on this organism. Studies by Jane Bradley (1991) in this laboratory suggested that energy starvation caused cells to change in morphology; cultures starved for H_2 and CO_2 contained cells that were smaller in size than those in cultures with excess H_2:CO_2. Such starved cells seemed to be better able to undergo genetic transformation, although reasons for this were not known. The purpose of the present study was to investigate the effect of energy and carbon starvation on *M. thermoautotrophicum*. Nucleobases and protein synthesis inhibitors were

used to determine if viability was further compromised, and perhaps to understand about a survival mechanism for this archaeon.

MATERIALS AND METHODS

Organism and growth conditions: *Methanobacterium thermoautotrophicum* Marburg was routinely cultivated in a liquid mineral medium of Balch *et al.* without yeast extract or vitamin supplements (Chapter 1) at 60°C in a shaking incubator.

Preparation of Gelrite plates: In addition to the ingredients used for making liquid medium, additional $CaCl_2$ (2 mL of 5% w/v), $MgSO_4$ (0.4 mL of 25% w/v) and Gelrite (0.8 g, Kelco, San Diego, Calif.) were added per 100 mL of medium.

Viability of *M. thermoautotrophicum* under different gas phases: Three 500 mL bottles containing 100 mL of liquid mineral medium were inoculated with actively growing culture of *M. thermoautotrophicum*, and pressurized to 170 kPa of H_2:CO_2 (80:20). The bottles were incubated horizontally at 60°C with shaking. The bottles were incubated for 48 h with flushing and refeeding with H_2:CO_2 at 19, 26 and 39 h of incubation to keep culture actively growing. After 48 h, an appropriate amount of sample was removed for viable counts. Then the H_2:CO_2 gas phase of all three bottles was replaced with H_2 (100%), N_2 (100%), and N_2:CO_2 (80:20%), respectively, by repeated flushing and evacuation for a total of 5-10 min. The bottles were incubated at 60°C and viable counts were performed after different intervals of incubation.

The ten-fold dilutions for viable counts were done inside the anaerobic chamber using sterile 96-well ELISA plates. The sterile liquid mineral medium was used as diluent and 100 µL was spread onto Gelrite plates. The plates were incubated in pressure vessels at 205 kPa H_2:CO_2 (80:20) with a

flush of H_2S. The plates were incubated for approximately 5-7 days until a drop in the vessel pressure was observed. Colonies were then counted and CFU/mL were calculated.

Effect of neomycin and 5-methyl tryptophan on viability of *M. thermoautotrophicum* under an N_2:CO_2 gas phase: To determine the MIC (smallest amount of antibiotic required to inhibit growth) of neomycin, different concentrations of antibiotic were added to the culture tubes. A tube without an antibiotic served as a positive control. The growth was monitored over a 72 h period. In survival experiments, *M. thermoautotrophicum* cells were grown in 160 mL serum bottles containing 40 mL of liquid mineral medium. Neomycin and 5-methyl tryptophan (Sigma Chemical Co., St. Louis, MO) at final concentration of 200 µg/mL and 50 µg/mL, respectively, were added to two separate experimental cultures just before changing the gas phase to N_2:CO_2. The control culture did not receive any additions. The viability of culture under different conditions was monitored for 7 days.

Effect of neomycin on *M. thermoautotrophicum* starvation under different gas phases and its recovery from such conditions: *M. thermoautotrophicum* culture (0.5 mL) was inoculated into 5 mL of mineral medium in Balch tubes. The tubes were pressurized to 275 kPa H_2:CO_2 (80:20). After 20 h of growth, 200 µg/mL of neomycin (Sigma Chemical Co., St. Louis, MO) was added to selected tubes, and the gas phase of some tubes was changed to N_2:CO_2 (80:20) by repeated flushing. The growth was monitored as absorbance for 120 h.

Effect of adenine nucleotides/nucleobases addition on growth of *M. thermoautotrophicum*: Filter-sterilized adenine derivatives (adenine, adenosine, 5'-AMP, ATP, cyclic AMP (cAMP), dibutyryl-cAMP (DB-cAMP); Sigma Chemical Co., St. Louis, MO) were added to liquid medium prior to inoculation. Growth was measured as absorbance (660 nm). After 27 h, tubes were repressurized; to some tubes an additional 2 mM compound was added (final concentration, 4 mM) and absorbance followed for additional 3 h.

Adenosine's effect on viability of cultures was tested as follows. After 6 h growth in mineral medium, duplicate cultures received 2 mM adenosine, controls received sterile water, and tubes were repressurized to 275 kPa with H_2:CO_2 (80:20). Tubes were incubated for 97 h and then used to inoculate quadruplicate tubes of medium as a measure of surviving cells. Growth was followed as absorbance.

