BIOCHEMICAL AND KINETIC CHARACTERIZATION OF METALLOPEPTIDASES FROM THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*

by

SHERRY V. STORY

(Under the Direction of Michael W. W. Adams)

ABSTRACT

The primary goal of this research project was to purify and characterize the biochemical and kinetic properties of three distinct zinc metallopeptidases and try to determine the physiological role of these enzymes in *Pyrococcus furiosus*. These enzymes include an aminoacylase, a lysine aminopeptidase, and an alanine aminopeptidase. None of these enzymes had been isolated or characterized from a hyperthermophile or from an archaeon, at the onset of this project. Aminoacylase catalyzes the hydrolysis of nonpolar N-acylated-L-amino acids (Met, Ala, Val, and Leu) and was purified by multistep chromatography. It contains three zinc atoms per subunit and is optimally active at 100°C. The gene (PF0597**)** encoding the enzyme was cloned but active recombinant enzyme was not produced in *Escherichia coli*. Based on its substrate specificity and kinetic properties, aminoacylase is proposed to have a role in peptide catabolism of *P. furiosus*

Lysine aminopeptidase (KAP) was purified by multistep chromatography from cell extracts of *P. furiosus*. It contains two zinc atoms per subunit and hydrolyzed only

basic N-terminal residues (Lys- and Arg-pNA). Surprisingly, its activity was stimulated four-fold by the addition of cobalt ions. The gene (PF1861) encoding the enzyme was was expressed in *Escherichia coli* and the recombinant protein was purified. Its properties, including molecular mass, metal ion dependence, pH and temperature optima were indistinguishable from those of the native form. Based on its amino acid sequence, KAP is annotated in the genome sequence as an endoglucancase, although such an activity could not be demonstrated, KAP is part of the M18 family of peptidases which includes yeast aminopeptidase I. KAP is the first lysine aminopeptidase to be purified from a prokaryotic source. Another gene (PF0369) annotated in the genome of *Pyrococcus furiosus* as encoding an endoglucanase was cloned and expressed in *Escherichia coli*. The recombinant enzyme also lacked endoglucanase activity and hydrolyzed tri- and tetrapeptides containing an Ala residue at the N-terminus indicating that it is an alanine aminopeptidase. It has an optimal temperature for activity of 100 ^{o}C using Ala-Ala-Ala-Ala [2mM] as the substrate. The enzyme contains two zinc atoms per subunit but its activity was strictly dependent on the addition of cobalt ions for reasons not known. The kinetic properties of both alanine and leucine aminopeptidase suggest that these two enzymes play roles in the degradation of peptide growth substrates of *P. furiosus.*

INDEX WORDS: *Pyrococcus furiosus*, hyperthermophilic archaea, aminoacylase, lysine aminopeptidase, alanine aminopeptidase, metallopeptidase, protein growth substrates.

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DEDICATION

This page is dedicated go my grandfather, Willie Gus Hines, who passed before I completed this degree. Although he never finished elementary school, he instilled in me the importance of education at an early age. He was my strength throughout my childhood and I will always appreciate and love him for his teachings about God and life. I also dedicate this to my husband, Micah Story, who was my greatest supporter over the past few years and to my children, Mikala and Jordan.

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A special thanks to my adopted mom and dear friend, Paula Halbert, who gave many words of wisdom, spiritual support, and encouragement over the years. I would like to thank Terris Thomas, my friend and sister in Christ, who has been there to support and encourage me from the beginning. I would like to thank my friend, my brother, Darin Thomas, for his support and encouragement in the last year. Finally, I would like to thank my dear friend, Denese Williams for all of her support and encouragement. These people really helped me to keep pushing forward during times in which I wanted to give up. I love and appreciate all of you.

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LIST OF ABBREVIATIONS

AAP-alalnine aminopeptidase

ACY-acylase I

ADP-adenosine 5’-triphosphate

AMP-adensosine 5’-monophosphate

ARGE-acetylornithine deacetylase

ATP-adenosine 5’-triphosphate

CoA-coenzyme A

CPG2-carboxypeptidase 2

DAP-deblocking aminopeptidase

DNA-deoxyribonucleic acid

DTT-dithiothreitol

DT-dithionite

EDTA-ethylene diamine-tetra-acetic acid

Fd-ferredoxin

GAPOR-glyceraldehyde-3-phosphate oxidoreductase

GAPDH-glyceraldehyde-3-phosphate dehydrogenase

IOR-indolepyruvate oxidoreductase

IPTG-isopropyl-β-D-thiogalactopyranoside

KAP-lysine aminopeptidase

KGOR-2-ketoglutarate oxidoreductase

MAP-methionine aminopeptidase

NTA-nitrilotriacetic acid

NMR-nuclear magnetic resonance spectroscopy

ORF-open reading frame

PCR-polymerase chain reaction

PMSF-phenylmethylsulfonyl fluoride

POR-pyruvate oxidoreductase

RNA-ribonucleic acid

Trx-thioredoxin

VOR-2-ketosisovalerate oxidoreductase

CHAPTER 1

INTRODUCTION

Hyperthermophilic microorganisms were first isolated in the early 1980s by Stetter and coworkers (Stetter 1996a). These organisms have optimum growth temperatures of at least 80 ^{o}C and maximum growth temperatures above 90 ^{o}C with a current upper limit of 113 ^{o}C. To date, more than 29 different hyperthermophilic genera are known and these are listed in Table 1.1. Currently, hyperthermophiles have been isolated mainly from aquatic terrestrial and marine geothermal environments, in which they form complex microbial communities (Stetter 2000). Of the 29 genera listed in Table 1.1 only four (*Thermoproteus*, *Thermofilum*, *Sulfophobococcus,* and *Methanothermus*) have been found exclusively in fresh water environments. Marine ecosystems include various hydrothermal systems located at shallow and abyssal depth (Huber et al. 2000). On land, natural biotopes consist of hot springs, solfataric fields, and mud-holes. Deep-sea vent formation occurs by the convective circulation of seawater through newly formed oceanic crust thereby generating sub seafloor volcanic activity. Temperatures in these vents can approach 400 ^{o}C and form 'black smoker' chimneys that discharge jets of water blackened by metal sulfide precipitates (Jannasch 1985). Smoker walls tend to show a steep temperature gradient created by the strong cooling of the surrounding cold seawater (about 3 ^{o}C). Hyperthermophiles can be isolated both from the porous smoker walls at temperatures well in excess of 100 ^{o}C and from the surrounding hot sediments (Huber, 2000). All of the marine hyperthermophiles require

high salt concentrations to grow, therefore it may seem improbable that the same genus can be isolated from both fresh water and marine environments (Adams 1999). Nonetheless, two species, *Desulphurococcus* and *Pyrobaculum* have been isolated from both freshwater and marine ecosystems. Similarly, species of *Pyrococcus* and *Methanococcus* have been found in shallow and deep sea vents. Of the known hyperthermophiles, none seem to be dramatically affected by growth under high pressures of their natural environment.

Of the 29 genera listed in Table 1.1, two are bacteria (*Aquifex* and *Thermatoga*) and the remainder are Archaea. Archaea constitutes the third domain of life and was first recognized in the early 1980s based on 16S rRNA analyses. The pioneering work of C. R. Woese led to a universal phylogenetic tree exhibiting a tripartite division of the living world based on 16S rRNA sequence comparisons (Woese and Fox 1977; Woese et al. 1990). The three domains are the Bacteria, the Archaea, and the Eukarya (Fig. 1.1). The hyperthermophiles represent the most slowly evolving organisms within both the archaeal and the bacterial domains (Olsen et al. 1994; Stetter 1996b). It is believed that present-day hyperthermophiles may be the key to understanding the biochemical evolution of the primary metabolic pathways found in the mesophilic organisms, especially those in higher life forms, in which hyperthermophiles may share a common ancestor (Woese et al. 1990).

Archaea are prokaryotes in which most of the genera are indistinguishable from bacteria by size and shape. A few have morphologies that are not found in bacteria, such as polygonal in halophilic Archaea or very irregular cocci in particular hyperthermophiles (Forterre et al. 2002). The Archaea consist of two major kingdoms: The Crenarchaeota

and the Euryarcheota. The latter kingdom consists of methanogens, extreme halophiles, sulfate-reducing species, and two types of thermophiles (*Thermoplasma* and the *Thermococcus-Pyrococcus* group) and this group is relatively heterogeneous. The Crenarchaeota are a physiologically homogenous group mainly composed of a compact clustering of hyperthermophiles including the *Sulfolobales-Thermoproteales* branch (Woese et al. 1990; Blochl 1995). A third phylum, Nanoarchaeota, has been discovered based on 16S ribosomal RNA sequence (Hohn et al. 2002). *Nanoarchaeum equitans* is the only organism that has been isolated and characterized. It is a spherical organism about 400 nm in diameter with a genome size of only 0.5 megabases. It is a symbiotic organism that is obligately dependent on a specific archaeal host belonging to the genus *Ignicoccus*.

