1-1-2014

Functional Studies Of 5-Carboxyaminoimidazole Ribonucleotide Synthetase And Mutase From Thermotoga Maritima

Mingyang Zhao
Wayne State University,

Functional Studies of 5-Carboxyaminoimidazole Ribonucleotide Synthetase and Mutase from *Thermotoga maritima*

by

MINGYANG ZHAO

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2014

MAJOR: PHARMACEUTICAL SCIENCES

Approved By:

_____

Advisor Date

DEDICATION

In dedication to my beloved mother and father

who give me courage and support.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Professor Steven M. Firestine for his patience, enthusiasm and insight. His guidance helped me a lot in my master's research, my course studying and thesis writing. Without his guidance, this thesis would not have been possible.

I would like to thank my committee members, Professor Christine Chow and Professor Timothy L. Stemmler, for their encouragement, insightful comments and constructive criticism.

I am also thankful to Professor David Oupicky of the University of Nebraska Medical Center, Professor Fei Chen and Professor Randall L. Commissaris for their encouragement and advice for my master's study and further career plan.

In addition, I would like thank my laboratory members, Cale Streeter, Qian Lin, Zhiwen Shi and Dr. Shiv Sharma. They are the best lab mates I've ever worked with.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

APRT	Adenine phosphoribosyltransferase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
PurF	Amidophosphoribosyltransferase
AICAR	Aminoimidazole-4-carboxamide ribonucleotide
PurH	5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase
AIR	5-Aminoimidazole ribonucleotide
PurM	Aminoimidazole ribonucleotide (AIR) synthetase
NH_3	Ammonia
Asp	Aspartic acid
bp	Base pair(s)
HCO_3^-	Bicarbonate ion
BSA	Bovine serum albumin
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
k_{cat}	Catalytic constant
CO_2	Carbon dioxide
CAIR	4-Carboxy-5-aminoimidazole ribonucleotide
N^5-CAIR	N^5-Carboxyaminoimidazole ribonucleotide

PurE	N^5-Carboxyaminoimidazole ribonucleotide (N^5-CAIR) mutase (Class I)
PurK	N5-Carboxyaminoimidazole ribonucleotide (N5-CAIR) synthetase
cAMP	Cyclic adenosine monophosphate
dNTP	Deoxyribonucleotide
DNA	Deoxyribonucleic acid
Em	Emission wavelength
U	Enzyme unit
Ex	Excitation wavelength
FAICAR	5-Formamido-4-imidazolecarboxamide ribonucleotide
FGAR	N-Formylglycinamide ribonucleotide
FGAM	N- Formylglycinamidine ribonucleotide
PurT	Formylglycinamide ribonucleotide (FGAR) synthetase (prokaryotic)
PurL	Formylglycinamidine ribonucleotide (FGAR) synthetase
GAR	Glycinamide ribonucleotide
PurD	Glycinamide ribonucleotide (GAR) synthetase
PurN	Glycinamide ribonucleotide (GAR) transformylase
GMP	Guanosine monophosphate
HEPES	N-(2-Hydroxuethyl)-piperazine-N-(2-ethanesulfonic acid)
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
IMP	Inosine monophosphate

PurJ	Inosine monophosphate (IMP) cyclohydrolase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LD	Lactate dehydrogenase
LB	Luria-Bertani media
V_{max}	Maximum velocity
T_m	Melting temperature
K_m	Michaelis constant
NADH	Nicotinamide adenine dinucleotide (reduced form)
OD	Optical density (absorbance)
PEP	Phosphoenolpyruvic acid
PRPP	5-Phosphoribosylpyrophosphate
PRA	5-Phospho-D-ribosylamine
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PK	Pyruvate kinase
PurB	SAICAR lyase
SAICAR	Succino 5-aminoimidazole-4-carboxamide ribonucleotide
PurC	N- Succinyl 5-aminoimidazolyl-4-carboxamide ribonucleotide synthetase
Taq	Taq polymerase
Tris	Tris-(hydroxymethyl)aminomethane

1. Introduction

1.1 Purines

1.1.1 Structure and function of purines

A purine is a heterocyclic compound which consists of a pyrimidine and an imidazole ring (Figure 1.1). Purines play significant roles in the genetic and metabolic processes of all living organisms. The purine bases, adenine and guanine (shown in Figure 1.1) are fundamental components of nucleotides, which are the building blocks of DNA and RNA. Purines are also crucial components of many other biomolecules such as the energy storage molecule ATP, signaling molecule cAMP and the cofactor NADH.

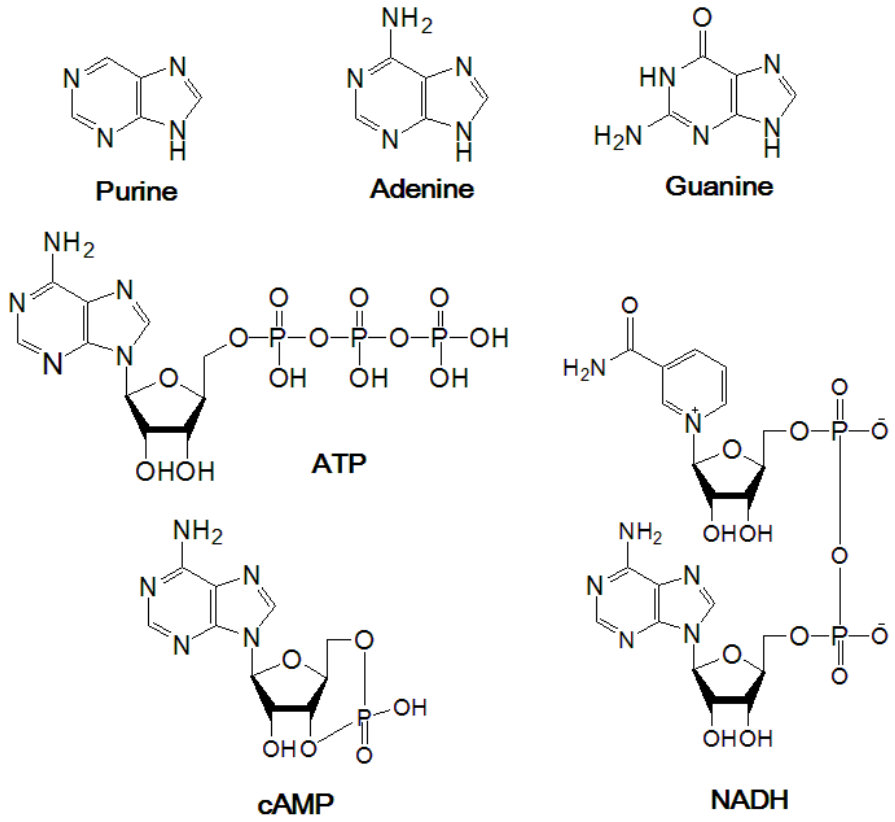


Figure 1.1 Structures of purine, adenine, guanine, ATP, cAMP and NADH.

1.1.2 Purine biosynthesis pathways

Given the critical roles played by purines, two pathways exist for their synthesis. Purines can be synthesized either by the *de novo* pathway or the salvage pathway.

The salvage pathway for purine synthesis is a recycling biochemical pathway in which the purine heterocycles created from the degradation of complex biomolecules, such as DNA, are reused to synthesize new purine nucleotides.[1] Phosphoribosyltransferases are criticial salvage pathway enzymes which catalyze the reaction between the bases and PRPP (Scheme 1.1).[2] In animals, this pathway is important for some cells which lack the enzymes required in the *de novo* pathway, such as the red blood cell.[3]

$$\text{PRPP} + \text{Adenine} \xrightarrow{\text{APRT}} \text{AMP} + \text{PP}_i$$

$$\text{PRPP} + \text{Hypoxanthine} \xrightarrow{\text{HGPRT}} \text{IMP} + \text{PP}_i$$

$$\text{PRPP} + \text{Guanine} \xrightarrow{\text{HGPRT}} \text{GMP} + \text{PP}_i$$

Scheme 1.1 Salvage pathway of purine biosynthesis.

De novo is a Latin phase meaning "from the beginning". *De novo* biosynthesis refers to the process of complex molecule synthesis from simple starting materials. The *de novo* purine pathway begins with PRPP (shown in Figure 1.2). Glutamine donates an amino group to PRPP at C-1 to yield PRA. This reaction is catalyzed by the enzyme, PurF. In the next step glycine is added to generate GAR. After a formylation reaction, GAR is converted into FGAR. In the following step, glutamine provides the source of nitrogen to generate FGAM. Dehydration and ring closure

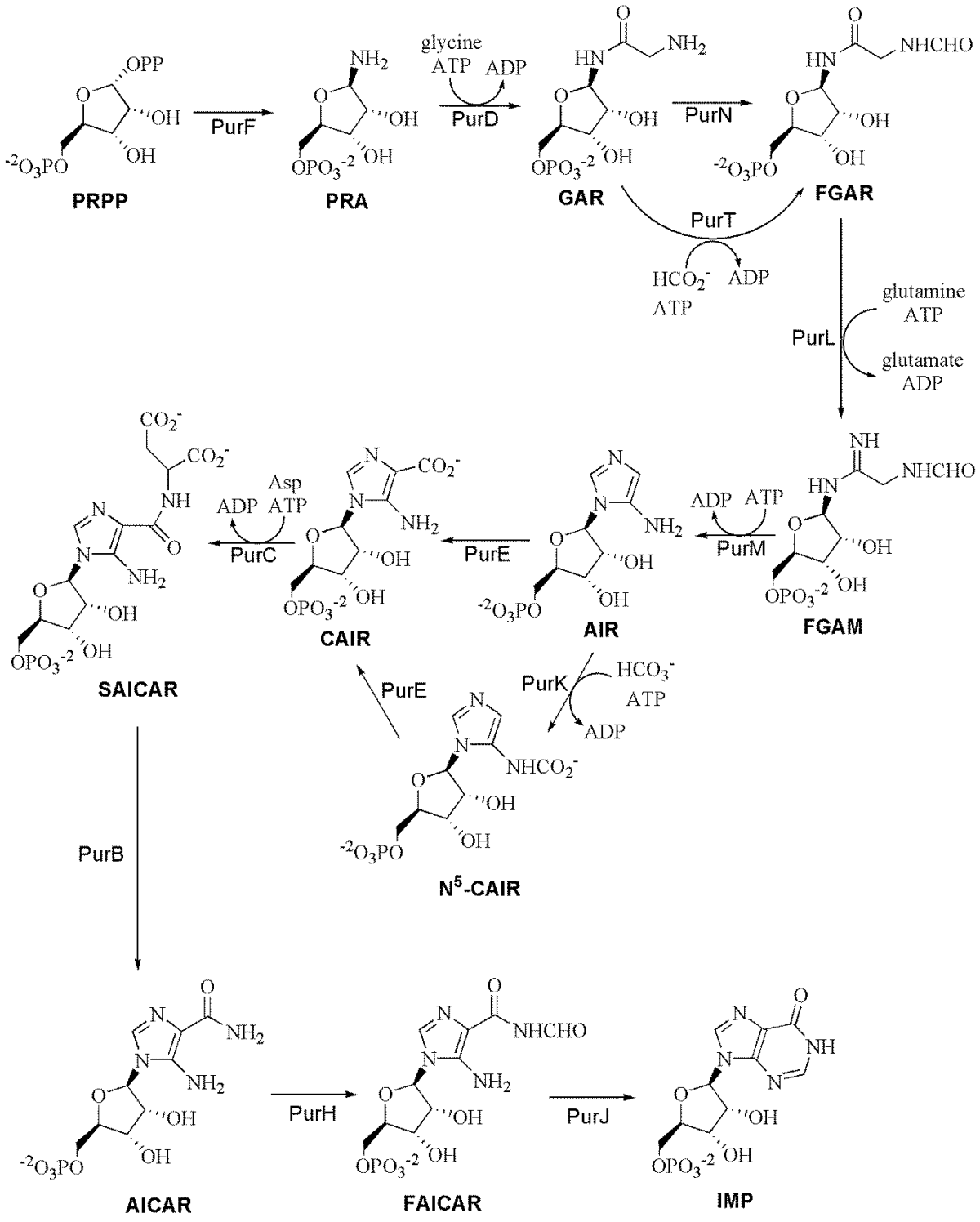


Figure 1.2 *De novo* purine biosynthesis pathway.

yields AIR. AIR is ultimately converted into CAIR by processes which differ depending on the organisms. Aspartic acid is then added to CAIR to yield SAICAR. In the following step, SAICAR is converted to AICAR, which is formylated and cyclized to yield IMP, which is the first ribonucleotide containing a purine ring. IMP is then converted to GMP and AMP to give the basic purine nucleotides.