RESULTS

Survival of *M. thermoautotrophicum* under H_2, N_2, and N_2:CO_2: Figure 4.1 shows the results of incubation of *M. thermoautotrophicum* cells under headspaces consisting of H_2, N_2, and N_2:CO_2 (These data are also summarized in Table 4.2). At time zero, the initial numbers of cells (3.7 X 10^8 cells/mL^{-1}) were equivalent under different gas phases. After 48 h, only 3.9% of cells were viable under H_2, and 61% were viable under N_2. Assuming exponential decay over the first two days, the log reductions in number per day were 0.66 and 0.1, respectively. In contrast, under N_2:CO_2 a 63% increase in viable count was observed. Sufficient energy reserves or carry over of H_2 from the chamber atmosphere may have sufficed for less than a doubling of cell number. After 144 h, viable counts under H_2, N_2, and N_2:CO_2 were 0.003%, 0.78%, and 21.2% of initial values, respectively. Between 48 and 144 h, the slopes of these exponential decay curves were 0.75, 0.48, and 0.21 log units per day. At the end of 240 h, no viable cells were found under H_2 or N_2 headspaces, whereas 2.6% of cells were viable under N_2:CO_2. No viable cells were found under N_2 and N_2:CO_2 after 360 h of incubation. The pH of culture medium under H_2 gas phase after 360 h of experiment was 9, and that of N_2 and N_2:CO_2 was 8. The prolonged survival of *M. thermoautotrophicum* under N_2:CO_2 gas phase prompted us to investigate the mechanism of survival. Preliminary experiments were designed to determine if protein synthesis was involved, using the protein synthesis inhibitors neomycin and 5-methyl tryptophan.

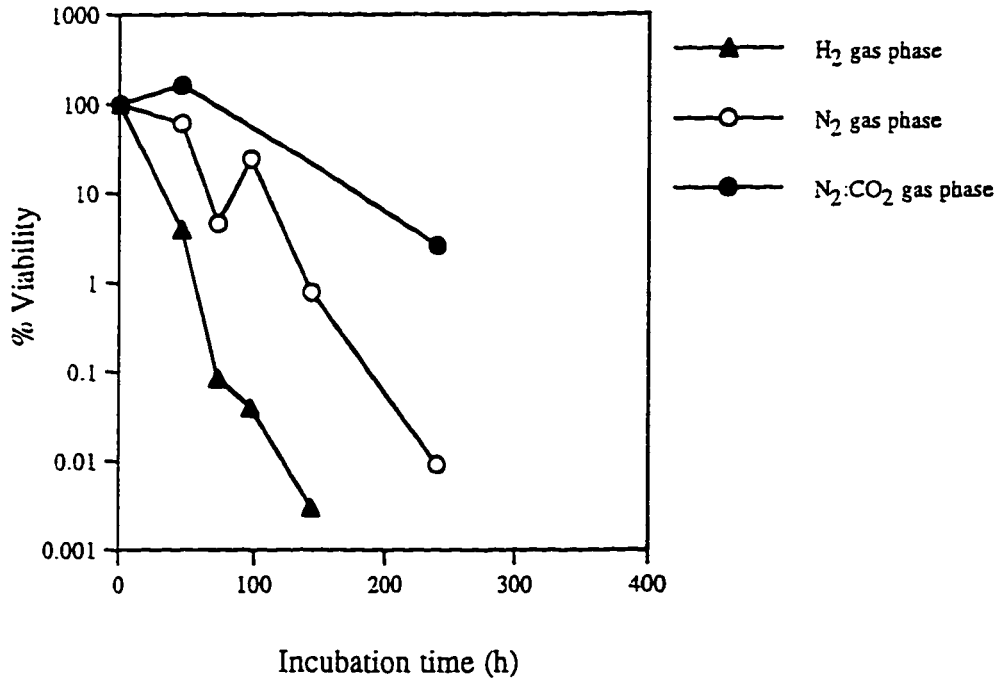

Fig. 4.1. Viability of *M. thermoautotrophicum* under different gas phases. % viability is expressed as ratio of viable counts after incubation to viable counts at time zero (3.7×10^8 mL^{-1}).

Table 4.2. Viability of *M. thermoautotrophicum* under different gas phases[a]

Incubation Time (h)	Gas Phase	Log Reduction in Cell number/day[b]	% Viable cells remaining[c]
0-48	H_2	0.66	3.9
	N_2	0.1	61.0
	N_2:CO_2	-0.095	163.3
48-144	H_2	0.75	0.003
	N_2	0.48	0.78
	N_2:CO_2	0.21	21.2
144-240	H_2	0.75	0
	N_2	0.47	0.009
	N_2:CO_2	0.2	2.6

[a] see also Figure 4.1.