1. Metabolism of Hyperthermophiles

Virtually all of the hyperthermophiles are strict anaerobes and in this regard represent the environments from which they have been isolated. However, there are a few exceptions. There are five genera containing aerobic species and three of them (*Pyrolobus*, *Aquifex*, and *Pyrobaculum*) are microaerophiles (Table 1.1). These species are capable of reducing nitrate thereby obtaining energy by a respiratory-type metabolism (Adams 1999). Since the ability of organisms to use O_2 is limited at temperatures above 90 ^{o}C, the predominant metabolic mode amongst the hyperthermophilic species is the use of elemental sulfur (S^{o}) as an electron acceptor, thereby generating H_2S. In fact, the majority of the currently known hyperthermophilic genera fall into the 'S^{o}-dependent category'. Nearly all of these species are restricted to obtaining energy for growth only

by S^o respiration (Adams 1999). The exceptions include some species of *Pyrococcus* and *Thermococcus* which are capable of growing by fermentative-type mechanisms without the presence of elemental sulfur. In addition, there are three S^o-independent genera that are unable to utilize elemental sulfur. These include *Sulfophobococcus* (Hensel 1997) which grows fermentatively, and *Aeropyrum* (Sako et al. 1996) which is an obligate anaerobe. The third genus is *Pyrolobus* which obtains energy by a respiratory metabolism in which H_2 is the electron donor and nitrate, thiosulfate or low concentrations of O_2 are the electron acceptor. One of the species, *P. fumarii* has a maximal growth temperature of 113 ^oC, the highest reported yet and amazingly has been shown to survive autoclaving (121 ^oC for 1 hour) (Blochl et al. 1997). Three genera of methanogens (Table 1.1) are included as another group of hyperthermophilic archaea which like their mesophilic relatives, produce methane from H_2 and CO_2. Two closely related genera, the sulfate-reducing *Archaeglobus* and the iron-reducing *Ferroglobus* also carry out S^o-independent modes of metabolism. The two bacterial genera *Thermotoga* and *Aquifex* have different metabolic properties. Species of *Thermotoga* are all fermentative and reduce S^o to H_2S, although they well in the absence of sulfur. In contrast, *Aquifex* species are microaerophilic denitrifiers.

The majority of hyperthermophiles are obligate heterotrophs with a few exceptions (Table 1.1). Surprisingly, the main source of carbon occurs via the breakdown of peptidolytic substrates rather than carbohydrates. Furthermore, significant growth is only obtained on complex proteinaceous substrates such as yeast, bacterial or meat extracts, peptone and tryptone. The true growth substrates for these organisms are unknown. There are a few species of *Pyrococcus*, *Thermococcus*, and *Desulphurococcus*

(Raven 1997; Adams 1999) that are capable of growing on mixtures of amino acids, however, the highest cell yields and lowest doubling times are only observed when complex media are used. This suggests that certain factors are required for growth, but is not provided by mixtures of amino acids and vitamins (Rinker 1996)]. It is shown in Table 1.1 that most of the heterotrophic species are strictly proteolytic; however, a few are sacchrolytic with a limited substrate range. Most are capable of using complex carbohydrates such as starch and glycogen, and disaccharides such as maltose and cellobiose. One exception is a *Thermococcus* species that grows on starch but not maltose (Miroshnichenko 1989). Virtually none of the hyperthermophiles are able to utilize monosaccharides except *Thermoproteus*, which uses glucose as the main carbon source, on the other hand, growth using monosaccharides is characteristic of the hyperthermophilic bacterium, *Thermotoga*. Pyruvate is capable of supporting growth of some of the hyperthermophilic archaea, including some *Pyrococcus* species. Cell yields are much lower using pyruvate in comparison to using complex peptides or carbohydrates as growth substrates (Adams 1999). Recently, a novel hyperthermophilic archaeon, *Geoglobus ahangari*, was isolated that is capable of growing not only on pyruvate, but acetate, palmitate, and stearate, all of which are coupled to reduction of Fe(III) (Kashefi et al. 2002). It is clear that most of the hyperthermophiles which have been isolated so far are typically strict anaerobic heterotrophs that are either obligately or facultatively dependent upon sulfur reduction for maximum growth.

Pyrococccus furiosus has been the model organism for studying the physiology and metabolism of hyperthermophilic archaea and this organism is the focus of studies discussed herein. *P. furiosus* was isolated from geothermally-heated marine sediments

Table 1.1. The hyperthermophilic genera: organisms that grow at $\geq$ 90° C[*]

Genus	T_{max}†	Metabolism‡	Substrates§	Acceptors
S^o-dependent archaea				
Thermofilum (c)¶	100°	hetero	Pep	S^o, H^+
Staphylothermus (d/m)	98°	hetero	Pep	S^o, H^+
Thermodiscus (d/c)	98°	hetero	Pep	S^o, H^+
Desulfurococcus (d/c)	90°	hetero	Pep	S^o, H^+
Thermoproteus (c)	92°	hetero (auto)	Pep, CBH (H_2)	S^o, H^+
Pyrodictium (d/m)	110°	hetero (auto)	Pep, CBH (H_2)	S^o, H^+
Pyrococcus (d/m)	105°	hetero	Pep	$\pm S^o$, H^+
Thermococcus (d/m)	97°	hetero	Pep, CBH	$\pm S^o$, H^+
Hyperthermus (m)	110°	hetero	Pep (H_2)	$\pm S^o$, H^+
Stetteria (m)	103°	hetero	Pep + H_2	S^o, $S_2O_3^{2-}$
Pyrobaculum (d/c)	102°	hetero (auto)	Pep (H_2)	$\pm S^o$, mO_2, NO_3^-
Acidianus (m/c)	96°	auto	S^o, H_2	S^o, O_2
Palaeococcus (d)	90°	hetero	Pep, Fe^{2+}	S^o, H^+
Ignicoccus (d/m)	98°	auto	H_2	S^o
Vulcanisaeta (c)	90°	hetero	Pep	S^o, $S_2O_3^{2-}$
Caldivirga (c)	92°	hetero	Pep, CBH	S^o, $S_2O_3^{2-}$, SO_4^{2-}
S^o-independent archaea				
Sulfophobococcus (c)	95°	hetero	Pep	-
Pyrolobus (d)	113°	auto	H_2	$S_2O_3^{2-}$, mO_2, NO_3^-
Aeropyrum (m)	100°	hetero	Pep	O_2
Thermosphaera (c)	90°	hetero	Pep	-
Sulfate-reducing archaea				
Archaeoglobus (d/m)	95°	hetero (auto)	CBH, H_2	$S_2O_3^{2-}$, SO_4^{2-}
Iron-oxidizing archaea				

Ferroglobus (m)	95^{o}	auto	Fe^{2+}, H_2, S_2^-	$S_2O_3^{2-}$, NO_3^-
Iron-reducing archaea				
Geoglobus (d)	90^{o}	hetero (auto)	Fe^{3+} + H_2, long-chain FAs	Fe_2O_3
Methanogenic archaea				
Methanococcus (d/c)	91^{o}	auto	H_2	CO_2
Methanothermus (c)	97^{o}	auto	H_2	CO_2
Methanopyrus (d/m)	110^{o}	auto	H_2	CO_2
Bacteria				
Thermotoga (d/m)	90^{o}	hetero	Pep, CBH	S^{o}, H^+
Aquifex (m)	95^{o}	auto	S^{o} (H_2)	mO_2, NO_3^-
Thermocrinis (c)	90^{o}	auto		$\pm S^{o}$, H^+

*Table adapted from Adams, 1999. Data taken from Kelly and Adams, 1994, Blochl et al., 1997,
Sako et al., 1996, Jochimsen et al., 1997, Kashefi et al., 2002, Itoh, 2002, Huber et al., 2000, Takai et al., 2000, Itoh et al., 1999, and Hensel et al., 1997.
†Maximum growth temperature. ‡Denotes heterotrophic (hetero) or autotrophic (auto) growth mode.
§Growth substrates include peptides (Pep), carbohydrates (CBH), hydrogen (H_2), elemental sulfur (S^{o}), and fatty acids (FA) as electron donors.
¶Species have been found in continental hot springs (c), shallow marine (m), and/or deep (d) sea environments.