In general, purine synthesis is mostly conserved in all living organisms.[4] The only known divergence in *de novo* purine biosynthesis occurs between microorganisms and higher eukaryotes.[5] The difference occurs in the sixth step of the pathway (shown in figure 1.3). In bacteria, fungi and yeast, AIR is converted into CAIR via the intermediate N^5-CAIR. Two enzymes are required, namely N^5-CAIR synthetase and N^5-CAIR mutase.[6] The enzymes are also frequently referred to as PurK and Class I PurE respectively. PurK converts AIR, ATP and bicarbonate to N^5-CAIR, ADP and Pi. Class I PurE transfers the carboxyl group of N^5-CAIR from N-5 to C-4 of the aminoimidazole ring. In higher eukaryotes, AIR is directly converted to CAIR in one step by the enzyme AIR carboxylase. AIR carboxylase is designated as a Class II PurE. Class II PurEs utilize carbon dioxide as the source of the carboxyl group.[7]

The divergence of the *de novo* purine biosynthesis pathways between microorganism and human suggests that the sixth step may be a good antimicrobial target. Inhibitors of Class I PurE and PurK are currently being studied as antimicrobial agents.[8]

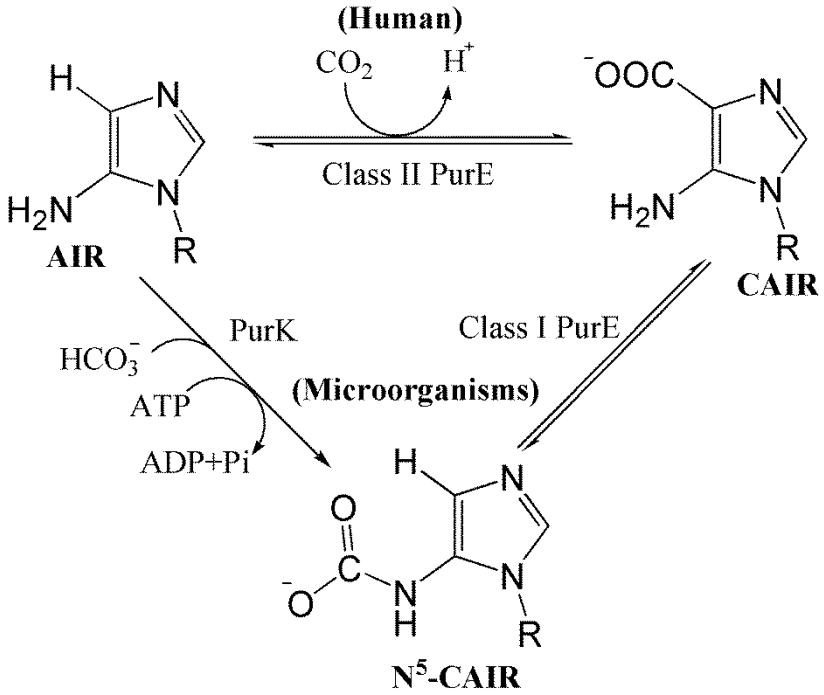


Figure 1.3 Divergence at the sixth step of purine synthesis in human and microorganisms. R is ribose 5'-phosphate.

1.1.3 Structure and function of PurK and PurE

The crystal structures of both PurK and PurE from various organisms have been published (Figure 1.4).[9,10,11,12] PurK is a functional dimer[6] which belongs to the ATP grasp superfamily of enzymes.[10] A single subunit of PurK contains three domains which are named the A, B and C domains.[10] The A domain contains the AIR binding site. Conserved residues in the B and C domains are responsible for the binding of ATP. In functional studies, PurK catalyzes the activation of bicarbonate by ATP to yield carboxyphosphate. Then the amino group of AIR attacks either CO_2 generated from carboxyphosphate or carboxyphosphate itself to give N^5-CAIR.[6]

Both Class I and Class II PurEs have been studied. The crystal structures of PurEs from a variety of organisms have been determined.[9,11,13] PurE is an octamer

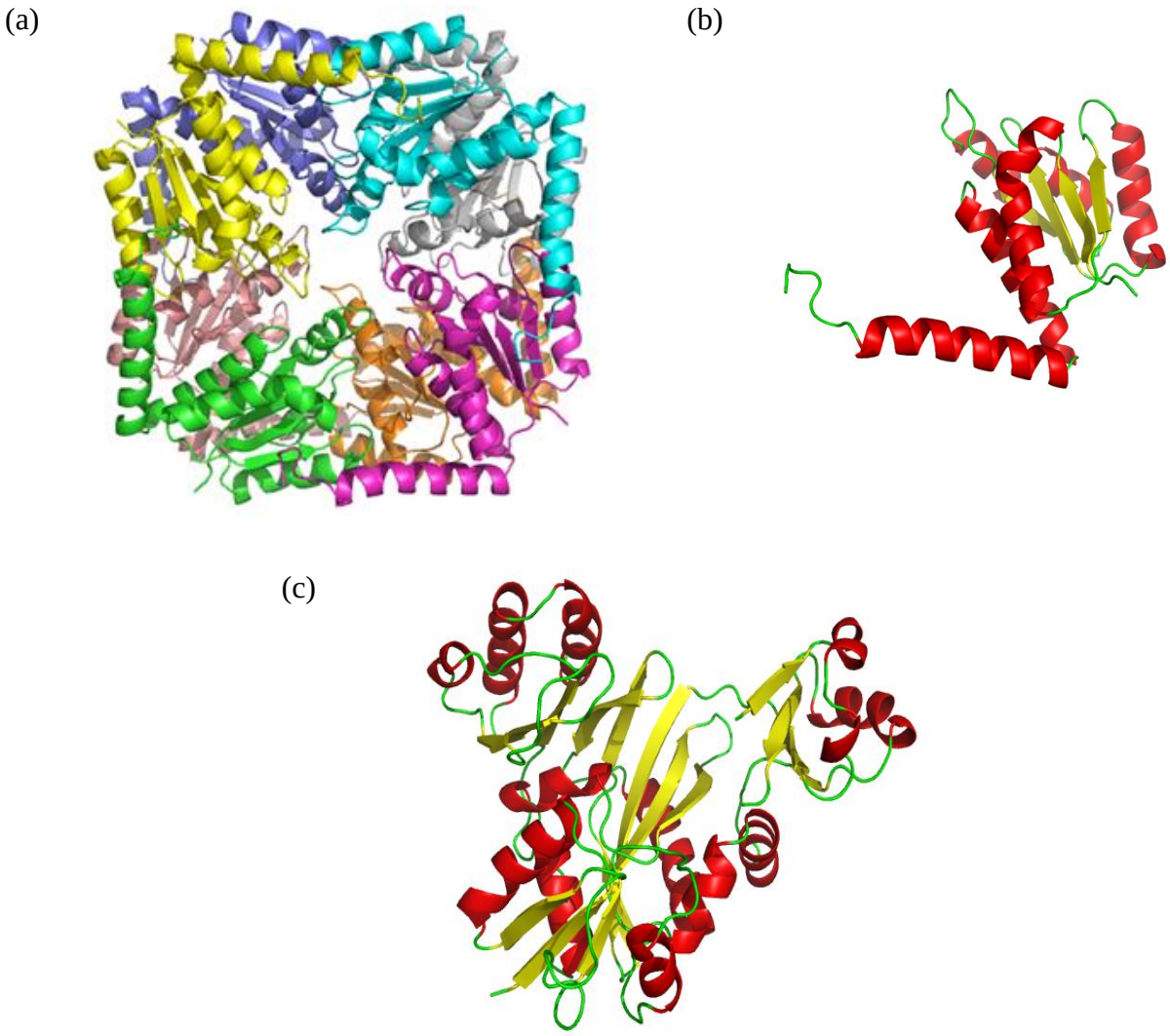


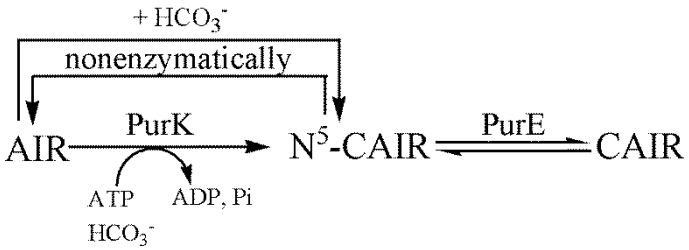
Figure 1.4 Crystal structures of (a) PurE (octomer), (b) PurE (monomer) and (c) PurK (monomer) from *E. coli*.

composed of eight identical subunits.[9,13] Class II PurEs, which are designated as AIR carboxylases, catalyze direct conversion of AIR into CAIR. In some organisms, such as the yeast *Cryptococcus neoformans*, a bifunctional enzyme containing both PurK and PurE activity is created.[14] In higher eukaryotes, like humans, the enzyme is also a bifunctional protein containing AIR carboxylase (Class II PurE) and SAICAR synthetase (PurC).[15] In humans, this bifunctional enzyme is called PAICS

(phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase). Sequence alignments between Class I and Class II PurEs show that these enzymes are evolutionarily related to each other. However, biochemical studies have shown the Class I and Class II PurEs are specific for their own reaction.[14]

1.1.4 Instability of N^5-CAIR

N^5-CAIR was an unknown intermediate in de novo purine biosynthesis until its discovery in the 1990's, some 40 years after most of the pathway was elucidated.[6] One reason for this is the fact that N^5-CAIR is a primary carbamate and thus unstable (shown in Scheme 1.2). N^5-CAIR has a $t_{1/2}$ of ~1 min at 30°C and pH 7.8 and it is nonenzymatically converted back to AIR.[15] The $t_{1/2}$ of N^5-CAIR varies depending upon pH and temperatures. The stability of N^5-CAIR decreases with decreasing pH and increasing temperature.[15] The $t_{1/2}$ at pH 8.4 is ~12 min, but at pH 7.6 is ~3 min.[6] At 5°C the $t_{1/2}$ is ~33 min. N^5-CAIR can also be produced from AIR nonenzymatically.[16] AIR and 1 M HCO_3^- produce N^5-CAIR so rapidly that it is difficult to monitor. The amount of N^5-CAIR generated from AIR and HCO_3^- is proportional to the concentration of HCO_3^-. In the presence of 1 M HCO_3^-, 75% of AIR is converted



Scheme 1.2 Instability of N^5-CAIR.

to N^5-CAIR.[16]

The instability of N^5-CAIR creates a significant challenge for the purine biosynthetic

pathway. If the production of N^5-CAIR is not regulated relative to its conversion to CAIR, ATP utilization can rapidly become non-stoichiometric. A key, unresolved question is how N^5-CAIR synthesis and utilization are regulated. One method to study this is to investigate the system under conditions in which N^5-CAIR stability is limited. Given the fact that N^5-CAIR is unstable at higher temperatures, studying N^5-CAIR synthesis with thermophilic proteins could provide insight into the problem.

1.2 *Thermotoga maritima*

1.2.1 Discovery and features of *T. maritima*

T. maritima is a non-spore forming, rod-shaped Gram-negative bacterium, which was originally isolated from geothermal heated marine sediments at Vulcano, Italy, and the Azores.[17] This extremely thermophilic bacterium grows between 80°C and 105°C, with an optimal growth temperature of 80°C.[17]

The genome of *T. maritima* has been sequenced.[18] Both purE and purK genes, which are designated TM0446 and TM0447 individually, have been found in the genome sequence. This suggests that *T. maritima* follows the *de novo* purine biosynthetic pathway commonly found in microorganisms. Such a result may be unexpected given the instability of N^5-CAIR noted above.

1.2.2 Purine biosynthesis in *T. maritima*

The TM0446 and TM0447 genes of *T. maritima* encode for N^5-carboxyaminoimidazole ribonucleotide mutase (TM0446, PBD code 1O4V) and synthetase (TM0447, PBD code

3AX6) respectively.

The crystal structure of PurE (TM0446) shows the protein is an octamer with an inner channel (Figure 1.5). The closest structural homologue of TM0446 protein is PurE from *E. coli*. The residues at the active site are the same between these two PurEs. The similarity between TM0446 and *E. coli* PurE in both gene sequence and protein structure indicates that TM0446 may have the same catalytic activity as the standard Class I PurEs previous studied.

The structure of TM0447 was studied as well (Figure 1.5).[8] It is a dimer and contains an ATP-grasp domain, indicating that TM0447 also belongs to the same superfamily as *E. coli* PurK. This provides a hint that TM0447 may have the same function as PurK from *E. coli*.

While the crystal structures of these proteins have been reported, no biochemical studies on these proteins have been published. Thus, while the available information suggests that these proteins have the same activity as bacterial enzymes, no verification of this has been presented.