[b] log reduction in cell number/day represents the slopes of lines in Figure 4.1 over the time period indicated.

[c] Viable counts were calculated from plates made from 3 dilutions in a series; the standard error for colony-forming units was in the range of $\pm$ 6-13.

Minimum inhibitory concentration of neomycin for *M. thermoautotrophicum*: Neomycin has been found to be stable at high temperature (72°C), pH of 7.3 for 3 days (Peteranderl *et al.*, 1990). Neomycin at a concentration of 50 μg/mL inhibited growth to some extent; 200 μg/mL neomycin completely inhibited the growth of *M. thermoautotrophicum* in liquid mineral medium (Figure 4.2). In contrast, 5-methyl tryptophan did not affect growth of wild type cells up to a concentration of 200 μg/mL (not shown).

Effect of neomycin and 5-methyl tryptophan on survival of *M. thermoautotrophicum* : Preliminary experiments were designed to test the possibility that neomycin and 5-methyl tryptophan (another protein synthesis inhibitor) would influence survival of *M. thermoautotrophicum*. To study the effect of neomycin on survival of *M. thermoautotrophicum* under N_2:CO_2, neomycin (200 μg/mL final concentration) was added to the starving culture. Neomycin had a strong negative effect on survival of cells under N_2:CO_2. As shown in Figure 4.3 and Table 4.3, after 3 days 12% of treated cells remained viable compared to 50% without neomycin. After 7 days, the effect was even stronger: 0.002% of treated cells were viable compared to 0.5% in control cultures. The magnitude of the effect is clear from the difference in slopes of survival curves with and without neomycin.

5-methyl tryptophan at a concentration of 200 μg/mL also had a negative effect on survival of cells under N_2:CO_2, even though it did not inhibit growing cells. After 7 days, only 1/77 as many cells treated with 5-methyl tryptophan survived as untreated controls (Figure 4.3 and Table 4.3). The observation that neomycin and 5-methyl tryptophan resulted in a significant loss of viability in N_2:CO_2-incubated *M. thermoautotrophicum*

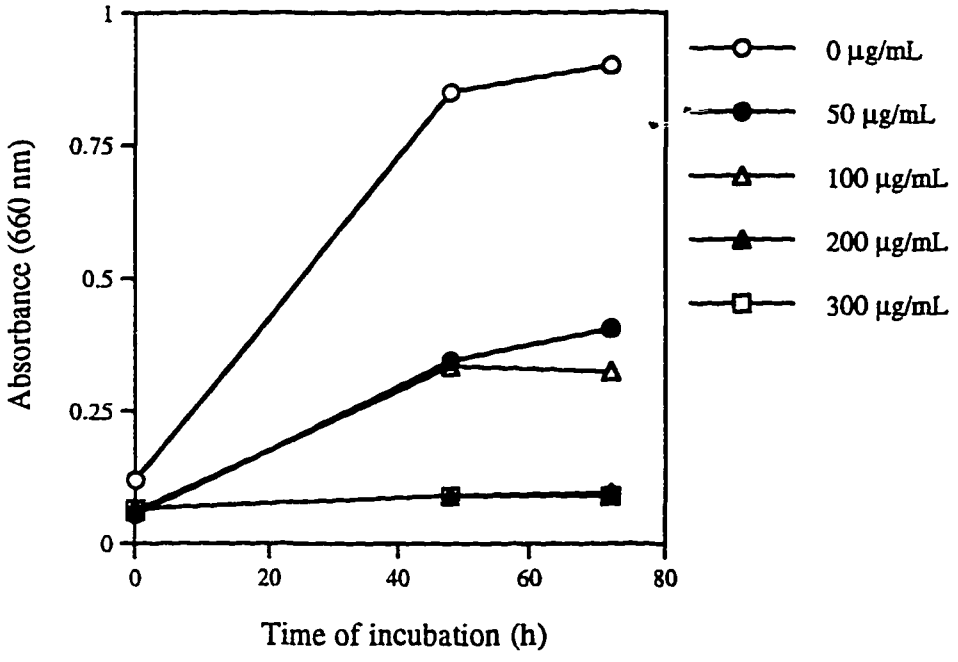

Figure 4.2. Inhibition of growth of *M thermoautotrophicum* by neomycin.