Fig 1.1.

Universal phylogenetic tree in rooted form, showing the three domains. Branching order and branch lengths are based upon 16s rRNA sequence comparisons. The root position was determined by comparing sequences of paralogous genes that diverged from each other before the three primary lineages emerged from a common ancestor. Red lines denote hyperthermophilic species. Modified from Stetter et al., 1992 and Woese et al., 1990.

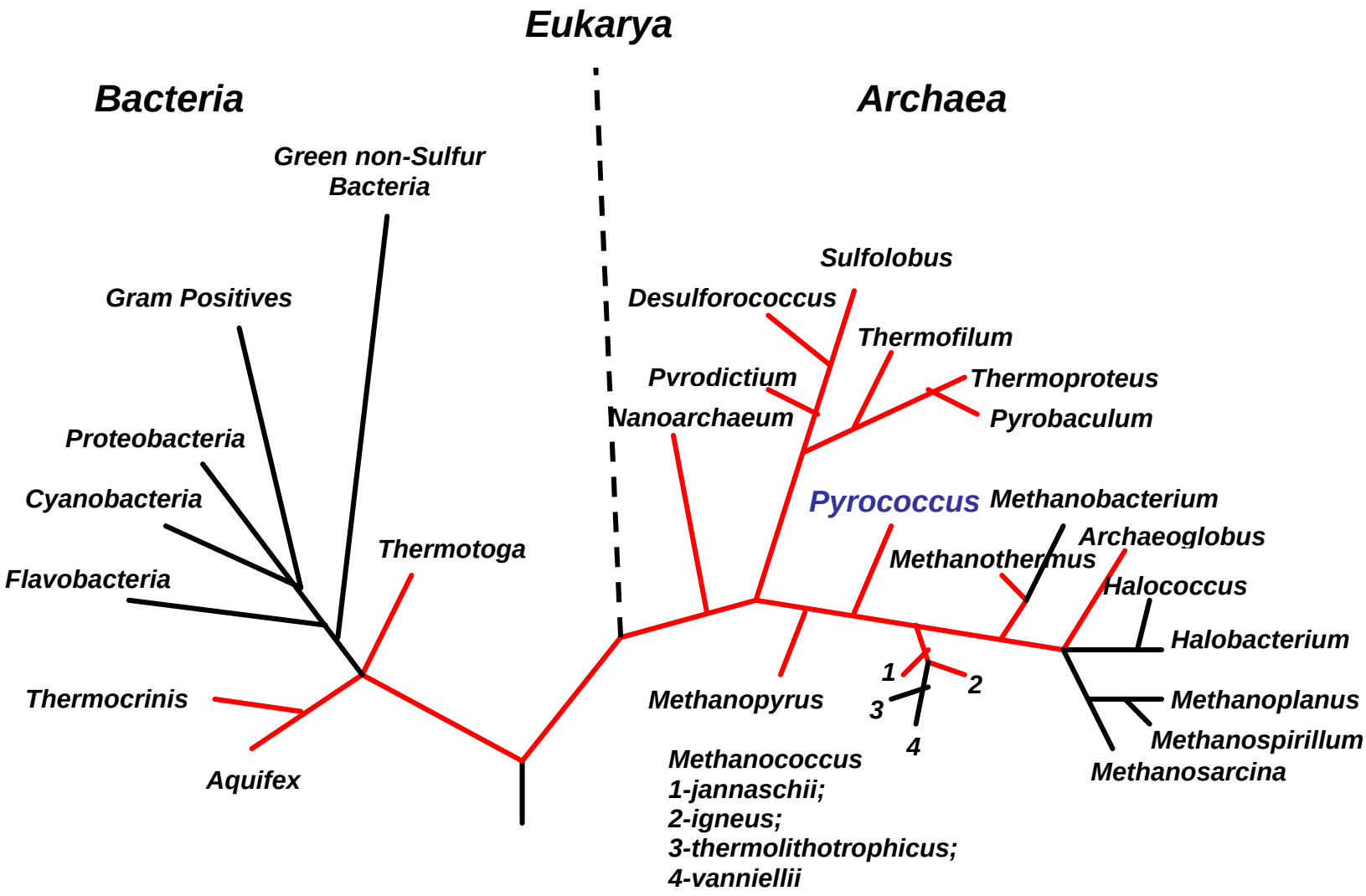
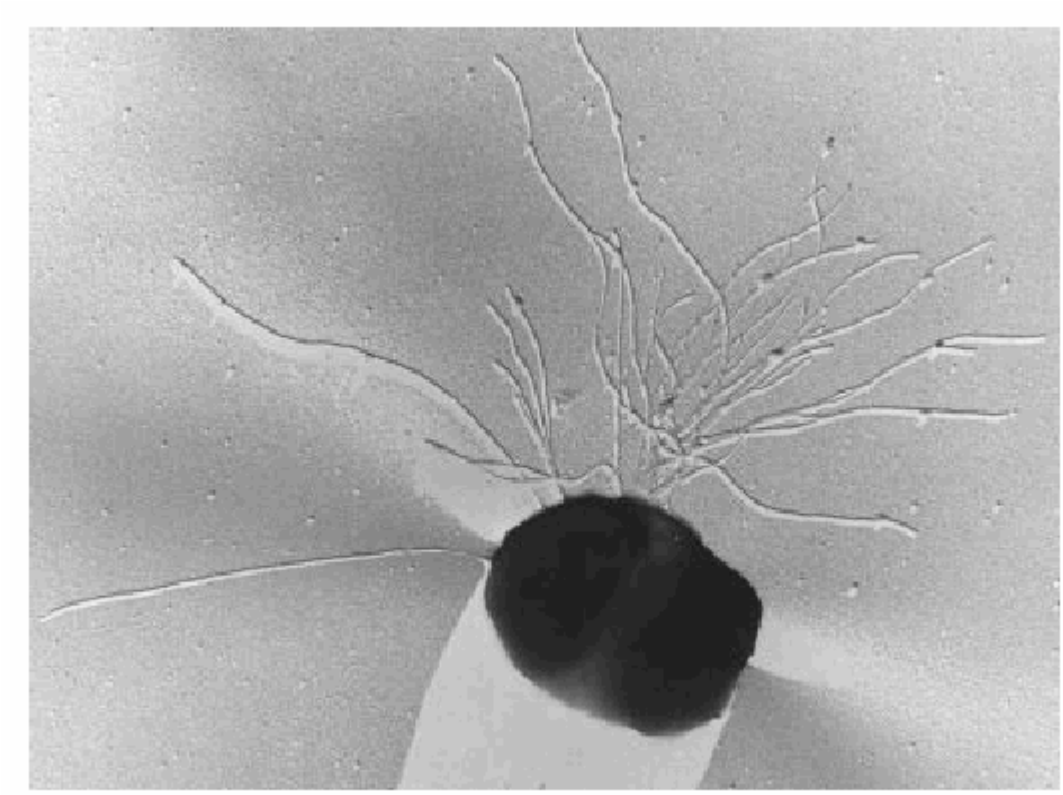

Eukarya
Bacteria
Archaea
Green non-Sulfur Bacteria
Gram Positives
Proteobacteria
Cyanobacteria
Flavobacteria
Thermotoga
Thermocrinis
Aquifex
Sulfolobus
Desulfurococcus
Thermofilum
Pvrodictium
Thermoproteus
Nanoarchaeum
Pyrobaculum
Pyrococcus
Methanobacterium
Archaeoglobus
Methanothermus
Halococcus
Halobacterium
Methanoplanus
Methanospirillum
Methanosarcina
Methanopyrus
1
2
3
4
Methanococcus
1-jannaschii;
2-igneus;
3-thermolithotrophicus;
4-vanniellii

Fig 1.2.