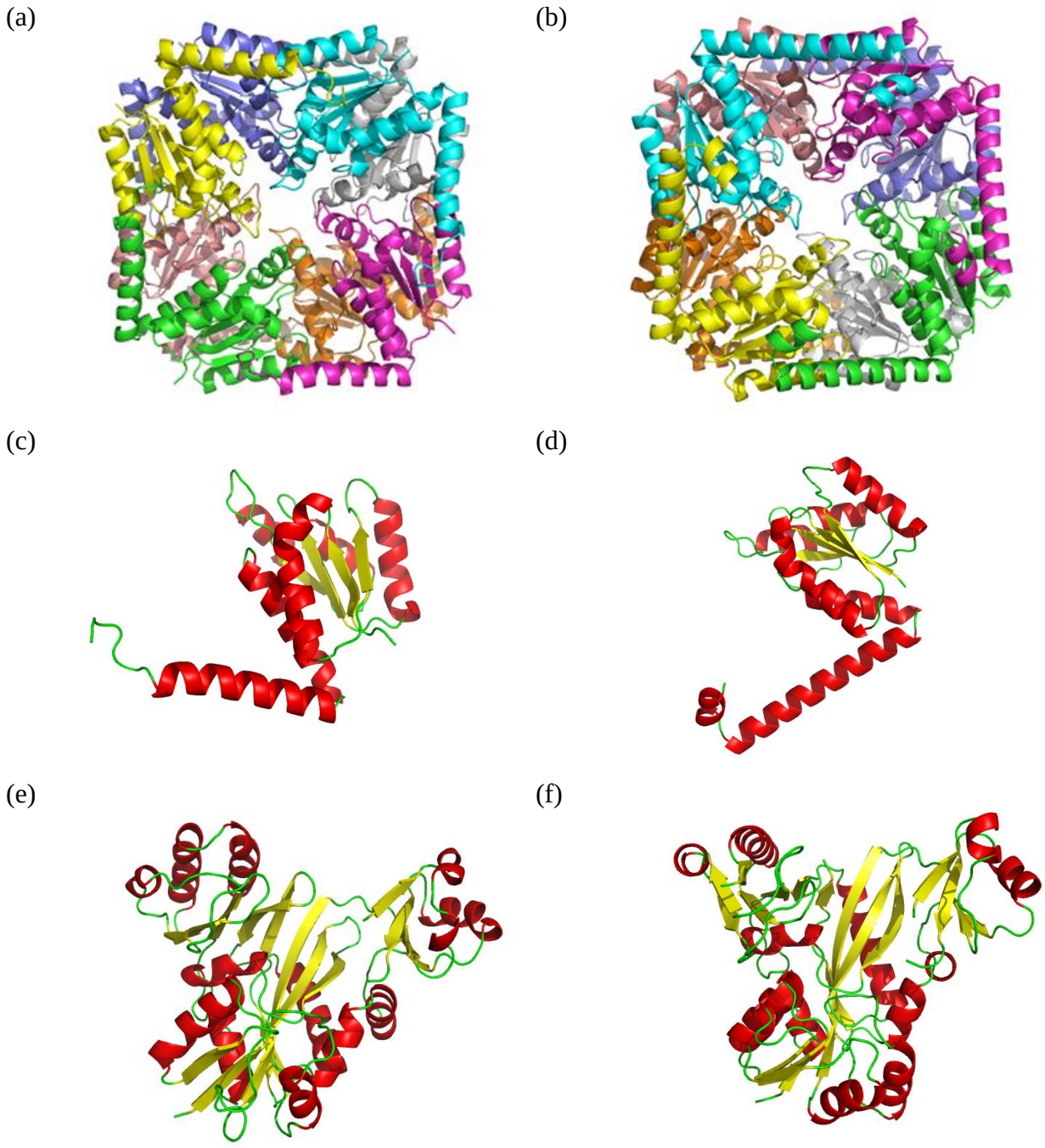


Figure 1.5 Crystal structures of PurE and PurK from *E. coli* and *T. maritima*. Monomer of PurE from *E. coli* (a) and *T. maritima* (b), monomer of PurE from *E. coli* (c) and *T. maritima* (d), and monomer of PurK from *E. coli* (e) and *T. maritima* (f).

1.3 Hypotheses

N^5-CAIR is so unstable that it is readily converted back into AIR even under standard biochemical conditions. Under severe conditions, such as high temperature, the utilization of N^5-CAIR is more troubling. The estimated $t_{1/2}$ at 80°C is about 80 ms. The conversion of AIR into N^5-CAIR consumes ATP. If the production of N^5-CAIR is not regulated relative to its conversion to CAIR, ATP utilization can rapidly become non-stoichiometric. ATP is the "molecular unit of currency" of intracellular energy transfer. If more ATP molecules are consumed in conversion of AIR to CAIR because of the chemical instability of N^5-CAIR, this would be a wasteful expense for purine synthesis. Therefore, we are interested in how bacteria deal with this problem. One mechanism to study this issue is to investigate how the problem is dealt with under conditions in which N^5-CAIR is even more unstable. Here we study the functions of enzymes PurK and PurE from *T. maritima*, a thermophilic bacterium, to try to understand how these two enzymes work to control N^5-CAIR synthesis and utilization at elevated temperatures.

Two hypotheses are proposed. One hypothesis assumes that there is a protein-protein interaction between PurK and PurE which allows for either direct transfer of N^5-CAIR from PurK to PurE or for the regulation of N^5-CAIR synthesis. The other one assumes PurE is highly efficient and thus able to convert N^5-CAIR to CAIR before it can decompose to AIR. In this thesis, we will investigate the catalytic activities of both enzymes and examine the synthesis and utilization of N^5-CAIR at elevated temperature and in the combination of the two proteins.

2. Materials and Methods

2.1 Gene cloning

2.1.1 PCR for purE and purK

T. maritima PurE and PurK genes were cloned from genomic DNA from strain MSB8 (ATCC 43589) by PCR. Each reaction mixture of a final volume of 50 µL contained 1 µM forward primer, 1 µM reverse primer, 1 µg template DNA, 1X Tag buffer, 1.25 U Taq, 1.5-4.0 mM $MgCl_2$ and 0.2 mM dNTP mix. Primers were synthesized commercially (Integrated DNA Technologies) (Table 2.1). The PCR was conducted for 30 cycles with each cycle including denaturation at 95 °C (1 min), annealing at 55 °C (1 min) and extention at 74 °C (1 min). Products were purified by agarose gel electrophoresis and isolated from gel slices using a genomic DNA purification kit (Promega).

Table 2.1 Sequences of primers for PCR.

genes	forward primers	reverse primers
purE (stop)	5'-AAACATATGCCAAGGGTAGGAATCATCA	5'-TTGCGGCCGCTTACTCTTTCTGGTTCAGA
purE (non-stop)	5'-AAACATATGCCAAGGGTAGGAATCATCA	5'-TTGCGGCCGCTGCCTCTTTCTGGTTCAGA
purK (stop)	5'-AAACATATGAAAAAGATCGGTATCATCG	5'-AAGCGGCCGCCTACCCTTGGCACATCGC
purK (non-stop)	5'-AAACATATGAAAAAGATCGGTATCATCG	5'-AAGCGGCCGCCATCCCTTGGCACATCGC

Underlined sequences are restriction sites: NdeI in forward primers and NotI in reverse primers.

2.1.2 Preparation of recombinant plasmids.

The purified purE and purK genes, which contained an A-tail, were ligated for 1 hr at room temperature into the pGEM-T vector (Promega) using T4 DNA ligase (Promega). Recombinant plasmids were transformed into *E. coli* DH5α and plated onto LB/X-gal for use in blue-white screening. Blue colonies were grown in LB medium with 100 µg/mL

ampicillin and plasmids were isolated by use of the Miniprep kit (Promega). The correct plasmid was verified by restriction digests using NotI and NdeI (BioLabs).

The pGEM plasmids, pET-22b(+) and pET-28a(+) vectors (Novagen) were digested with NotI and NdeI at 37 °C for one hour. The digested genes and plasmids were purified by agarose gel electrophoresis and the genes were ligated into the plasmids. The genes ligated into pET-22b(+) vector generated C-terminal His-tagged proteins. The genes ligated into pET-28a(+) vector yielded N-terminal His-tagged proteins. Successful ligation was validated by restriction digest with NotI and NdeI. Plasmids were amplified and sequenced by the Applied Genomic Technology Center (Wayne State University) with T7 forward and T7 reverse primers. Sequence analysis gave the expected products with no mutations. Expressed proteins will have the following additional sequences: HHHHHHSSGLVPRGSH at the N-terminus or AAALEHHHHHH at the C-terminus.

2.2 Protein expression and purification

Bacteria with recombinant pET plasmids were incubated overnight in 5 mL of LB media at 37 °C in the presence of 100 μg/mL ampicillin (pET-22 vectors) or 100 μg/mL kanamycin (pET-28 vectors). The overnight culture was used to inoculate to 500 mL of LB medium containing the corresponding antibiotics. The culture was incubated at 37 °C until an OD_{600} of 0.4 was obtained. IPTG (1 mM) was added to the medium and the incubation was continued for 4 hours. Bacteria were collected by centrifugation at 4000 rpm, and stored at -80 °C until use.

For protein purification, bacteria were lysed using the B-PER protein extraction reagent

(Thermo). The weight of bacteria pellet was determined by a balance and 4 mL B-PER reagent was added for per gram of cell pellet. The suspension was incubated for 10 min at room temperature followed by centrifugation at 4 °C at 13,000 rpm. The supernatant was treated with streptomycin sulfate to a final concentration of 1% and again centrifuged at 4 °C at 13,000 rpm to remove nucleic acids.

Protein purification was conducted at 4 °C. A 5 mL cobalt resin column (Gold Biotech) was washed with 3 mL water and 3 mL of 10 mM imidazole buffer (containing 50 mM sodium phosphate, 300 mM NaCl, pH 7.4) 3 times before the supernatant prepared above was applied to the column. The column was washed with 3 x 3 mL of 10 mM, 25 mM, 150 mM and 400 mM imidazole containing buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7.4). Fractions were collected and analyzed by 12% SDS-PAGE using Coomassie blue staining, which showed fractions containing 150mM and 400mM imidazole possessed pure protein. Fractions contained pure protein were collected and concentrated by dialysis centrifugal concentration (EMD). The protein was dissolved in buffer (containing 1 mM Tris-HCl and 50 mM NaCl, pH 8.0) and concentrated several times to remove imidazole. The concentration of the protein was determined by Bradford assay using BSA as a standard. The concentrations of PurEs and PurKs ranged from 1.5 mg/mL to 14 mg/mL. Proteins were stored in 5 µL aliquots at -80°C.

2.3 Thermal stability of PurE and PurK from thermophilic bacteria

Melting points of PurEs and PurKs from both *E.coli* and *T. maritima* were measured using a real-time PCR instrument (Stratagene Mx3005p, Agilent). Reactions in a final

volume of 20 µL contained 150 mM NaCl, 10 mM HEPES buffer (pH 8.0), SYPRO Orange and 75 µg/mL of protein. For PurE and PurK from *T. maritima*, melting studies were also conducted with 500 mM guanidine in 10 mM HEPES buffer (pH 5.0 or 8.0). Fluorescence of SYPRO Orange (Ex 492 nm and Em 610 nm) was determined as a function of temperature from 25 °C to 95 °C. Melting curves were plotted as temperature vs. fluorescence. T_m is determined by denaturation midpoint.

2.4 Stability of N^5-CAIR

The equilibrium of AIR to N^5-CAIR was determined from 20 °C to 80 °C using the Varian UV-vis Cary 100 spectrophotometer. The equilibrium was determined in a total volume of 1000 µL containing 250 mM $NaPO_4$ buffer (pH 8.0) with 200 mM sodium bicarbonate and 100 µM AIR. The mixture was added to a stoppered cuvette and was preincubated at 20 °C for 15 min. The equilibrium was monitored at 260nm as the temperature was ramped from 20 °C to 80 °C at a rate of 1 °C/min. The concentration of N^5-CAIR was calculated using the extinction coefficient of 970 $M^{-1}cm^{-1}$.[20]

2.5 Enzyme kinetics assay

Enzyme assays were conducted using the Cary 100 spectrophotometer equipped with a temperature controller.

2.5.1 The activity of PurE and PurK

2.5.1.1 CAIR decarboxylation assay for PurE from E. coli and T. maritima

The assay for PurE was conducted in a total volume of 1000 µL containing 100 mM Tris-HCl buffer (pH 8.0) and PurE (10 ng) from *E. coli* or PurE (450 ng for 21 °C, and

200ng for both 37 °C and 50 °C) from *T. maritima*. The cuvette was incubated at 21, 37 or 50 °C for 2 min, and the reaction was initiated by addition of CAIR (12.5 - 200 µM). The conversion of CAIR was monitored at 260 nm ($\Delta\varepsilon$=8930 $M^{-1}cm^{-1}$).

2.5.1.2 AIR carboxylation assay for PurK from T. maritima

The assay was conducted in a final volume of 1000 µL containing 50 mM HEPES buffer (pH7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 1 mM $NaHCO_3$, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase and PurK (500 ng). The cuvette was incubated at 37°C or 50°C for 2 min prior to the initiation of the reaction by addition of AIR (12.5-200 µM). The reaction was monitored by change in absorbance at 340 nm. The concentration of AIR consumed was calculated using the extinction coefficient of 6200 $M^{-1}cm^{-1}$.