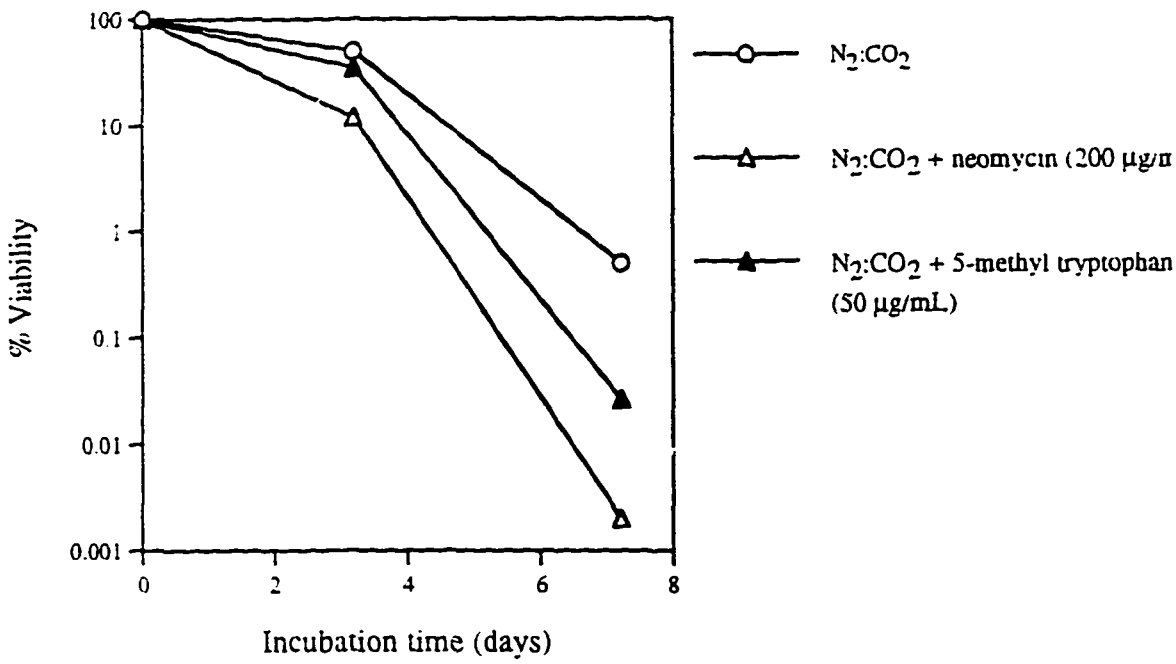

Figure 4.3. Viability of *M. thermoautotrophicum* under N_2:CO_2 with other additions. % viability is expressed as ratio of viable counts after incubation to viable counts at time zero (1.4 X 10^8 mL^{-1})

Table 4.3. Effect of neomycin (200 μg/mL) and 5-methyl tryptophan (50 μg/mL) on viability of *M. thermoautotrophicum* when incubated in presence of N_2:CO_2[a]

Incubation Time	Condition	Log reduction in cell number/day[b]	% Viability[c]
3 days	N_2:CO_2	0.16	50
	+ Neomycin	0.32	12
	+ 5-methyl tryptophan	0.21	35
Between 3-7 days	N_2:CO_2	0.5	0.5
	+ Neomycin	0.955	0.002
	+ 5-methyl tryptophan	0.755	0.026

[a] See also Figure 4.3.

[b] Log reduction in cell number/day represents slopes of lines in Figure 4.3 over the time periods shown.

[c] The viability counts were done on duplicate plates and Standard Deviation for colony-forming units was in the range of ±0-13.

cells suggested that protein synthesis may be involved in survival. To further confirm our observation, another set of experiments were carried out where we compared recovery and growth of N_2:CO_2-incubated cells with and without neomycin in fresh medium.

Recovery of *M. thermoautotrophicum* from prolonged incubation under various conditions: The recovery of *M. thermoautotrophicum* cultures pregrown under various conditions (H_2:CO_2, H_2:CO_2 plus neomycin, N_2:CO_2, and N_2:CO_2 plus neomycin) was studied by inoculating another set of tubes of liquid mineral medium and the growth was compared (Figure 4.4). The new set of medium did not receive any additions. The H_2:CO_2 and H_2:CO_2 plus neomycin pregrown cultures did not grow after 3 days when transferred to liquid mineral medium, suggesting no cells were viable. But N_2:CO_2 preincubated culture grew without any lag phase. The N_2:CO_2 plus neomycin pregrown culture also grew but with a lag phase of approximately 10 h (Figure 4.4), suggesting that viable cell numbers were much less.

Effect of exogenous cAMP and adenine nucleotides/nucleobases: The putative control molecule cAMP and its presumably permeable lipophilic analog DB-cAMP were tested as possible inhibitors of growth. At low concentrations, there was no effect on growth; at 4 mM cAMP and DB-cAMP, growth was reduced to 88% and 79% of untreated cultures, respectively (Table 4.4 and Table 4.5).