Electron micrograph of *P. furiosus*. The image shows monopolar polytrichous flagellation. It is platinum shadowed. Taken from Fiala and Stetter, 1986.



off the coast of Vulcano, Italy by Stetter and co- workers in 1986 (Stetter 1986). It is spherical in shape, 0.8 to 2.5 μm in width and has monopolar, polytrichous flagellation. It grows between 70 and 103 ^{o}C with an optimal growth temperature of 100 ^{o}C with a doubling time of 37 minutes. *P. furiosus* is a strict anaerobic heterotroph that grows both proteolytically on peptides (and requires elemental sulfur) and saccharolytically using maltose, starch, glycogen, and cellobiose as its primary carbon source. The metabolic products are CO_2, and H_2, alanine, and acetate (Kengen 1996). It is evident from the numerous studies that have been done to determine a defined growth medium that complex media such as yeast extract or tryptone are essential for obtaining high density cultures (Hoaki et al. 1994; Krahe 1996; Raven 1997; Adams et al. 2001).

2. Major Metabolic Pathways of *Pyrococcus furiosus*

2.A. Saccharolytic Pathway

P. furiosus is capable of utilizing starch, cellobiose, glycogen, and maltose as its primary carbon source, but not monosaccaharides. Several of the enzymes involved in the breakdown of these compounds to glucose have been characterized (Sunna et al. 1997). By using ^{13}C NMR spectroscopy, it was discovered that *P. furiosus* breaks down sugars via a modified Embden-Meyerhof pathway (Kengen et al. 1994). The pathway was further confirmed by the isolation of glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR). GAPOR replaces GAPDH, which is the enzyme involved in the first step of sugar breakdown (Mukund and Adams 1995). GAPOR differs from GAPDH in two ways. First, ferredoxin rather than NAD is used as an electron carrier. Second, it produces 3-phosphoglycerate rather than 1, 3-bisphosphoglycerate (the product

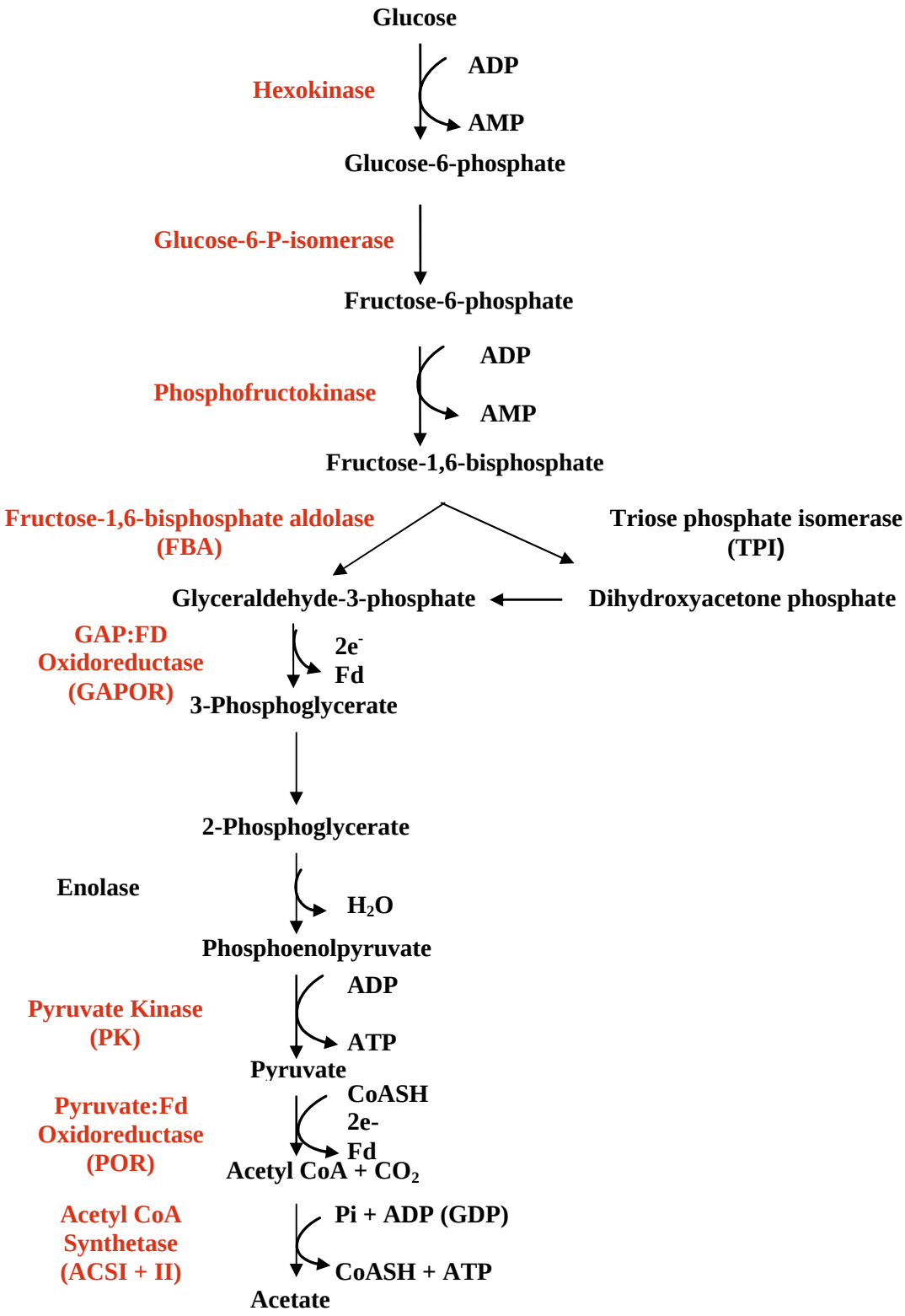
of the GAPDH reaction). Surprisingly, three other unusual enzymes are involved in this pathway. These are ADP- rather than ATP-dependent hexokinases and phosphofructokinase (Kengen et al. 1994) and an acetate-producing acetyl CoA synthetase (Schafer 1993). An enzyme that uses ferredoxin as the electron acceptor catalyzes the second oxidation step in the conversion of glucose to acetate. This enzyme is pyruvate ferredoxin oxidoreductase (POR) which produces acetyl CoA (Adams 1999). Oxidation of ferredoxin is coupled to the reduction of protons to produce H_2 or the reduction of elemental sulfur, if present, to produce hydrogen sulfide. The pathways by which this occurs are not clear at present.

2.B. Proteolytic Pathway

The majority of the hyperthermophilic archaea are capable of utilizing only protein-based substrates as their sole source of carbon and nitrogen. *P. furiosus* efficiently uses peptides for growth only in the presence of elemental sulfur. The first step in the proteolytic pathway is the breakdown of peptides. *P. furiosus* has high intracellular and extracellular protease activities (Bauer et al. 1996). Specific aliphatic and aromatic aminotransferases convert the amino acids produced by protease action to 2-keto acids (Fig. 1.4). *P. furiosus* has four distinct 2-keto acid oxidoreductases to convert transaminated amino acids to their corresponding acyl CoA derivative. These are abbreviated as VOR (2-ketoisovalerate oxidoreductase) which oxidizes branched chain 2-keto acids, IOR (indolepyruvate oxidoreductase) which uses aromatic 2-keto acids, KGOR (2- ketoglutarate oxidoreductase), and POR (pyruvate oxidoreductase). All four of these enzyme reactions are coupled to ferredoxin reduction (Fig. 1.4). VOR has the

Fig. 1.3

Modified Embden-Meyerhof Pathway for glycolysis and production of acetate by *P. furiosus*. Metabolism of glucose to acetate. Enzymes that are unique to *P. furiosus* are highlighted. Fd represents ferredoxin. Modified from Mukund and Adams, 1995. (Note: glycolysis is only from glucose ⟶ pyruvate).


Glucose
Hexokinase
ADP
AMP
Glucose-6-phosphate
Glucose-6-P-isomerase
Fructose-6-phosphate
Phosphofructokinase
ADP
AMP
Fructose-1,6-bisphosphate
Fructose-1,6-bisphosphate aldolase (FBA)
Triose phosphate isomerase (TPI)
Glyceraldehyde-3-phosphate
Dihydroxyacetone phosphate
GAP:FD Oxidoreductase (GAPOR)
2e-
Fd
3-Phosphoglycerate
2-Phosphoglycerate
Enolase
H2O
Phosphoenolpyruvate
Pyruvate Kinase (PK)
ADP
ATP
Pyruvate
Pyruvate:Fd Oxidoreductase (POR)
CoASH
2e-
Fd
Acetyl CoA + CO2
Acetyl CoA Synthetase (ACSI + II)
Pi + ADP (GDP)
CoASH + ATP
Acetate

highest specificity for 2-ketoacids derived from valine, leucine, and isoleucine, whereas IOR oxidizes derivatives of tyrosine, phenylalanine, and tryptophan (Adams, 1999). Acyl CoA intermediates are generated by the 2-ketoacid oxidoreductases and are further converted to acids by two distinct acyl CoA synthetases (Fig. 1.4) termed ACS I and II (Mai 1996). The products of the POR and VOR reactions, acetyl CoA and isobutaryl CoA are used by acyl CoA synthetases I (ACS I) which cannot use aryl CoAs. On the other hand, ACS II, can utilize acetyl CoA, branched chain acyl CoAs, and aryl CoAs. Neither enzyme can oxidize succinyl CoA, which is generated by KGOR. It is thought that succinyl CoA is used for biosynthesis and not energy conservation (Adams 1999). Hence, *P. furiosus* produces a variety of organic acids during growth on peptides.