2.5.1.3 Michaelis-Menten kinetics

The initial velocity vs concentration of substrate was plotted to generate a Michaelis-Menten plot. The steady-state kinetic parameters of the enzymes were obtained by non-liner regression of the plot to the Michaelis-Menten equation (1). Errors reported are those obtained from curve fitting.

$$v = \frac{v_{max}[S]}{K_m + [S]} \quad (1)$$

2.5.2 Assays of PurE and PurK in the presence of the other protein for PurE-PurK from *T. maritima*

2.5.2.1 CAIR decarboxylation assay for PurE in the presence of PurK

The assay was conduted in a total volume of 1000 µL containing 100 mM Tris-HCl (pH 8.0), PurE (100 ng) and PurK (the molar ratios of PurK/PurE monomer were 0, 0.25, 0.5, 1 and 2). The mixture was incubated at 37°C or 50°C for 2 min and initiated by addition of 100 µM CAIR. The reaction was monitored by change in absorbance at 260 nm.

2.5.2.2 AIR carboxylation assay for PurK-PurE

The assay was conducted in a final volume of 1000 µL containing 50 mM HEPES buffer (pH 7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 1 mM $NaHCO_3$, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, PurK (500 ng) and PurK (the molar ratios of PurK/PurE monomer are 0, 0.25, 0.5, 1 and 2). The cuvette with the mixture was incubated at 37°C or 50°C for 2 min prior to initiation by addition of 100 µM AIR. The reaction was monitored by change in absorbance at 340 nm.

2.5.3 Activity of PurC from *E. coli*

PurC (*E. coli*) was the kind gift of Dr. Kappock (Purdue University). The activity of PurC was determined by coupled assay monitoring ATP consumption and SAICAR production. In the ATP consumption assay, the assay mixture contained the following in a final volume of 1000 µL: 50 mM HEPES buffer (pH 7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, 0.1 mM CAIR and PurC (50, 75, 100 and 125 ng). The cuvette with the mixture was

preincubated at 37°C prior to initiation by addition of 10 mM aspartic acid. The reaction was monitored by change in absorbance at 340 nm ($\Delta\varepsilon$=6200 $M^{-1}cm^{-1}$). In the SAICAR production assay, the reaction contained the following in a final volume of 1000 µL: 50 mM HEPES buffer (pH 7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 0.1 mM ATP, 2.0 mM PEP, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, 0.1 mM CAIR and PurC (50 ng, 75 ng, 100 ng and 125 ng). The reaction was monitored by change in absorbance at 282 nm ($\Delta\varepsilon$=8480 $M^{-1}cm^{-1}$).

2.5.4 N^5-CAIR is the substrate for PurE

In the PurC coupled assay, SAICAR production was monitored to determine the substrate of PurE (*T. maritima*). In the assay mixture of 1000 µL total volume, 50 mM HEPES buffer (pH7.8), 20mM KCl, 6.0mM $MgCl_2$, 0.1 mM ATP, 2.0mM PEP, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, 0.1 mM AIR, 200 ng PurE and 800ng PurC were included. In another reaction, 700 ng PurK was also added to the same reaction mixture described above. The mixture was preincubated at 37°C prior to initiation by addition of 10 mM aspartic acid. These reactions were monitored at 282 nm.

2.5.5 Stoichiometry assay

The amounts of enzymes were calculated according to their specific activity so that the reaction could be completed rapidly. The reaction mixture in a total volume of 1000 µL contained the following: 50 mM HEPES buffer (pH7.8), 20 mM KCl, 6.0 mM $MgCl_2$, 0.1 mM ATP, 2.0 mM PEP, 5 units of pyruvate kinase, 17.8 µM AIR, 1000 ng or 500 ng PurE, 2220 ng PurK and 1260 ng PurC. PurE and PurK were added in the molar ratios of 1:1 or

1:2. The reactions were incubated at 37°C or 50°C and initiated by addition of 10 mM aspartic acid. The reactions were monitored at 282 nm for SAICAR production and the change in the absorbance was used to calculate the amount of SAICAR produced ($\Delta\varepsilon$=8480 $M^{-1}cm^{-1}$). To determine the amount of ATP, the same assay as above was repeated expect 0.2 mM NADH was added and ATP level was increased to 1.1 mM. The reaction was monitored at 340nm and the concentration of ATP was calculated from the change of absorbance ($\Delta\varepsilon$=6200 $M^{-1}cm^{-1}$).

3. Results

3.1 Gene Cloning

Since both PurE and PurK from *T. maritima* had been crystalized, we initially attempted to express the proteins from vectors used in these studies. We were unable to obtain the PurK expression plasmid from the authors. For PurE, we obtained the recombinant plasmid pMH1 containing the purE gene fused to a 6X His-tag. The plasmid was transformed into *E.coli* LMG194 for protein expression induced by arabinose. Protein expression studies with various concentration of arabinose in a variety of media failed to generate PurE as determined by SDS-PAGE and a his-tag western blot. Attempted purification by a cobalt column also failed to produce the desired protein.

Given these challenges, we elected to clone these genes ourselves. The purE and purK genes were cloned from genomic DNA of *T. maritima* by PCR (Figure 3.1). For each gene, we prepared two constructs. The first placed the his-tag on the N-terminus while the second placed the tag on the C-terminus. PCR was conducted with different concentrations of $MgCl_2$ to determine the optimum concentration of magnesium. The optimal concentration of $MgCl_2$ was determined to be 1.5 mM.

The purified PCR products were ligated into the pGEM-T (Promega) vector for amplification. Vectors containing the purE and purK inserts were determined by restriction mapping (Figure 3.2). The purE and purK were removed from the vector

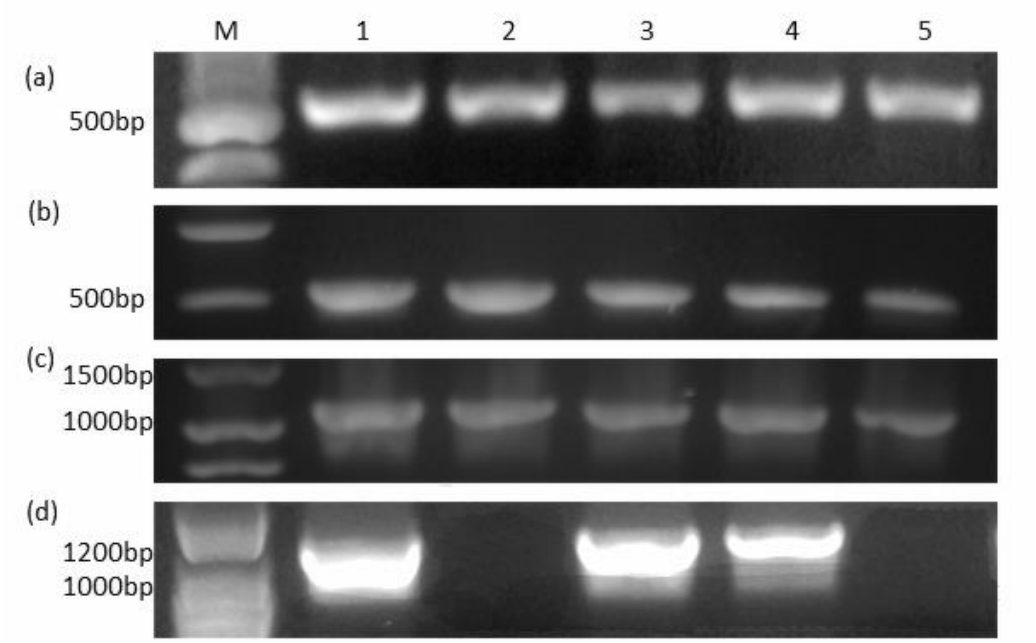


Figure 3.1 PCR products: (a) purE without stop codon, (b) purE with stop codon, (c) purK without stop codon and (d) purK with stop codon. M: molecular weight marker. 1-5: 1.5, 2.0, 2.5, 3.0 and 4.0 mM magnesium chloride.

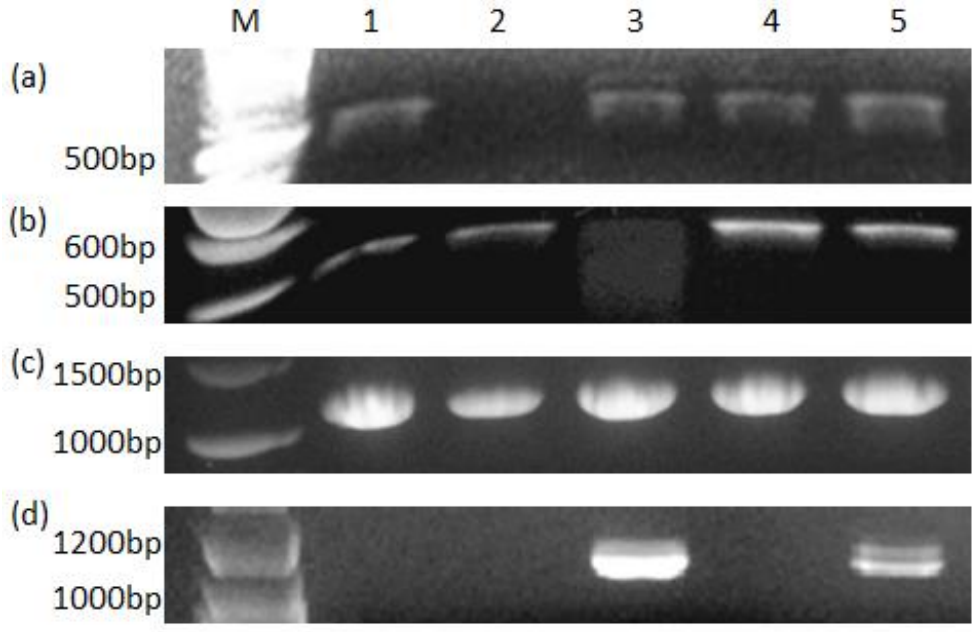


Figure 3.2 Genes digested from recombinant pGEM-T vectors: (a) purE without stop codon, (b) purE with stop codon, (c) purK without stop codon and (d) purK with stop codon. M refers to standard DNA marker, 1-5 refers to plasmids isolated from different colonies.

by NotI and NdeI, and the inserts were gel purified and ligated into either pET-22b(+) (for C-terminal His-tagged proteins) or pET-28a(+) (for N-terminal His-tagged proteins) for protein expression. The recombinant expression plasmids were verified by restriction mapping (Figure 3.3) and DNA sequencing.

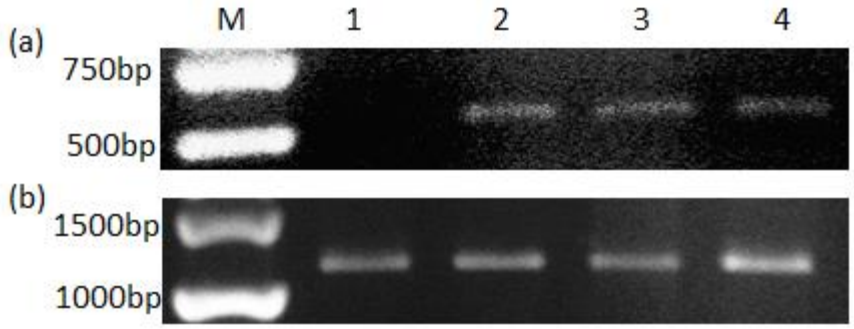


Figure 3.3 Recombinant pET plasmids were double digested by the NotI and NdeI restriction enzymes to verify ligation of genes in pET vectors: (a) purE genes and (b) purK genes. M refers to standard DNA marker, 1-4 refers to different plasmids isolated from different colonies.