In a pilot experiment, other adenine nucleotides and nucleobases derivatives were tested. After 27 h, there were few differences between treated and untreated cultures with the exception of the adenosine-treated

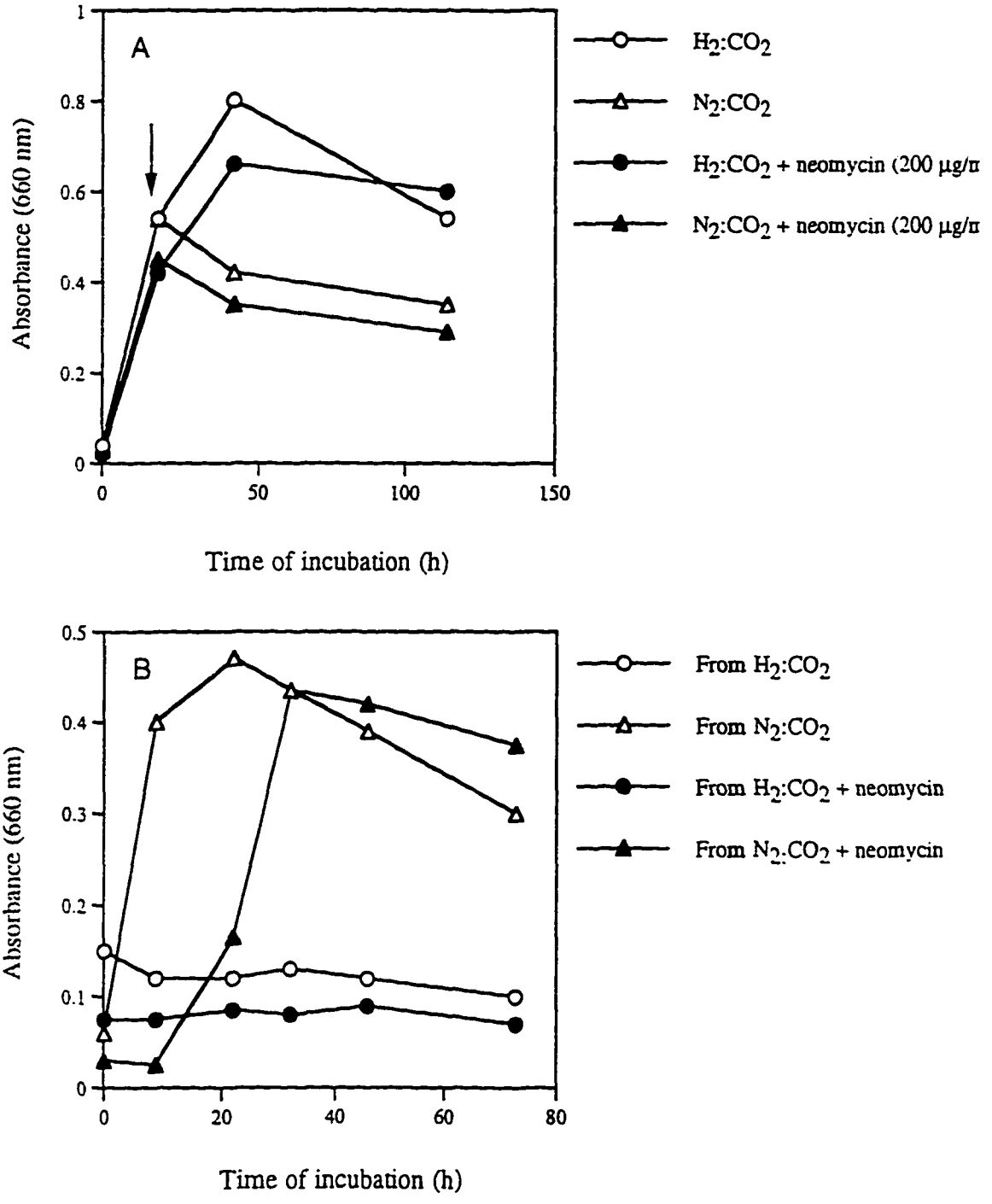

Figure 4.4. Recovery of *M. thermoautotrophicum* under various conditions.

A. (top) Preincubation conditions. The *arrow* indicates the time when additions were made and gas phases were replaced.

B. (bottom) Outgrowth experiments. Symbols indicate the source of 118 h cells from fig. 4.3A used to inoculate fresh medium. Data represent the average of duplicate tubes.