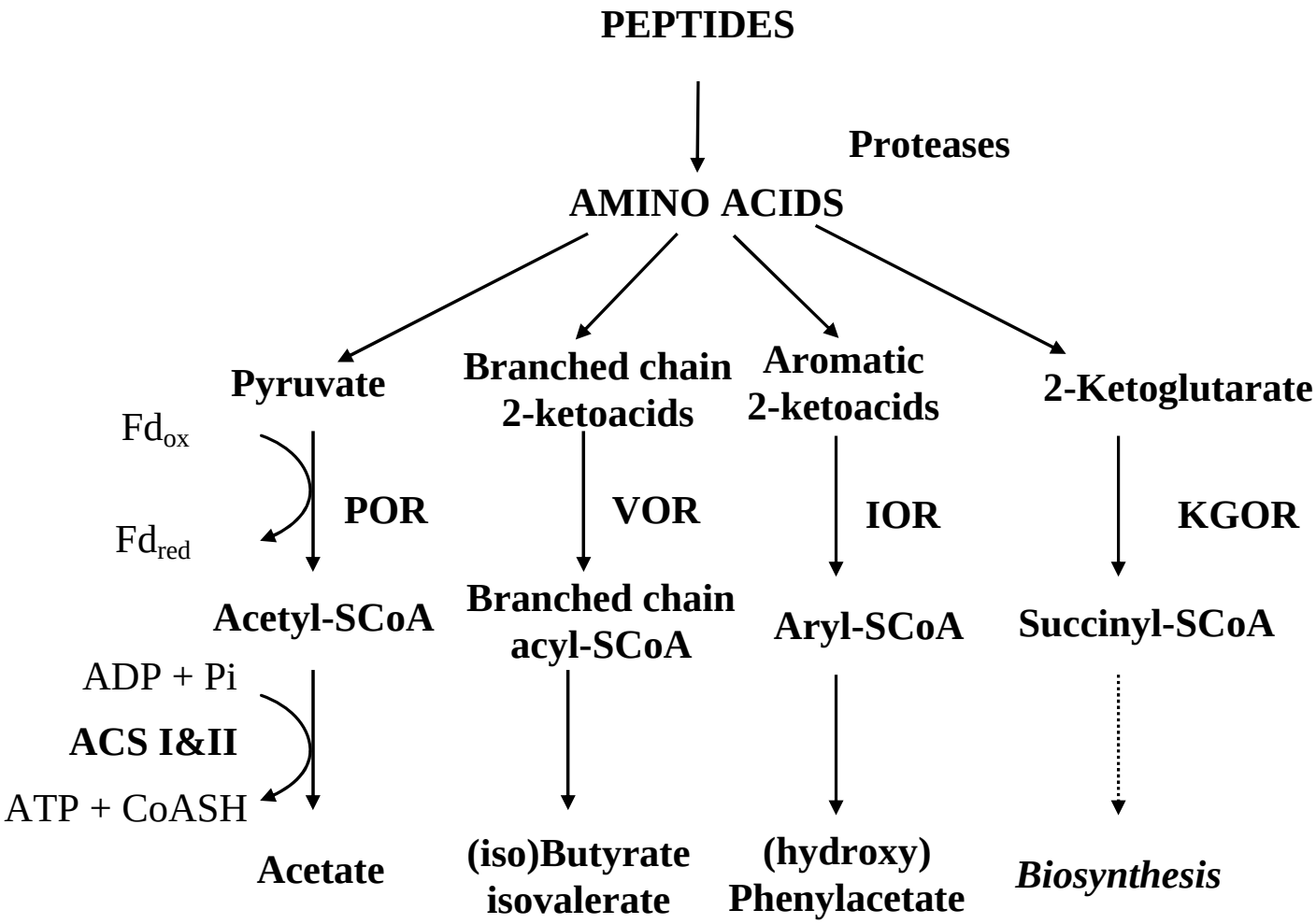
3. **Proteases**

3A. Classes of Proteases

Proteases are classified according to three major criteria: 1) type of reactions catalyzed, 2) chemical nature of the catalytic site, and 3) evolutionary relationship with reference to structure (Barrett 1994). They are subdivided into two distinct groups based on their site of action (i.e. exopeptidases and endopeptidases). Exopeptidases hydrolyze the peptide bond proximal to the amino or carboxy termini of the substrate (i.e. aminopeptidase or carboxypeptidase, respectively), whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (Rao et al. 1998). Proteases have been further categorized into five major classes based on their catalytic mechanisms that can involve serine, threonine, cysteine, aspartate, and metals. (Barrett 1998). There are a few proteases that are excluded from this standard classification, such as the ATP-

Fig 1.4.

Proposed proteolytic pathway for peptide catabolism in *P. furiosus*. POR, VOR, KGOR, and IOR represent ferredoxin-dependent oxidoreductases that use pyruvate, 2-ketoisovalerate, 2-ketoglutarate, and indolepyruvate, respectively. ACS represents acyl CoA synthetases I and II, and Fd_{ox} and Fd_{red} are the oxidized and reduced forms of ferredoxin, respectively. Adapted from Mai and Adams, 1996.


PEPTIDES
Proteases
AMINO ACIDS
Pyruvate
Branched chain 2-ketoacids
Aromatic 2-ketoacids
2-Ketoglutarate
Fdox
Fdred
POR
VOR
IOR
KGOR
Acetyl-SCoA
Branched chain acyl-SCoA
Aryl-SCoA
Succinyl-SCoA
ADP + Pi
ACS I&II
ATP + CoASH
Acetate
(iso)Butyrate isovalerate
(hydroxy) Phenylacetate
Biosynthesis

dependent proteases that require ATP for activity. Proteases can be divided into different families depending on their amino acid sequence and further divided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Argos 1987; Rawlings 1993). Each family of peptidases has a code letter indicating the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively (Rao et al. 1998).

Metallopeptidases are the most diverse of the catalytic types of proteases (Barrett 1995). These enzymes require the presence of a divalent cation for their activity. They include enzymes ranging from collagenases from higher organisms to thermolysin from bacteria (Hibbs 1985; Okada 1986). Chelating agents such as EDTA and o-phenanthroline inhibit metallopeptidases, but sulfhydryl agents do not affect them. Zinc is the cation most frequently associated with the enzymes and is typically bound by a conserved HEXXH motif (Jongeneel et al. 1989).

3B. Mechanism of Catalysis of Proteases

The catalytic site of proteases contain specificity subsites which can accommodate the side chain of a single amino acid residue from the substrate (Rao et al. 1998). The catalytic mechanism for serine, aspartic, cysteine, and metallo- proteases have been intensely studied and are well understood. The serine proteases contain a Ser-His-Asp catalytic triad, and the hydrolysis of the peptide bond involves an acylation step followed by a deacylation step. Aspartic proteases are characterized by an Asp-Thr-Gly motif in their active site and by an acid-base catalysis as their mechanism of catalysis. Cysteine proteases also have a general acid-base formation followed by hydrolysis of an

acyl-thiol intermediate. Metalloproteases require the binding of a divalent metal cation to metal-binding sequence, i.e. His-Glu-Xaa-Xaa-His (Rao et al. 1998).

3C. Role of Proteases

Proteases and peptidases play an important role in the maintenance of cellular function of organisms from all domains of life. They play a vital role in many physiological and pathological processes such as protein catabolism, cell growth and migration, blood coagulation, tissue arrangement, morphogenesis in development, activation of zymogens, and transport of secretory proteins across membranes. These enzymes catalyze the cleavage of peptide bonds. Extracellular proteases hydrolyze large proteins to peptide fragments for subsequent absorption by the cell whereas intracellular proteases play an important role in metabolic regulation. They also recognize and breakdown unnecessary or abnormal polypeptides, the latter produced as a result of environmental stress, mutation or errors in biosynthetic processes (Tomoyasu et al. 2001).