3.2 Protein expression and purification

The recombinant pET plasmids were transformed into BL21 DE3. A single colony was incubated overnight at 37 °C in 5 mL LB medium in the presence of either ampicillin or kanamycin. This was used to innoculate 500 mL of fresh LB medium containing ampicillin or kanamycin and the culture was incubated until an OD_{400} of 0.4 was obtained. Bacteria were incubated for 4 hours either in the absence or presence of 1 mM IPTG. Bacteria were collected by centrifuging, and stored at -80 °C until use. For purification, the bacteria were thawed and incubated with B-PER lysis buffer (Promega) before being applied to a 5 mL cobalt resin column (Gold Biotech). The column was washed with phosphate buffer

containing 10 mM imidazole followed by a step gradient of buffer containing 10, 25, 150 and 400 mM imidazole. Fractions were collected and analyzed for purity by SDS-PAGE. Fractions containing pure protein were collected and concentrated. Imidazole was removed by repeated dilution and concentration. Proteins were stored in 5 µl aliquots at -80 °C. The purity of each protein was determined by SDS-PAGE and was judged to be at least 98% pure (Figure 3.4)

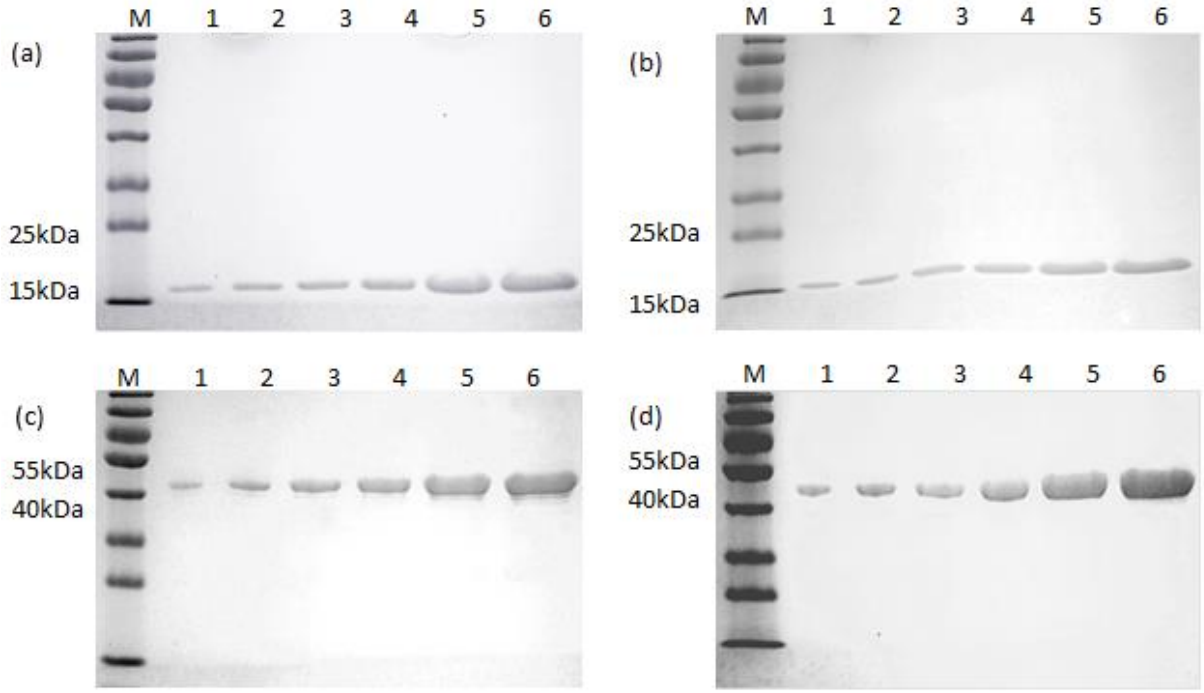


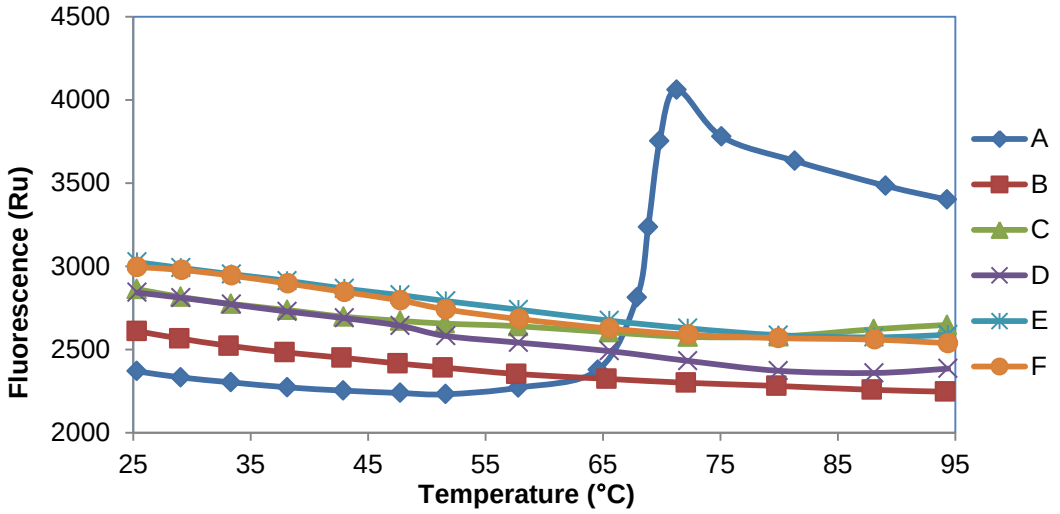
Figure 3.4 SDS-PAGE analysis of pure protein: (a). PurE-His, (b). His-PurE, (c). PurK-His, (d). His-PurK. M: protein ladder. 1-6: 0.5, 1, 2, 4, 8 and 10 µg proteins.

3.3 Thermal stability of PurE and PurK from thermophilic bacteria.

The thermostability of PurEs from *E. coli* and *T. maritima* were measured using a thermal shift assay. In this experiment, unfolded protein is detected by Sypro Orange, a fluorescent dye that binds to exposed hydrophobic surfaces in the denatured protein. This results in an increase in fluorescence as a function of protein unfolding. To promote unfolding, the temperature is increased. A real-time PCR instrument was used to detect the thermal melting of the protein. Melting curves of both PurEs are shown in Figure 3.5 a. The melting temperature for PurE (*E.coli*) in 300 mM NaCl at pH 8.0 is 72 °C. In contrast, no change in fluorescence intensity for PurE from *T. maritima* was observed under any conditions. Changes in pH and the addition of denaturant also failed to give a measurable melting temperature. These studies indicate that PurE from *T. maritima* is significantly more stable than *E. coli* PurE.

The thermal stability of PurKs from *E. coli* and *T. maritima* were also measured using the thermal shift assay (Figure 3.5 b). The melting temperature of PurK (*E. coli*) in 300 mM NaCl at pH 8.0 is 68 °C. The changes of fluorescence intensity for PurK from *T. maritima* under all conditions tested were similar with PurE (*T. maritima*). These results indicate that the PurK from *T. maritima* is again remarkably more stable than *E. coli* PurK.

(a)



(b)

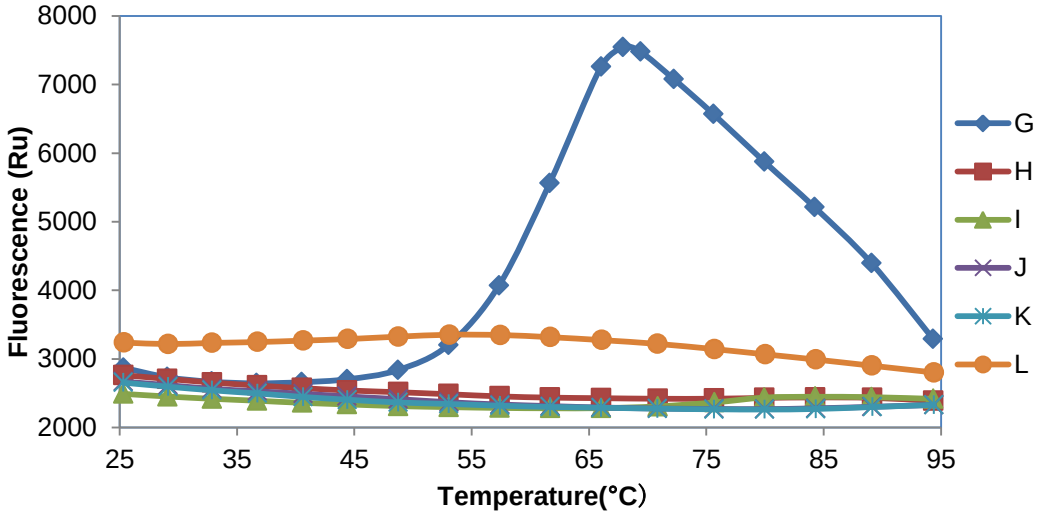


Figure 3.5 (a). Melting curves of PurE (*E. coli*) and PurE (*T. maritima*) from 25 °C to 95 °C: A. PurE (*E. coli*) at pH 8, B. PurE (*T. maritima*) at pH 8, C. PurE (*T. maritima*) at pH 5, D. PurE (*T. maritima*) at pH 5 in the presence of 500 mM guanidine, E. PurE (*T. maritima*) at pH 10, F. PurE (*T. maritima*) at pH 10 in the presence of 500 mM guanidine. Data from one experiment only in shown for clarity.
(b). Melting curves of PurK (*E. coli*) and PurK (*T. maritima*) from 25 °C to 95 °C: G. PurK (*E. coli*) at pH 8, H. PurK (*T. maritima*) at pH 8, I. PurK (*T. maritima*) at pH 5, J. PurK (*T. maritima*) at pH 5 in the presence of 500 mM guanidine, K. PurK (*T. maritima*) at pH 10, L. PurK (*T. maritima*) at pH 10 in the presence of 500 mM guanidine. Data from one experiment only in shown for clarity.

3.4 Enzyme kinetics

3.4.1 Activity of PurE and PurK from *T. maritima*.

Although both PurE and PurK from *T. maritima* had been crystallized, no studies on the activity of these enzymes have been reported. Thus we conducted a full characterization of these proteins. For PurE, we examined the activity of the reverse reaction, CAIR converted to N^5-CAIR, using the standard CAIR decarboxylation assay.[6] This assay was chosen because of the instability of N^5-CAIR. For PurK, we utilized the AIR carboxylation assay. We examined the activity of both the N and C-terminal His-tagged PurEs and PurKs at various temperatures (Table 3.1). For PurK, we did not examine the kinetic constant of bicarbonate because the equilibrium of this substrate is affected at different temperatures.

Both the C-terminal His-tagged PurE and PurK from *T. maritima* showed higher V_{max} and k_{cat} at higher temperature, indicating that these enzymes prefer to operate at high temperatures. However, the effect on K_m of various substrates for PurE and PurK were opposite. The increasing K_m of CAIR for PurE at high temperature suggested that the temperature affected substrate binding. In contrast, the K_m of AIR for PurK decreased when the temperature increased. The position of His-tag also affected the k_{cat} and K_m of PurE. N-terminal His-tagged PurE showed a lower K_m and k_{cat}, but the efficiency was roughly same with C-terminal His-tagged PurE. Comparing the k_{cat}/K_m value at different temperatures indicated that there was no big difference among the efficiency of PurEs at different temperatures, while PurK was more efficient as the temperature increased.

Table 3.1 Steady-state kinetic constants for PurE and PurK

enzyme	temp (°C)	substrate	Vmax (μmol/min·mg)	kcat (min^{-1})	Km (μM)	kcat/Km ($min^{-1}M^{-1}$)
PurE (*E.coli*)C[a]	37	CAIR	270±17	38200±2500	30±6.7	$1.3±0.31x10^{9}$
PurE (*E.coli*)C	50	CAIR	240±7	34000±1000	11±1.8	$3.2±0.79x10^{9}$
PurE (*T.maritima*)C	21	CAIR	5.2±0.2	870±30	11±2.3	$0.83±0.26x10^{8}$
PurE (*T.maritima*)C	37	CAIR	18±2	2688±367	36±8.0	$0.75±0.34x10^{8}$
PurE (*T.maritima*)C	50	CAIR	38±6	5700±900	74±17	$0.78±0.38x10^{8}$
PurE (*T.maritima*)C[c]	80	CAIR	69	10300	135	$0.76x10^{8}$
PurK(*T.maritima*)C	21	AIR	0.8±0.1	640±40	77±13	$0.83±0.23x10^{7}$
PurK (*T.maritima*)C	37	AIR	5.2±0.1	4400±60	19±1.6	$2.4±0.83x10^{8}$
PurK (*T.maritima*)C	50	AIR	11±1	9100±680	18±0.4	$5.2±2.51x10^{8}$
PurK (*T.maritima*)C[c]	80	AIR	21	17800	18	$1.2x10^{9}$
PurK (*T.maritima*)C	37	ATP	2.2±0.1	3700±100	56±5.1	$0.66±0.1x10^{8}$
PurK (*T.maritima*)C	50	ATP	4.9±0.2	8350±390	84±11	$1.0±0.20x10^{8}$
PurE (*T.maritima*)N[b]	21	CAIR	6.0±0.3	900±51	8.5±2.6	$1.1±0.49x10^{8}$
PurE (*T.maritima*)N	37	CAIR	13±0.3	1930±46	2.7±0.5	$0.71±0.1x10^{8}$
PurE (*T.maritima*)N	50	CAIR	25±1	3740±140	2.1±0.6	$1.8±0.86x10^{8}$
PurE (*T.maritima*)N[c]	80	CAIR	43	6400	2.1	$3.0x10^{8}$
PurK (*T.maritima*)N	37	AIR	2.3±0.1	3800±82	23±1.9	$1.7±0.19x10^{8}$
PurK (*T.maritima*)N	37	ATP	2.7±0.2	4600±300	50±10	$0.93±0.3x10^{8}$

a. C terminus 6X His-tag. b. N terminus 6X His-tag. c. Constants at 80 °C are estimated.