Table 4.4. Effect of exogenous cAMP on growth of *M. thermoautotrophicum*[a]

cAMP concentration (mM)	Absorbance (660 nm)[b]	Growth (% control)
0	0.80	100
1	0.90	119
2	0.80	100
3	0.80	100
4	0.70	88

[a] The cells were grown in liquid mineral medium under H_2:CO_2.
[b] n=1

Table 4.5. Effect of exogenous adenine nucleotides/nucleobases on growth of *M. thermoautotrophicum* under H_2:CO_2

Compound	Concentration (mM)[a]		Absorbance[b]		Growth % Control	
	Initial	27 h	27 h	30 h	27 h	30 h
Control	0		0.52	0.90	100	100
DB-cAMP	0.5		0.53	0.95	102	105
	1		0.52	0.90	100	100
	2	4	0.49	0.74	94	82
ATP	1		0.51	0.95	98	105
	2	4	0.51	0.90	98	100
5'-AMP	1		0.54	0.95	104	105
	2	4	0.54	0.85	104	94
Adenine	1		0.52	1.00	100	111
	2	4	0.52	0.85	100	94
Adenosine	1		0.54	0.80	104	89
	2	4	0.66	0.75	127	83

[a] At 27 h, tubes were repressurized with H_2:CO_2, and additional compounds were added (to achieve 4 mM total concentration).

[b] Data represent the average of duplicate determinations (S.D. $\pm$ 0.02).

cultures which had grown to 127% of control; such slight stimulation by adenosine was seen in other experiments. When additional compounds were added at 27 h, there was slight inhibition (6-17%) by AMP, adenine and adenosine.

The results of adenosine treatment (Table 4.5) suggested there were viability differences in treated cultures. In the experiment of Figure 4.5, cultures with 2 mM adenosine grew to higher absorbance values and maintained higher absorbance after 97 h. When the 97 h cultures were transferred into fresh, unsupplemented medium, adenosine-grown cells grew with little or no lag whereas untreated cells lagged for more than 20 h. This result suggests that more viable cells may have remained in the adenosine-treated culture. Viability counts for this experiment were not carried out.