Cells contain numerous proteases that process proteins and polypeptides (Arsene et al. 2000). Proteolytic reactions must be tightly regulated to prevent destruction of the cell's metabolic machinery. In nature, a variety of protease regulation mechanisms are found, for example, ATP-driven protein degradation, active site access restriction, high substrate specificity, and activation cascades (Vandeputte-Rutten and Gros 2002). Proteases can range from simple monomeric hydrolases to complex, multi-subunit structures with molecular masses high as 1 MDa (Ward 2002).

4. **Proteases in *Pyrococcus furiosus***

Analyses of the genome sequence of *P. furiosus* reveals that it contains three groups of proteases (Table 1.2), ATP-dependent, ATP-independent, and peptidases (Ward 2002). ATP-dependent proteases play a role in protein and peptide turnover and stress response (Gottesman 1996). *P. furiosus* contains five such enzymes including a Lon protease, an ATP-dependent regulatory subunit, two proteasome beta subunits, and a proteasome alpha subunit. Based on the amino acid sequence of the Lon protease, it is unique in archaea due to its lack of an ATP-binding domain; however, there is no biochemical evidence to support the enzyme's function. The 20S proteasome is a cylindrically shaped protease and it acts in a processive manner, cleaving protein substrates at multiple sites to yield peptide fragments of three to thirty amino acids in length (Kisselev 1998). Little is known about the metabolic roles and regulation of Lon and the proteasome in Archaea.

ATP-independent proteases include serine, aspartic, cysteine, and metallo-proteases. *P. furiosus* can grow on proteinaceous substrates as primary carbon and energy sources. These substrates must initially be broken down by extracellular proteases that may or may not be associated with the cell (Ward 2002). Peptides can then be transported into the cell via an ABC-type transporter where they can be further degraded to individual amino acids by intracellular proteases and peptidases. In *P. furiosus*, peptides are fermented to free acids, i.e. acetate, isovalerate, butyrate, and phenylpyruvate, producing ATP by substrate-level phosphorylation (Schafer 1993).

Based on homologs found in *P. furiosus* genome, this organism contains five ATP-dependent proteases, thirteen ATP-independent proteases, and twenty-three

Table 1.2. Proteases found in *P. furiosus*.

Locus	Function
ATP-dependent proteases	
pf_481472	ATP-dependent LA (Lon)
pf_119316	ATP_dependent regulatory subunit
pf_169930	Proteasome beta subunit (PSMB-2)
pf_1318448	Proteasome beta subunit (PsmB-1)
pf_1467425	Proteasome alpha subunit
ATP-independent proteases	
pf_301754	Pyrolysin[a]
pf_251719	Periplasmic serine protease, putative
pf_176949	Metalloprotease
pf_401364	Metalloprotease
pf_472643	Metalloprotease
pf_699579	Subtilisin-like protease
pf_1479679	Protease IV
pf_1553191	Alkaline serine protease
pf_1757236	Putative protease
pf_1599202	Intracellular protease I (PfpI)[b]
pf_637303	Hydrogenase maturation protease (hyc I)
pf_754933	Hypothetical protein
pf_1136394	Conserved hypothetical protein (bacteriocin homolog)
Peptidases	
pf_471039	Carboxypeptidase I[c]
pf_334012	Protein similar to acylaminoacyl peptidase
pf_181525	O-sialoglycoprotein Endopeptidase (gcp-1)
pf_488067	O-sialoglycoprotein Endopeptidase (gcp-2)
pf_561253	Methionine aminopeptidase (MAP)[d]
pf_707740	Proline-dipeptidase related protein
pf_743892	Putative proline-dipeptidase
pf_803543	Prolyl Endopeptidase[e]
pf_848157	Membrane dipeptidase
pf_1775248	D-aminopeptidase
pf_1898123	Putative aminopeptidase
pf_1902688	Putative aminopeptidase
pf_1906416	Putative aminopeptidase

pf_1262501	XAA-Pro dipeptidase (proline dipeptidase)[f]
pf_1224347	Pyroglutamyl-peptidase[g] I
pf_383398	Endoglucanase/peptidase
pf_1444497	Endoglucanase (celM)/peptidase[h]
pf_1717470	Endo-1,4-beta-glucanase/peptidase[i]
pf_1131554	Acetylornithine deacetylase (ArgE)/peptidase
pf_1890964	Succinyl-diaminopimelate desuccinylase/peptidase
pf_329163	Signal sequence peptidase I (SEC11)
pf_679507	Hypothetical protein
pf_619035	IAA-amidohydrolase (aminoacylase)[j]

Table adapted from Ward et al., 2002.
[a]Voorhorst et al., 1996.
[b]Halio et al., 1997.
[c]Cheng et al., 1999.
[d]Tsunasawa et al., 1997.
[e]Harwood et al., 1997.
[f]Ghosh, 1998.
[g]Tsunasawa, 1998
[h]Tsunasawa, 1998.
[i]Story, to be submitted.
[j]Story,2001.

peptidases (Table 1.2). Furthermore, nine of the proteases contain a putative signal sequence thereby suggesting that they are exported from the cell. Of the total forty-one genes, only the products of eleven have been isolated and characterized (Blumentals et al. 1990; Eggen 1990; Connaris 1991; Halio 1997; Harwood et al. 1997; Tsunasawa et al. 1997; Voorhorst et al. 1997; Ghosh et al. 1998; Tsunasawa 1998; Tsunasawa et al. 1998; Cheng et al. 1999; Chang et al. 2001; de Vos et al. 2001; Story et al. 2001). Thus, still much has to be done to understand the properties of protease-type enzymes in this organism.

5. **Properties of the Transition Element Zinc**

Zinc is unique among first-row transition metals because it contains a filled *d* orbital and therefore is not involved in redox reactions (Williams 1987; McCall et al. 2000). Zinc functions as a Lewis acid to accept a pair of electrons, therefore it binds to nitrogen and oxygen as readily as it binds to sulfur. The fact that zinc lacks redox activity makes it a stable ion in a biological medium whose potential is typically in constant flux. Thus, the zinc ion is an ideal metal cofactor for reactions that require a redox-stable ion to function as a Lewis acid-type catalyst, i.e, in proteolysis and the hydration of carbon dioxide (Butler 1998). In addition, due to the filled *d*-shell orbitals, zinc has a ligand-field stabilization energy of zero in all liganding geometries, therefore, no geometry is inherently more stable than another (Huheey 1993). Zinc is stereochemically flexible which allows it to assume multiple coordination geometries. In virtually all zinc-containing metalloenzymes that have been studied, the binding geometry mostly occurs as a slightly distorted tetrahedral with the metal ion coordinating

three or four protein side chains. Some enzymes have been found that contain five-coordinate distorted trigonal bipyramidyl geometry, including astacin, purple acid phosphatase, and phospholipase [(Hough et al. 1989; Ippolito and Christianson 1994; Klabunde et al. 1996). In the metal-binding sites, different combinations of protein side chains, including the nitrogen of histidine, the oxygen of aspartate or glutamate, and the sulfur of cysteine, coordinate the zinc ion. Of these, histidine is the most commonly observed, followed by cysteine (Gregory et al. 1993). Rarely seen ligands include the hydroxyl of tyrosine, the carbonyl of the protein backbone, and the carbonyl of either asparagine or glutamine.

In metalloenzymes containing zinc, the primary role of the zinc ion can be catalytic, cocatalytic, or structural. In catalytic sites, the zinc ion is directly involved in the bond-making or bond-breaking step whereas in cocatalytic sites, several metal ions are bound to the protein, but only one plays a catalytic role while the other ions enhance the catalytic activity (Vallee and Auld 1993a). Zinc also has a structural role in which it stabilizes the tertiary structure of the enzyme in a way that is analogous to disulfide bonds. In all these cases, removal of the bound zinc results in a loss of catalytic activity. A detailed analysis of the structure and function of a number of zinc proteins has demonstrated distinct features of catalytic and structural zinc sites as shown in Table 1.3 (Arnold and Haymore 1991; Coleman 1992; McCall et al. 2000).