T. maritima grows optimally at 80 °C. Attempts to investigate the activity of these proteins above 60 °C proved challenging due to the instability of the substrates used in the assay. To estimate the activity at 80 °C, we studied each enzyme at different temperatures. A plot of activity vs temperature gave a straight line. Thus we can estimate the activity of the enzymes at higher temperatures (Figure 3.6). The calculated V_{max} of C-terminal His-tagged PurE (*T. maritima*) at 80 °C is 69 μmol/min·mg, k_{cat} is 10,300 min^{-1} and K_m is 135 μM. The estimated value of k_{cat}/K_m

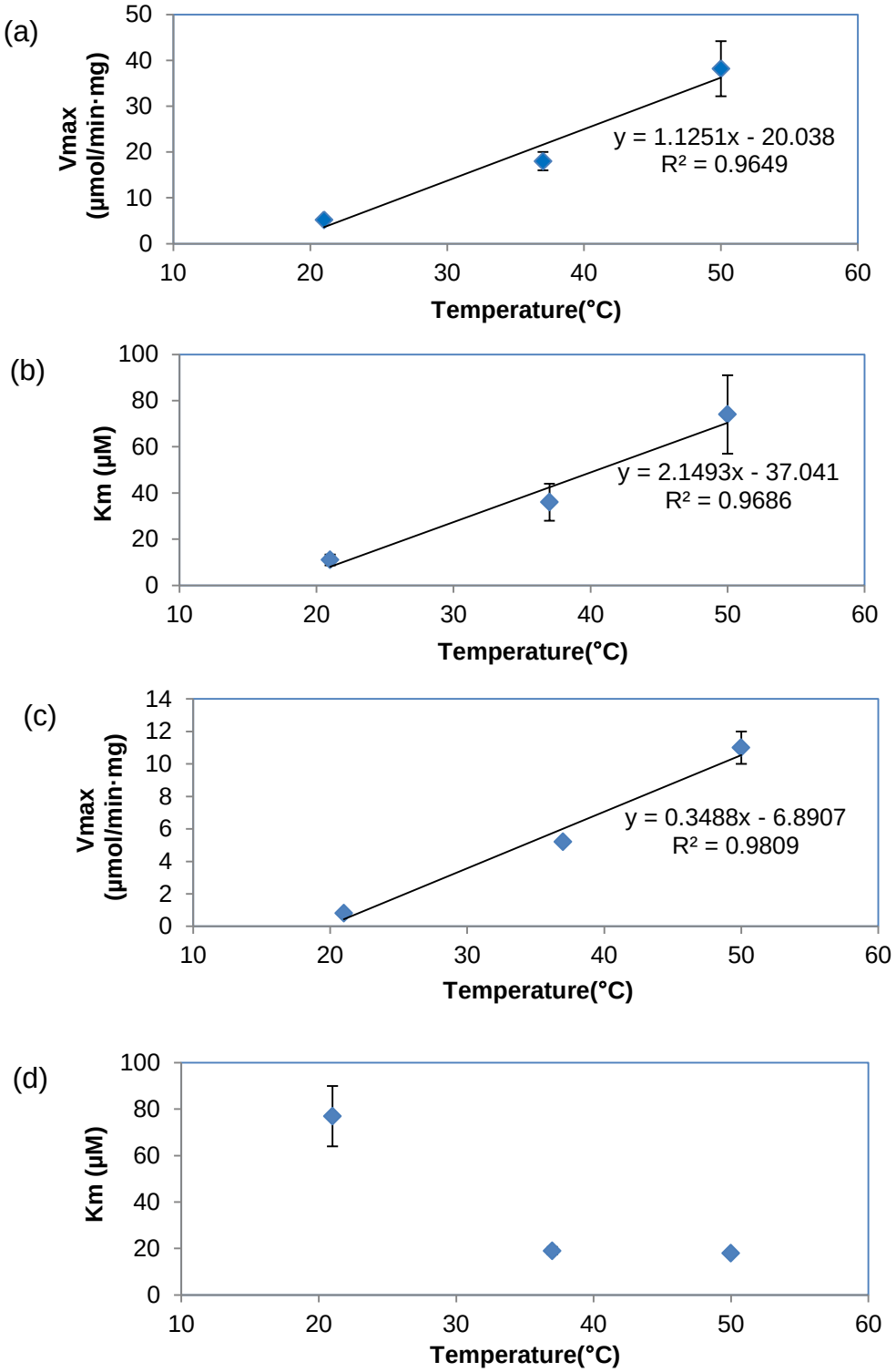
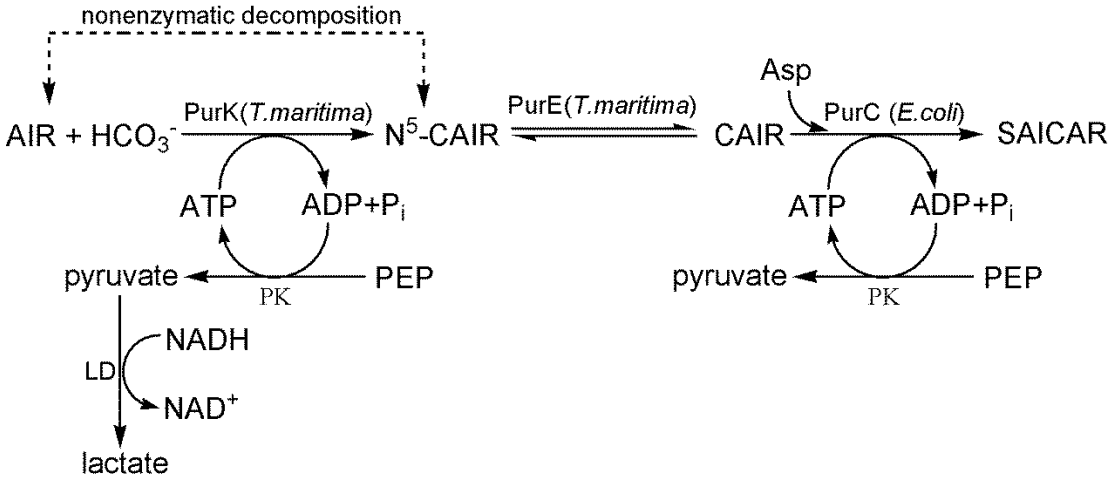


Figure 3.6 Relationship between activity of enzymes (*T. maritima*) and temperature. (a) V_{max} of PurE (*T. maritima*). (b) K_m of PurE (*T. maritima*). (c) V_{max} of PurK (*T. maritima*). (d) K_m of PurK (*T. maritima*).

for this PurE (*T. maritima*) at 80 °C is $0.76x10^8$ $min^{-1}M^{-1}$. The estimated V_{max} of C-terminal His-tagged PurK (*T. maritima*) at 80 °C is 21 μmol/min·mg, and k_{cat} is 17,800 min^{-1}. There is no linear relationship between K_m and temperature for PurK. An examination of the data suggests that the K_m at 80 °C is likely the same as that at 50 °C. Therefore the estimated value of k_{cat}/K_m for PurK (*T. maritima*) is $1.2x10^9$ $min^{-1}M^{-1}$. These estimated values indicate that both enzymes are highly efficient at 80 °C.

To determine whether AIR or N^5-CAIR is the substrate of PurE, a coupled AIR carboxylase assay (Scheme 3.1) was conducted with PurE (*T. maritima*) and PurC (*E. coli*) in the absence or presence of PurK (*T. maritima*).



Scheme 3.1 Coupled AIR carboxylase assay with *E. coli* PurC.

SAICAR production in the presence of PurK (*T. maritima*) is greater than in the absence of PurK (Figure 3.7). This indicates that PurK catalyzed AIR into N^5-CAIR which is utilized by PurE as a substrate. Therefore, as predicted, N^5-CAIR is the substrate for PurE. Thus, the PurK and PurE enzymes from *T. maritima* are functionally identical to these from *E. coli*.

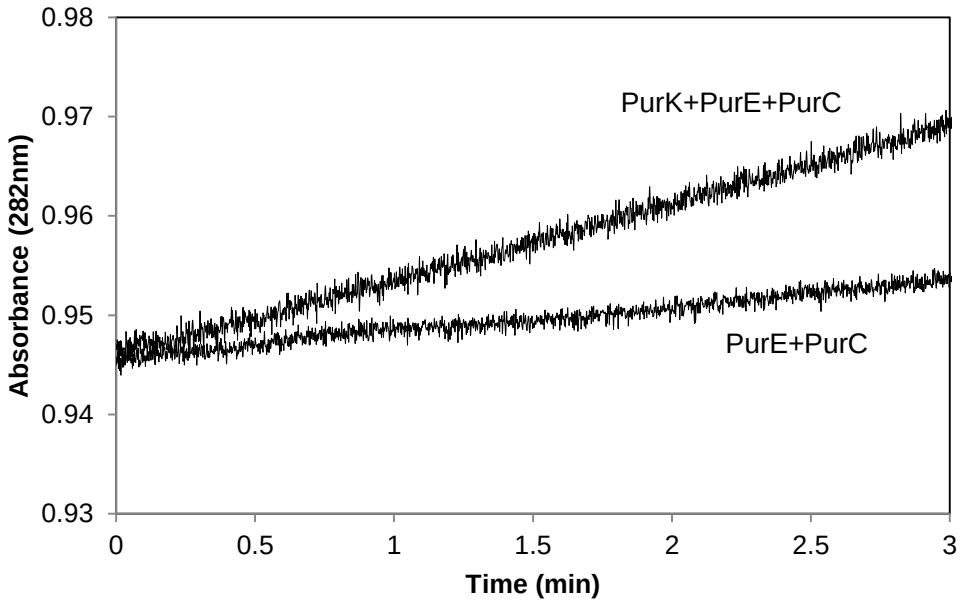


Figure 3.7 Change of absorbance at 282 nm (for SAICAR production) from AIR catalyzed by PurE (*T. maritima*) and PurC (*E. coli*) in the presence and absence of PurK (*T. maritima*) at 37 °C.

3.4.2 Stability of N^5-CAIR

In the absence of enzymes, AIR and bicarbonate can be nonenzymatically converted into N^5-CAIR in minutes or CAIR after more than 70 hours.[16] To study the conversion of AIR into N^5-CAIR at different temperatures, we conducted a thermal assay that monitored N^5-CAIR production at pH 8.0 from 20 °C to 80 °C (Figure 3.8). Previous studies indicated that the AIR/ N^5-CAIR equilibrium was rapidly reached and therefore we expect that the value represents the equilibrium value for that temperature.[16] N^5-CAIR was monitored at 260 nm where the extinction coefficient of AIR (1570 $M^{-1}cm^{-1}$) is much higher than that of N^5-CAIR (710 $M^{-1}cm^{-1}$). We observed that the percentage of N^5-CAIR decreased as the temperature increased. This result is also consistent with previous studies which show that

N^5-CAIR is more unstable at higher temperatures.[16] These studies validate that N^5-CAIR is highly unstable at 80 °C and the concentration of this intermediate in solution is very low. At 80 °C, only 2% of AIR is converted into N^5-CAIR. Thus, this intermediate must be carefully utilized and/or stabilized.

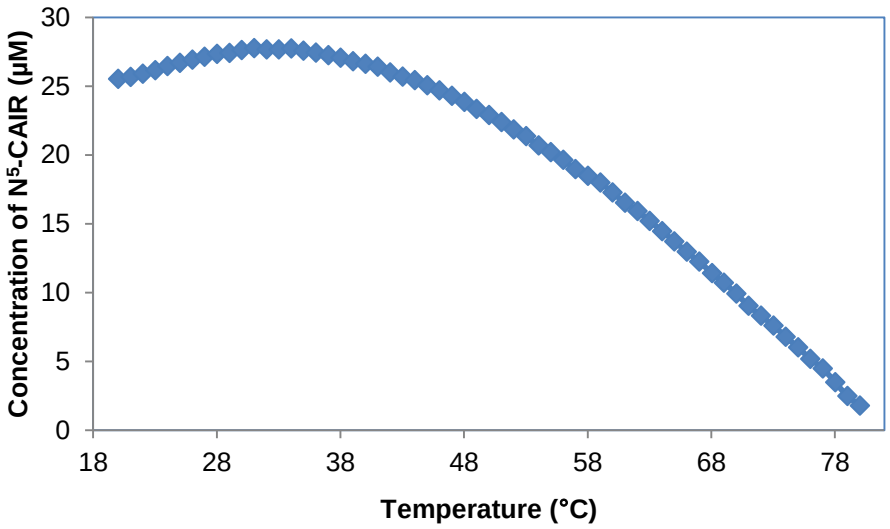


Figure 3.8 Equilibrium of AIR/HCO_3^-/N^5-CAIR as a function of temperature. Data from one experiment only is shown for clarity.