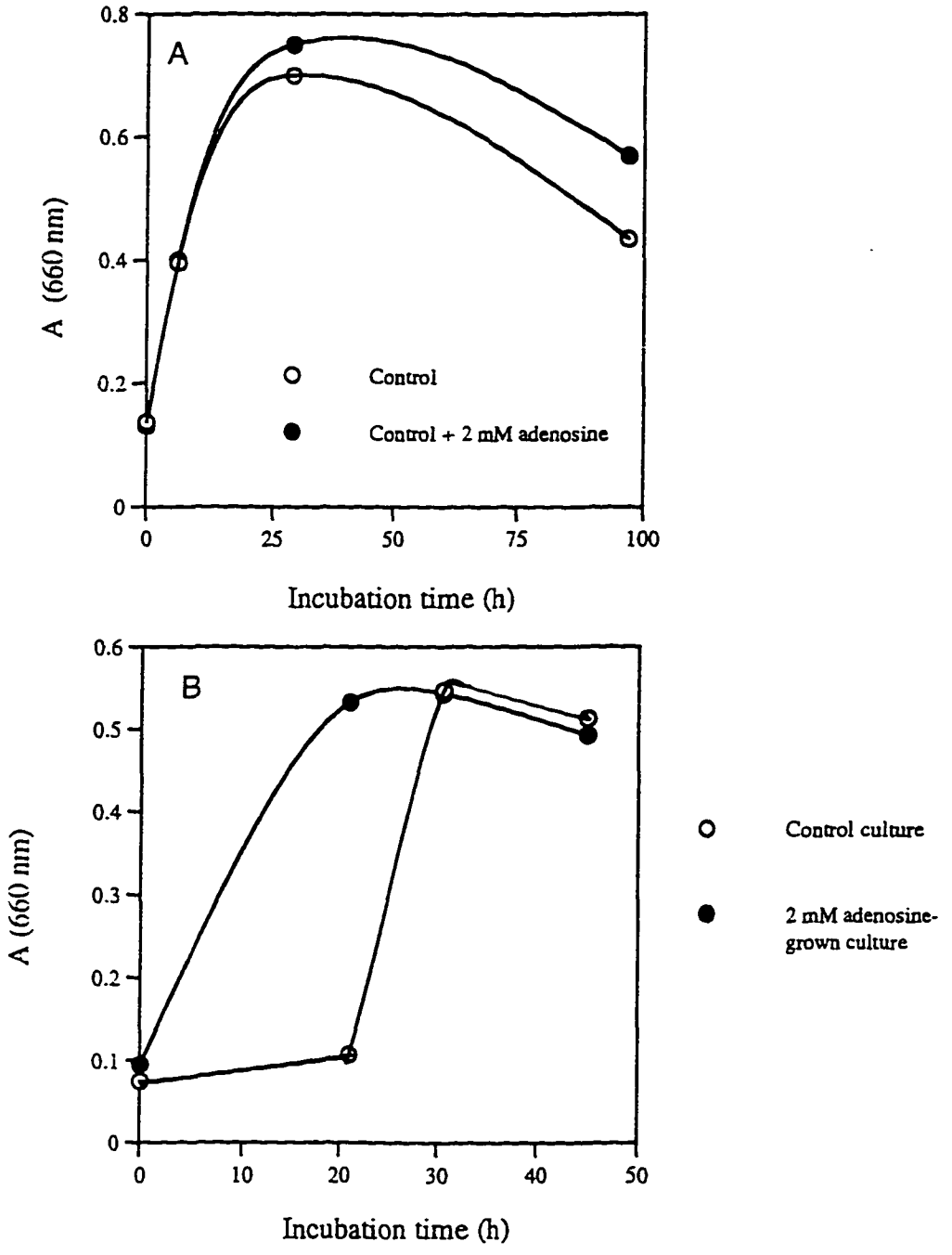

Figure 4.5. Effect of adenosine on growth and recovery of *M. thermoautotrophicum* .
A (top) Growth of *M. thermoautotrophicum* in presence of adenosine.
B (bottom) Symbols indicate the source of inoculum from fig. 4.5A used to inoculate fresh unsupplemented medium after 97 h.

DISCUSSION

The data indicated that starvation of *M. thermoautotrophicum* in the presence of H_2 gas-phase resulted in a greater loss of viability than under N_2 or N_2:CO_2 gas-phase. Under H_2 there were no viable cells after 7 days, whereas under N_2 or N_2:CO_2 cultures lost viability more slowly. The N_2:CO_2 gas-phase was the most favorable of all three gas-phases for survival of *M. thermoautotrophicum*. In studies with another methanogen, a similar pattern was observed. *M. hungatii* lost viability at a faster rate under H_2 than under N_2 (Breuil and Patel, 1980). Although *M. thermoautotrophicum* was able to maintain some viability for at least 7 days in presence of H_2, a drastic effect of pH on viability under H_2 gas phase can not be ruled out. The optimum pH for growth of *M. thermoautotrophicum* is around 7.5, and pH of starving culture after 10 days under H_2 was 9. It may be necessary to test if pH was responsible for drastic viability loss; pregrown *M. thermoautotrophicum* cells can be suspended in a carbonate-free medium adjusted to various pH values and viabilities under H_2 can be compared.

Survival under N_2:CO_2, the most favorable gas phase, may have involved protein synthesis. Both neomycin (aminoglycosidic antibiotic) and 5-methyl tryptophan (amino acid analog) adversely affected the survival of *M. thermoautotrophicum*. The former, a non-bacteriocidal compound (Weisberg and Tanner, 1982) irreversibly combines with translating ribosomes preventing protein synthesis (Elhardt and Bock, 1982). The latter, an amino acid analog, would make faulty proteins if incorporated into them.

In the recovery experiment, N_2:CO_2 plus neomycin preincubated culture was slower to recover in the fresh mineral medium than N_2:CO_2 preincubated culture, suggesting that the number of viable cells was decreased in presence of neomycin. This was confirmed by viable count data

for cells incubated under N_2:CO_2 in presence of neomycin, as there was a significant decrease in survival of *M. thermoautotrophicum*. One possible explanation for the effect of neomycin on *M. thermoautotrophicum* cells incubated under N_2:CO_2 is that new proteins must be synthesized for survival. *M. thermoautotrophicum* cells were also less viable when starved in the presence of 5-methyl tryptophan. However, studies with 5-methyl tryptophan raised an important question. 5-methyl tryptophan did not affect the growing cells of *M. thermoautotrophicum*, but it did seem to reduce the viability in starving culture under N_2:CO_2. At this point it is not clear how cells are affected by 5-methyl tryptophan under an energy-depleted conditions and not under energy-replete conditions. It is conceivable that a subset of proteins being synthesized during starvation might contain sufficient 5-methyl tryptophan to become poorly functional. Under the energy replete condition, cells might ignore utilization of this compound, or synthesize sufficient *L*-tryptophan to overcome inhibition at the concentration used. It would be interesting to see if 5-methyl tryptophan has any effect on an *M. thermoautotrophicum* auxotroph requiring *L*-tryptophan (Rechsteiner *et al.*, 1986). The results would indicate whether 5-methyl tryptophan can act as a tryptophan analog in *M. thermoautotrophicum*, and may help to explain the adverse effect of 5-methyl tryptophan seen on starving wild type cells in this study.