5A. Catalytic zinc sites

Zinc ions involved in catalysis are found at the active site and are directly involved in the catalytic mechanism, interacting with the substrate molecules undergoing

the reaction (Fig. 1.5). The catalytic zinc site has an open coordination sphere that contains at least one water molecule plus three or four protein ligands (Vallee and Auld 1992a; 1992b). This feature is distinct from the structural zinc site in which the zinc-binding polyhedron is saturated with protein side chains. This difference can be seen by numerous spectroscopic and structural techniques. In catalytic sites, histidine is most frequent observed ligand, followed by glutamate, aspartate, and then cysteine. A water molecule completes the tetrahedral coordination sphere (Christianson 1991; Jernigan 1994). Most zinc sites commonly surround the metal ion by a shell of hydrophilic groups that is embedded within a larger shell of hydrophobic groups (Yamashita 1990).

5B. Cocatalytic zinc sites

In enzymes containing two or more zinc ions (or other metals), the metals act together to enhance catalysis. A class of cocatalytic zinc sites has been defined in which two or more zinc atoms are in close proximity to one another (Vallee and Auld 1993b). This group includes phospholipase C (three zinc ions) (Hough et al. 1989), nuclease P1 (three zinc ions) (Volbeda et al. 1991), alkaline phosphatase (two zinc ions and one magnesium ion) (Kim and Wyckoff 1991), and leucine aminopeptidase (two zinc ions) (Kim and Lipscomb 1994). An example of a cocatalytic site is shown in Fig. 1.6. In Fig. 1.5, the first zinc ion denoted as the catalytic zinc (Zn_1) contains a bound water that is needed for catalysis and has a histidine$_2$-glutamate metal polyhedron similar to those found in single catalytic zinc sites. The difference is seen in the second and third metals which may be coordinated by the oxygen of serine or threonine or the nitrogen of the N-terminal amino group. Another distinct feature is the presence of

Table 1.3. Characteristics of catalytic and structural zinc sites[a].

	Catalytic Zn[e]	Cocatalytic Zn[e]		Structural Zn[e]
		Catalytic Zn	Noncatalytic Zn	
Coordination sphere	Open	Open	Closed or bridging H_2O	Closed
Ligands[b]	H, D, E, C, H_2O	H, D, E, H_2O	H, D, S, N/O atoms, H_2O	C, H, (D)[d]
Ligands *(n)*	4, 5	4	5	4
Binding geometry	Asymetrical: distorted tetrahedral or distorted trigonal bipyramidyl	Distorted tetrahedral or octahedral minus one geometry	Slightly distorted trigonal bipyramidyl[c]	Symmetrical: tetrahedral
Location of ligands	Rigid: α-helix or β-sheet			Nonrigid: loop, α-helix or β-sheet
Spacing between ligands	Regular	Regular	Irregular	Irregular

[a]Table adapted from McCall et al., 2000.
[b]Most common ligands are highlighted.
[c]Mg is octahedral.
[d]D is observed in only one case: ferredoxin from *Sulfolobus* sp.
[e]Vallee and Auld 1990b.

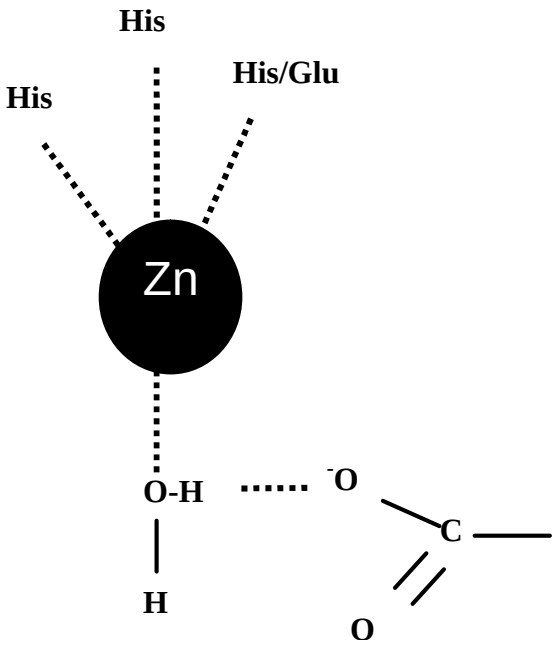
one or more bridging ligands (usually aspartate, water, or both) between the second zinc ion and the third zinc or magnesium ion that forms pentacoordinate geometry. For phosphate ester hydrolytic enzymes, the phosphate produced interacts with all three metals, displacing the weak uncharacteristic ligands in the second and third zinc sites to carry out catalysis (Vallee and Auld 1993b). Thus the name cocatalytic sites because all three metals play an important role in catalysis irrelevant of the fact that the first zinc ion activates the attacking water.

5C. Structural zinc sites

Four amino acid side chains coordinate the metal ion in structural zinc sites, usually forming a tetrahedral geometry, thereby excluding the solvent as an inner sphere ligand (Vallee and Auld 1990b; Vallee et al. 1991)]. The most frequently seen amino acid residue for structural sites is cysteine whereas histidine is present in many cases and aspartate has been observed (John et al. 1994; Lovejoy et al. 1994). Recently, the crystal structure of an alcohol dehydrogenase from *Sulfolobus solfataricus*, revealed an unusual coordination of its structural zinc ions by three cysteine residues and one glutamic acid residue. The glutamatic acid residue replaces one of the four cysteine residues that are highly conserved throughout the structural zinc-containing dimeric alcohol dehydrogenases (Esposito et al. 2002). Unlike in catalytic sites, the ligands that bind structural zinc sites are not in a regular pattern or motif, and they can be located on a flexible loop rather than a rigid secondary structure. Structural stability occurs due to the high stability constants of the tetradentate zinc complexes. This overall stability is similar to that provided by disulfide bonds (Vallee and Auld 1990b).

Fig 1.5.

Catalytic sites. Simplified schematic of the activation of a water nucleophile and the substrate-binding mode to mononuclear zinc peptidases (Lipscomb, 1996).