3.4.3 Stoichiometry

A key question in these studies is whether at elevated temperature, the N^5-CAIR that is produced can be effectively utilized by the system before it is broken down to AIR. This would lead to a non-stoichiometric consumption of ATP. To measure this, we examined the stoichiometry of the PurK and PurE enzymes using the coupled PurC assay system (Table 3.2). We measured ATP consumption using the NADH coupled system and AIR consumption by the production of SAICAR at 282 nm. In the stoichiometry assays, enough enzymes were used to make sure AIR (17.8 μM) could be completely converted in several

minutes.

Table 3.2 Stoichiometry assay[a]

Temp(°C)	Molar ratio of PurE to PurK	AIR(μM)	ATP(μM)	SAICAR(μM)
37	1:1	17.9±0.7[b]	35.2±2.4	17.1±1.4
50	1:1	17.7±0.4	38.1±6.6	17.7±1.8
50	1:2	17.9±0.1	52.4±4.1	17.5±3.3

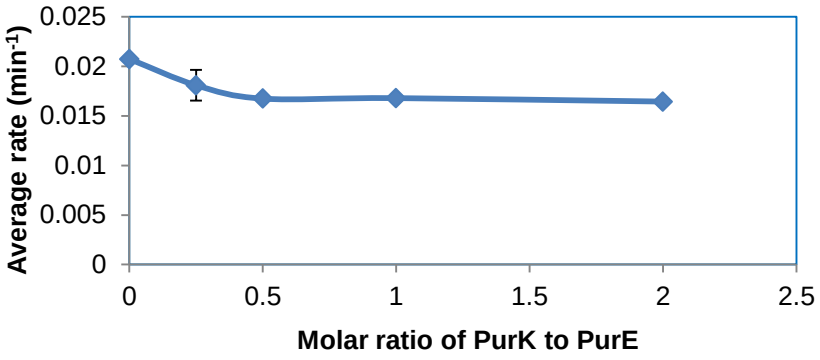
a. Each assay was done in triplicate. b. AIR concentration was determined at 250nm (ε=3830 $M^{-1}cm^{-1}$, pH 7.0).

When the molar ratio (monomer) of PurE to PurK was 1:1, the reaction showed the expected stoichiometry of 1:2:1 at 37 °C. Increasing the temperature to 50 °C gave the same result. Decreasing the concentration of PurE lead to a non-stoichiometric consumption of ATP, presumably because the nonenzymatic conversion of N^5-CAIR to AIR was faster than the conversion of N^5-CAIR. This indicates that the ratio of PurE to PurK is critical for preventing the waste of ATP at the expense of N^5-CAIR synthesis.

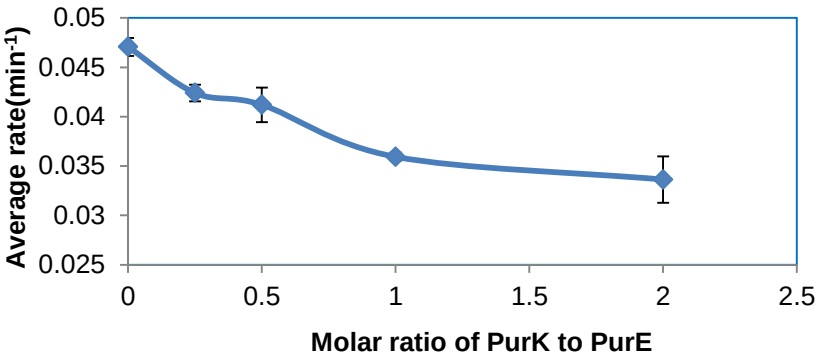
3.4.4 Protein-protein interaction

One mechanism by which N^5-CAIR could be stabilized is by binding to PurK until PurE is available for catalysis. In addition, channeling between PurK and PurE could also occur. To examine this, we determined the rate of each enzyme in the presence of the other protein. Different monomer molar ratios (0.25, 0.5, 1 and 2) of these two proteins were mixed and either the AIR carboxylation or CAIR decarboxylation assays were conducted (rates shown in Figure 3.9).

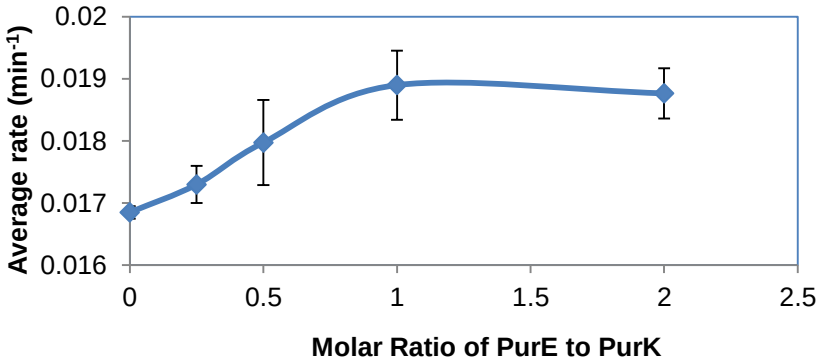
(a)



(b)



(c)



(d)

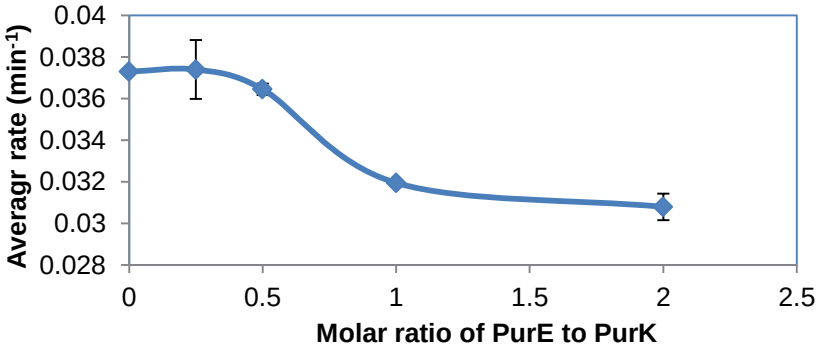


Figure 3.9 The average rate (triplicate) for CAIR decarboxylation assay at 37°C (a) and 50°C (b), and AIR carboxylation assay at 37°C (c) and 50°C (d).

In the CAIR decarboxylation reaction for PurE, PurK was added in the mixture without its substrates, such as ATP and AIR. Therefore, PurK was inactive in this assay. The inactive PurK decreased the activity of PurE by 21% at 37 °C and 29% at 50 °C. The result suggests that PurK can regulate PurE; however, the mechanism by which this occurs is unknown. Repeating this assay in the presence of BSA resulted in no change.

For PurK, we utilized the AIR carboxylation reaction with PurE added in different molar ratios. In this assay, PurE was active since there was N^5-CAIR produced by PurK. At 37 °C, the activity of PurK increased by 11% in the presence of PurE. And again, BSA had no change. This could suggest that the complex is formed. At 50 °C, the reaction showed the opposite result, the PurK activity decreased. We believe that the reason for this result at 50 °C can be explained by the stoichiometry results. For the molar ratio of PurE to PurK at 0 or 1:2, the reaction is nonstoichiometric and consumes more ATP because the N^5-CAIR is decarboxylated back to AIR. As the amount of PurE increases, the N^5-CAIR produced is converted to the more stable CAIR and thus the rate of ATP consumption decreases.

The protein-protein interaction study shows that the PurE and PurK from *T. maritima* can regulate each other. It suggests that these two enzymes probably form a protein complex.

4. Discussion

4.1 Properties of PurE and PurK from *T. maritima*

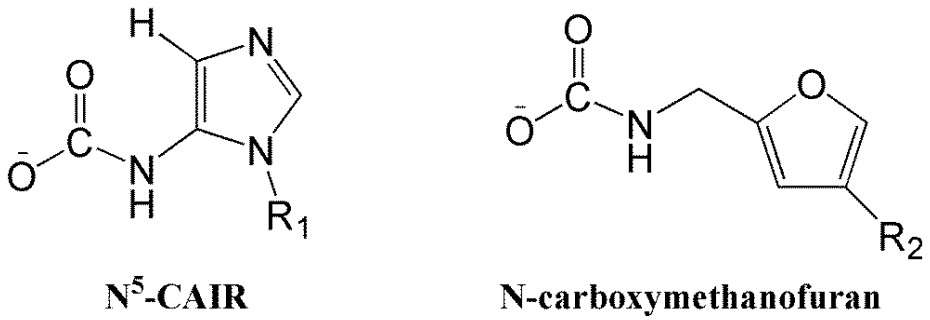
The roles of PurK and PurE from *E. coli* have been studied by Meyer[6] and Mueller[16]. Sequence comparisons of *T. maritima* with *E. coli* have indicated that *T. maritima* purE and purK genes are similar to those from *E. coli*, and the protein structure similarities with *E. coli* PurE and PurK have also been noted.[6,9,10,13,19] We have studied the substrate specificity for PurK and PurE from *T. maritima*. *T. maritima* PurK was studied by the standard AIR carboxylation assay, which indicated that this enzyme utilized the same substrates as the enzyme from *E. coli*. Previous studies have shown that there are at least two classes of PurEs: N^5-CAIR mutase and AIR carboxylase. To investigate PurE from *T. maritima*, we conducted a coupled assay with *E. coli* SAICAR synthetase in the absence or presence of PurK from *T. maritima*. In the absence of PurK, SAICAR synthesis was minimal indicating that *T. maritima* PurE was unable to catalyze the conversion of AIR into CAIR. However, in the presence of PurK, SAICAR synthesis was robust revealing that N^5-CAIR was the substrate of *T. maritima* PurE. Both of these results indicate that *T. maritima* follows the same pathway previously determined for *E. coli*.

By studying the functions of PurK and PurE from *T. maritima*, we have shown that these enzymes have a functional similarity to the enzymes from *E. coli*. We have investigated the activity of these enzymes at different temperatures and have shown that they have higher activity at higher temperature. This is not surprising for an enzyme from a thermophilic organism. Thermal studies for other enzymes from thermophilic organisms have been

published.[21,22] For example, in the study of S-adenosylhomocysteine hydrolase from *T. maritima*, the enzyme was most active at 75 °C.[22] The enzyme also showed thermostability at 75 °C after incubating for 120 min. In another study, the thermostability of *T. maritima* dihydrofolate reductase was investigated by incubating the enzyme at high temperatures for 15 min.[21] The results showed that 100% of enzyme activity are retained at 75 °C while 65% remained at 90 °C.

4.2 The problem with N^5-CAIR

Our studies on PurK and PurE from *T. maritima* indicate that this organism follow the same pathway for purine biosynthesis as *E. coli.* Such a result presents a potential problem. N^5-CAIR is an unstable intermediate. The $t_{1/2}$ of N^5-CAIR at 37 °C is 1.3 min.[16] The estimated $t_{1/2}$ at 80 °C is about 80 ms. This rapid rate of decarboxylation suggests that N^5-CAIR may not be a viable intermediate in purine biosynthesis. However, it is instructive to look at other unstable carbamates found in microbes. The compound N-carboxymethanofuran is an intermediate in the methanogenesis pathway found in methanogenic bacteria. A study of N-carboxymethanofuran shows that the $t_{1/2}$ of this primary carbamate is 1 s at 17 °C and 88 ms at 37 °C in solution of pH 8.0.[23] Thus, this critical compound is even more unstable than N^5-CAIR, yet is still a key component is methanogenesis.



N^5-CAIR **N-carboxymethanofuran**

Figure 4.1 Structures of N^5-CAIR and N-carboxymethanofuran. R_1=ribose 5'-phosphate. R_2=4-[N-(4,5,7-tricarboxyheptanoyl-γ-L-glutamyl-γ-L-glutamyl)-p-(β-aminoethyl) phenoxymethyl]

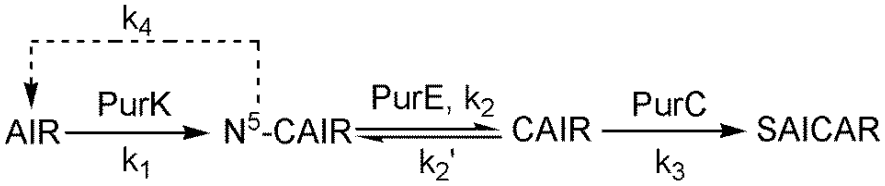
The instability of N^5-CAIR presents a challenge for *de novo* purine biosynthesis in *T. maritima* because ATP is consumed in the conversion of AIR to N^5-CAIR. If N^5-CAIR decomposes back to AIR, more ATP will be consumed. Thus, under certain conditions, a futile cycle can be created where the consumption of ATP is much greater than the synthesis of IMP from the pathway.

In the study of the equilibrium of AIR and N^5-CAIR, the results show that 30% of AIR is converted into N^5-CAIR at 37 °C, but only 2% at 80 °C. The equilibrium and kinetic data clearly indicate that the amount of N^5-CAIR present at 80 °C is low. Of course, the actual level of N^5-CAIR would be dependent upon the concentration of CO_2 in the bacteria as well as the temperature.