Protein synthesis was required in some eubacteria for survival from starvation, as shown with antibiotics (protein synthesis inhibitors) and amino acid analogs. In well-studied organisms, duration of starvation and mechanisms for survival are well known. In pure culture survival experiments, *Escherichia coli* was found to be viable from several weeks to 1 year (Kolter, 1992; Siegele and Kolter, 1992). Nitrifying bacteria,

Pseudomonas cepacia , and *Thiobacillus* sp. were found to be able to survive for 5 years, 14 years, and 54 years, respectively. In a study by Reeve *et al.* (1984b), addition of chloramphenicol or amino acid analogs (*S*-2-aminoethylcysteine, 7-azatryptophan, and *p*-fluorophenylalanine) to glucose-starved *E. coli* culture resulted in a rapid loss of viability. Similarly, addition of chloramphenicol at the onset of starvation to *Vibrio* sp. resulted in significant decrease in viability (Nystrom *et al.*, 1990). The induction of stress proteins has been reported to occur in *E. coli* (Reeve *et al.*, 1984a), *Salmonella typhimurium* (Spector *et al.*, 1986) *Vibrio* sp. (Nystrom *et al.*, 1990) in response to nutritional starvation. Genetic studies have allowed identification of genes that are not essential during growth but whose products may be required to maintain viability during starvation. Tormo *et al.* (1990) isolated the *E. coli surA* (survival) gene that was functional during stationary phase, and an *E. coli* mutant lacking this gene (surA$^-$) was not capable of survival.

cAMP is a global regulator in *E. coli* involved in regulation of protein synthesis during starvation (Schultz *et al.*, 1988). cAMP has been shown to exist in archaea (Leichtling *et al.*, 1986), but its function in these organisms is not documented. In our studies, there was no evidence for significant inhibition of growth of *M. thermoautotrophicum* by cAMP, DB-cAMP or other adenine nucleoside/nucleotide compounds.

Adenosine seemed to extend viability of starving *M. thermoautotrophicum* cells, and allowed growth in fresh mineral medium without any lag. Moore *et al.* (1995; ASM abstract) reported that *M. thermoautotrophicum* strain ΔH converted adenosine into hypoxanthine *via* inosine, and that hypoxanthine was not degraded after several days of incubation. Extracts of *M. thermoautotrophicum* Marburg were shown to

contain adenosine deaminase activity (26 U/mg) converting adenosine to inosine and also measurable inosine phosphorylase activity (Worrell and Nagle, 1990). The role that adenosine plays in survival of *M. thermoautotrophicum* is unknown. Further experiments such as viability counts of *M. thermoautotrophicum* incubated in the presence of adenosine are needed to confirm our results.

The preliminary studies on starvation survival of *M. thermoautotrophicum* suggest that the organism utilizes a mechanism(s) to survive in an energy-limiting situations which is not understood at present. The ribosome (Davis *et al.*, 1986; Dawes, 1976) and protein degradation (Reeve *et al.*, 1984b) in starving bacteria may provide some energy to maintain viability, whereas storage granules are involved in other bacteria to supply carbon and phosphate during starvation periods (Dawes, 1985; Morita, 1988). *M. thermoautotrophicum* under normal growth conditions has been shown to contain a phosphate compound, cyclic diphosphoglycerate (cyclic DPG) (Seely and Fahrney, 1983), however its role during growth or starvation is not known (Ferry, 1993). The high energy phosphate compound, pyrophosphate (PP_i), seems to provide energy during starvation in many bacteria (Reeves, 1987; Wood, 1987). Although cell-free extracts of *M. thermoautotrophicum* ΔH contained pyrophosphatase activity, ^{31}P-NMR did not detect PP_i in *M. thermoautotrophicum* strain Marburg or strain ΔH (Ferry, 1993). Another reserve material, glycogen, has been found in *Methanolobus* sp. and *Methanococcus* sp. (Konig *et al.*, 1985), but its presence in *Methanobacterium* sp. is unknown.

The starvation survival study can have important implications in an ecological and basic context since *M. thermoautotrophicum* may have to survive in natural environments with little or no energy or carbon source.

Additional studies will be required to see if starvation-specific proteins are made in *M. thermoautotrophicum* upon onset of starvation. It will be interesting to perform pulse-chase experiments by adding radiolabelled amino acid to cells at the onset of starvation, and resolve proteins on two-dimensional polyacrylamide gels by the method of O'Farrell. Polypeptide patterns of a culture after imposition of starvation conditions can be compared to a nonstarved culture, and would show whether new proteins are synthesized or any newly synthesized proteins are increased or decreased in amount relative to the nonstarved control. Such starvation-specific proteins can be compared to eucaryotic and eubacterial proteins, to look for the possibility of functional and evolutionary relationships. Stress proteins are found to have high degree of conservation from eucaryotic to eubacterial species. Some studies have suggested that the stress response in archaea is similar to that in eucaryotes and eubacteria (Daniels *et al.*, 1984).

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