Despite the challenge with N^5-CAIR, the stoichiometry data at 50 °C showed that the reaction of AIR to SAICAR is stoichiometric with conditions in which the molar ratio of PurE and PurK are equal. However, when PurE is less than PurK, the reaction is not stoichiometric presumably due to the decomposition of N^5-CAIR which leads to more ATP

consumption. In a stoichiometry study of *C. neoformans* ADE2, which is a bifunctional enzyme containing both PurE and PurK (thus both enzymes are locked into a 1:1 ratio), the reaction was stoichiometric.[14] However, N^5-CAIR was also released from the enzyme.[14] Studies on the *E. coli* PurK and PurE enzymes in a 1:1 ratio gave non-stoichiometric consumption of ATP.[14]

In the pathway for the conversion of AIR to SAICAR, the concentration of N^5-CAIR depends on the rate constants for PurK (k_1), PurE (k_2) and the decomposition rate k_4 (Scheme 4.1). There are two factors that affect the reaction rate: the activity of the enzymes and the quantity of enzymes in the reaction. In our stoichiometric studies, non-stoichiometry happened when the molar ratio of PurE/PurK is 1:2. Although PurE is highly active, the lower amount of PurE required a longer time to convert N^5-CAIR to CAIR, which lead to the decomposition to AIR and thus more ATP consumption. A PurE/PurK complex could help to regulate these two enzymes in the pathway to prevent the wasteful decomposition of N^5-CAIR by tightly controlling its synthesis and use.



Scheme 4.1 Conversion of AIR to SAICAR.

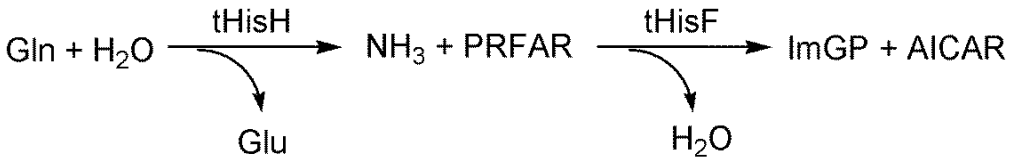
Our studies have revealed that the use of N^5-CAIR as an intermediate is not a problem as long as the amount of monomer PurE and PurK are equal. The reason for this is unclear as it may be due simply to kinetics or it could be due to the nature of the function of a complex

between the PurE and PurK.

4.3 Is there evidence for a protein-protein interaction between PurE and PurK?

The challenge of N^5-CAIR in the middle of the pathway could be dealt with by the formation of a PurK and PurE complex in which each enzyme is regulated by the other. Our studies have shown that the activities of either PurE or PurK are different when the other enzyme is present. Such a result indicates that PurE and PurK may form a complex which affects the kinetics of each enzyme.

An example of a protein-protein interaction that regulates a metabolic function is imidazole glycerol phosphate (ImGP) synthase from *T. maritima*.[24] ImGP synthase is a necessary enzyme which links histidine and *de novo* purine biosynthesis.[24] It is a bifunctional enzyme complex, consisting of a glutaminase subunit (tHisH) and a synthase subunit (tHisF). tHisH produces ammonia which reacts with the unstable substrate, N'-((5'-phosphoribulosyl) formimino-5-aminoimidazole-4-ribo- nucleotide (PRFAR)(Scheme 4.2).[24] Results of association state assays showed that tHisH and tHisF form a complex and enzyme kinetic assays for tHisF and the tHisH-tHisF complex showed that the kinetic constants of tHisF were affected in the presence of tHisH.[24] The quaternary structure of the complex provided a hypothesis in which the protein-protein interaction forms a hydrophobic channel facilitating the transfer of ammonia from tHisH to tHisF for attack on PRFAR.



Scheme 4.2 The reaction of ImGP synthase (tHisH-tHisF).

Another study on protein-protein interactions involving an unstable intermediate, carbamoyl phosphate (CP), in hyperthermophile *Pyrococcus furiosus* also has been done.[25] CP is produced by carbamoyl-phosphate synthetase (CKase) and is utilized by ornithine carbamoyltransferase (OTCase). The $t_{1/2}$ of CP is about 5 min at 37 °C[26], while at 100 °C, the optimal growth temperature of *P. furious*, the $t_{1/2}$ is less than 2 s.[25] The product of CP decomposition is cyanate, whose accumulation is harmful to the bacteria. A channeling cluster formed by CKase and OTCase was confirmed by two approaches including affinity electrophoresis and co-immunoprecipitate techniques.[25] This channeling for CP is significant for the bacteria because it not only allows for an efficient reaction, but also protects the cells from cyanate.

Organisms utilize dramatic approaches to overcome enormous challenges from both internal and external environments. The mechanism of PurE and PurK enzymes from *T. maritima* for dealing with the problem of the unstability of N^5-CAIR is still not clear so far. Our studies provide a hint that an enzyme complex is probably formed. More evidence is required to confirm our hypothesis.

5. Future Study

To confirm our hypothesis in which PurE and PurK from T. maritima form a complex to stabilize N^5-CAIR, techniques to determine a protein-protein interaction will be applied, such as chemical cross-linking, gel filtration or chromatography. The complex can also be determined by CD spectroscopy.

REFERENCES

1. Murray, A. W. "The Biological Significance of Purine Salvage". *Annu. Rev. Biochem.* 40, 811-826 (1971).
2. Murray, A. W. et al. "Nucleotide biosynthesis from preformed purines in mammalian cells: regulatory mechanisms and biological significance". *Prog Nucleic Acid Res Mol Biol.* 10, 87-119 (1970).
3. Fontenelle, L. J., Henderson J. F. "An enzymatic basis for the inability of erythrocytes to synthesize purine ribonucleotides de novo". *Biochim. Biophys. Acta.* 177, 175-176 (1969).
4. Brown, A. M. et al. "Purine biosynthesis in archaea: variations on a theme". *Biology Direct.* 6: 63 (2011)
5. Tranchimand, S. et al. "*Treponema denticola* PurE Is a Bacterial AIR Carboxylase". *Biochemistry.* 50, 4623-4637 (2011)
6. Meyer, M. et al. "Purification and Characterization of the *purE*, *purK*, and *purC* Gene Products: Identification of a Previously Unrecognized Energy Requirement in the Purine Biosynthetic Pathway". *Biochemistry.* 31. 5022-5032 (1992).
7. Firestine, S. M. et al. "Reactions catalyzed by 5-Aminoimidazole Ribo- nucleotide Carboxylases from *Escherichia coli* and *Gallus gallus*: A case for Divergent Catalytic Mechanisms?" *Biochemistry.* 33, 11927-11934 (1994).
8. Maria, V. F. "Novel inhibitors of the bacterial de novo purine biosynthesis enzymes, n5-carboxyaminoimidazoleribonucleotide synthetase and mutase". Master's Thesis,

Wayne State University, 2012.

9. Mathews, I. et al. "Crystal structure of *Escherichia coli* PurE, an unusual mutase in the purine biosynthetic pathway". *Structure.* 7, 1395-1406 (1999).

10. Thoden, J. B. et al. "Three-Dimensional Structure of N^5-Carboxyamino- imidazole Ribonucleotide Synthetase: A Member of the ATP Grasp Protein Superfamily". *Biochemistry.* 38, 15480-15492 (1999).

11. Brugarolas, P. et al. "Structural and biochemical characterization of N^5-carboxyaminoimidazole ribonucleotide synthetase and N^5-carboxy-aminoimidazole ribonucleotide mutase from *Staphylococcus aureus*". *Acta Cryst.* 67, 707-715 (2011).

12. Tuntland, M. L. et al. "Structure of N^5-carboxyaminoimidazole ribonucleotide synthase (PurK) from *Bacillus anthracis*". *Acta Cryst.* 67, 870-874 (2011).

13. Schwarzenbacher, R. et al. "Crystal Structure of a Phosphoribosyl- aminoimidazole Mutase PurE (TM0446) From Thermotoga maritima at 1.77-Å Resolution". *PROTEINS: Structure, Function and Bioinformatics.* 55, 474-478 (2004).

14. Firestine, S. M. et al. "Biochemical Role of the *Crypococcus neoformans* ADE2 Protein in Fungal *de novo* Purine Biosynthesis". *Archives of biochemistry and biophysics.* 351, 123-134 (1998).

15. Li, S. et al. "Octameric Structure of the Human Bifunctional Enzyme PAICS in Purine Biosynthesis". *J. Mol. Biol*. 366, 1603-1614 (2007).

16. Mueller, E. J. et al. "N^5-Carboxyaminoimidazole Ribonucleotide: Evidence for a

New Intermediate and Two New Enzymatic Activities in the *de Novo* Purine Biosynthetic Pathway of *Escherichia coli*". *Biochemistry*. 33, 2269-2278 (1994).

17. Huber, R. et al. "*Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C". *Arch Microbiol*. 144, 324-333 (1986).

18. Nelson, K. E. et al. "Evidence for lateral gene transfer between Archaea and Bacteria from genome sequence *Thermotoga maritima*". *Nature*. 399, 323-329 (1999).

19. Okada, K. et al "Crystal structures of N5-carboxyaminoimidazole ribo- nucleotide synthetase, PurK, from thermophilic bacteria". To be published.

20. Meyer, E. "Two new activities and a new intermediate in the purine pathway". PhD's thesis, Massachusetts Institute of Technology, 1996.

21. Wilquet, V. et al. "Purification and characterization of recombinant *Thermo- toga maritima* dihydrofolate reductase". *Eur. J. Biochem.* 255, 628-637 (1998).

22. Lozada-Ramírez, J. D. et al. "Recombinant S-Adenosylhomocysteine Hydrolase from *Thermotoga maritima*: Cloning, Overexpression, Characterization, and Thermal Purification Studies". *Appl Biochem Biotechnol.* 170, 639-653 (2013).

23. Bartoschek, S. et al. "N-Carboxymethanofuran (carbamate) formation from methanofuran and CO_2 in methanogenic archaea". *Eur. J. Biochem.* 267, 3130-3138 (2000).

24. Beismann-Driemeyer, S. et al. "Imidazole Glycerol Phosphate Synthase from *Thermotoga maritima*. Quaternary structure, steady-state kinetics, and reaction

mechanism of the bienzyme complex". *J. Bio. Chem*. 276, 20387 -20396 (2001).

25. Massant, J. et al. "Metabolic Channeling of Carbamoyl Phosphate, a Thermolabile Intermediate: Evidence for Physical Interaction between Carbamate Kinase-like Carbamoyl-phosphate Synthase and Ornithine Carbamoyltransferase From The Hyperthermophile *Pyrococcus furiosus*". *J. Biol. Chem*. 277, 18517-18522 (2002).

26. Allen, C. M., Jr, Jones, M. E. "Decomposition of Carbamylphosphate in Aqueous Solution". *Biochemistry.* 3, 1238–1247 (1964).

ABSTRACT

Functional Studies of 5-Carboxyaminoimidazole Ribonucleotide Synthetase and Mutase from *Thermotoga maritima*

by

MINGYANG ZHAO

August 2014

Advisor: Dr. Steven M. Firestine

Major: Pharmaceutical Sciences

Degree: Master of Science

De novo purine biosynthesis is divergent depending upon the organism. In bacteria, yeasts and plants, 5-aminoimidazole ribonucleotide (AIR) is converted to 4-carboxyaminoimidazole ribonucleotide (CAIR) by N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) synthetase (PurK) and N^5-CAIR mutase (Class I PurE). In animals, AIR is converted into CAIR by AIR carboxylase (Class II PurE). The distinction makes PurK and Class I PurE good targets in design of antimicrobial drugs. N^5-CAIR is chemically unstable with $t_{1/2}$ of about 0.9 minutes at pH 7.8 and 37 °C. If the production of N^5-CAIR is not regulated relative to its conversion to CAIR, ATP utilization can rapidly become non-stoichiometric. One mechanism to study this problem is to investigate how the problem is dealt with under conditions in which N^5-CAIR is even more unstable, such as at higher temperature. *Thermotoga maritima*, originally isolated from geothermal heated marine sediment, is a thermophilic bacterium whose optimal growth temperature is 80°C.

The TM0446 and TM0447 genes of Thermotoga maritima encode a phosphoribosylaminoimidazole mutase (PurE) and synthetase (PurK) individually. We cloned genes and expressed both proteins. Enzyme kinetic analyses for each enzyme indicate that these enzymes follow the same pathway as those found in *E. coli*. Studies of the stoichiometry of the reaction reveal that at elevated temperature, the reactions are stoichiometric as long as the ratio of PurE and PurK is 1:1. When this ratio is lower, ATP consumption becomes non-stoichiometric. We have also examined whether there is a complex formed between these enzymes. Our data indicate that each enzyme is capable of regulating the activity of the other. This suggests that a complex is formed by these two enzymes.

AUTOBIOGRAPHICAL STATEMENT

MINGYANG ZHAO

EDUCATION

2014 M.S. Pharmaceutical Sciences, Wayne State University, Detroit, Michigan, U.S.A.
2012 B.S. Pharmacy, China Pharmaceutical University, Nanjing, Jiangsu, China.