His
His/Glu
His
Zn
O-H
H
-O
C
O

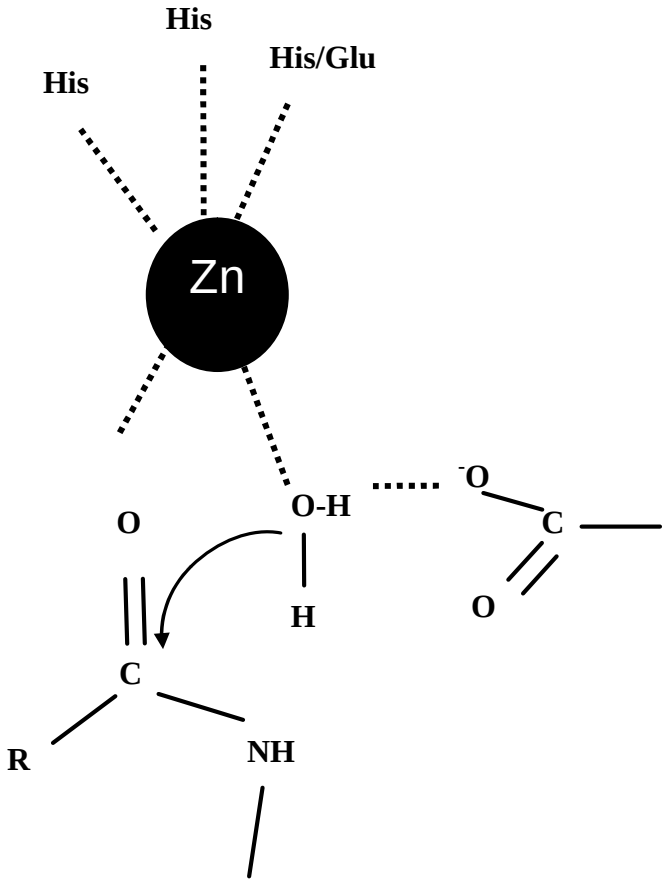
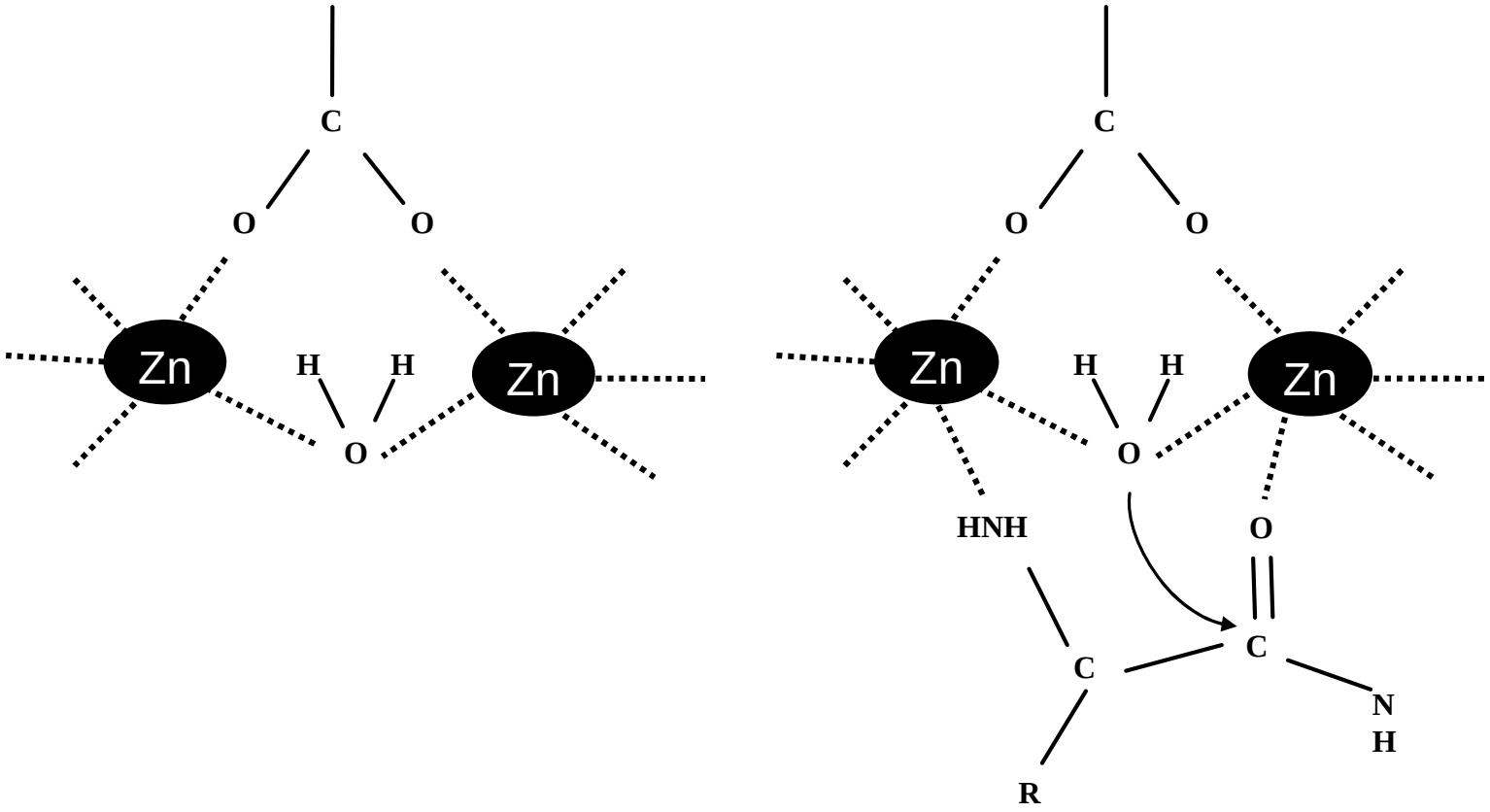

His
His/Glu
His
Zn
O-H
H
-O
C
O
O
C
R
NH

Fig 1.6.

Catalytic sites. Simplified schematic of the activation of a water nucleophile and the substrate-binding mode to dizinc aminopeptidases. Dizinc amino- and carboxypeptidases also contain a catalytic side chain (not shown) (Lipscomb, 1996).


C
O
O
Zn
H
H
Zn
O
C
O
O
Zn
H
H
Zn
O
HNH
O
C
C
N
H
R

6. **Zinc Metallopeptidases**

There are more than 50 families of metallopeptidases and about 50% of these fall into three so-called clans (MA, MB, and MX/MBA) (Hooper 1994; Rawlings and Barrett 1995). The term family is used to describe peptidases in which each member has an evolutionary relationship to at least one other, either throughout their entire sequences or at least in the part of the sequence that play a role in catalytic activity. A clan is a group of families that are believed to have common ancestry and the members are usually recognized by similarities in tertiary structure. Zinc is almost exclusively the preferred metal ion used by peptidases for the hydrolysis of peptide bonds. Zinc peptidases form the largest group of zinc enzymes and they are widely distributed amongst prokaryotes and eukaryotes (Coleman 1998). The first zinc protease to be described was carboxypeptidase A from mammalian pancreas which was characterized more than fifty years ago (Vallee 1954). Zinc metallopeptidases are divided into five families containing specific metal-binding motifs: 1) gluzincins (HEXXH + E) in which a glutamate residue plays an important role in catalysis; 2) metzincins (HEXXH + H) where a third histidine is coordinated to the catalytic metal ion; 3) inverzincins (HXXEH) which contain an inverted zinc binding motif in which the third zinc binding ligand is a glutamate located 82 amino acid residues C-terminal to this motif; 4) carboxypeptidases where the metal ligands are not part of the signature HEXXH motif, but they do contain histidine-glutamate residues (HXXE) involved in metal-binding as well as a third ligand, a histidine located 108-135 residues towards theC-terminal of this motif; and 5) DD-carboxypeptidases which contain three histidines, two of which occur in the sequence

DHXHV and the third one is located 42 residues on the N-terminal side of this motif (Rawlings 1993; Hooper 1994).

Thermolysin is the prototype of the family, gluzincins and in this enzyme glutamate is 20 amino acid residues closer to the C-terminal than the second histidine residue. Its catalytic mechanism involves a nucleophilic attack on the substrate carbonyl by zinc hydroxide, aided by a hydrogen bond from the water molecule to an adjacent carboxylate (Matthews and Goulding 1997). The second family, metzincins, includes subfamilies of astacins which are animal proteases that have various functions ranging from digestion to morphogenesis. This family contains a conserved methionine residue, fourteen residues closer to the C-terminal than the third histidine ligand and this is a part of a turn necessary for formation of the active site structure, hence the name metzincins. The inverzincins includes insulin-degrading enzymes that have been found in human, rat, and *Drosophila* (Hooper 1994). This family also includes pitrilysin and a yeast processing-enhancing protein. Other families that do not contain the signature HEXXH sequence include the following (examples of their enzymes are given in parentheses): MC (carboxypeptidase A and H), MD (D-Ala-D-Ala carboxypeptidases), MF (leucine aminopeptidase), MG (methionine aminopeptidase), MH (glutamate carboxypeptidase and yeast aminopeptidase I), MJ (*E. coli* β-aspartyl peptidase), MK (O-sialoglycoprotein endopeptidase), ML (hydrogenase maturation peptidase), and MM (*B. subtilus* sporulation factor IVB) (Rawlings and Barrett 1995). The remainder of this review will focus on two particular enzymes that belong to families of zinc metallopeptidases and they do not contain the signature HEXXH metal-binding sequence. These enzymes

include aminoacylase and two aminopeptidases (lysine aminopeptidase, and alanine aminopeptidase), the characterization of which are the focus of this thesis.

7. **Aminoacylases**

Aminoacylases hydrolyze N-acyl amino acids to produce fatty acids and amino acids. Such activities are also exhibited by certain carboxypeptidases, aminopeptidases, and dipeptidases. Aminoacylases are a family of soluble zinc-dependent amidohydrolases (Pittelkow et al. 1998). Based on sequence homology, these enzymes belong to the MH clan of metallopeptidases. Prokaryotic enzymes showing aminoacylase activity are composed of subunits of similar molecular mass (approximately 43 kDa), such as carboxypeptidase G_2 (Boyen et al. 1992), *E. coli* acetylornithine deacetylase (Meinnel et al. 1992), *B. thermoglucosidius* aminoacylase (Cho 1987), *B. stearothermophilus* dipeptidase (Cho et al. 1988), and *B. stearothermophilus* aminoacylase (Sakanyan et al. 1993). Although the properties of aminoacylases have been studied for many years, the physiological roles of these enzymes is still very poorly understood (Anders 1994). It has been suggested that aminoacylases may play a role in the catabolism of N-acylpeptides or in the salvage of N-acetylated amino acids (Endo 1980), but strong evidence for this is lacking. One problem in identifying a function is that the substrate specificities of aminoacylases are very different, varying from broad to very specific. These enzymes are chiral specific and utilize either D- or L- amino acids as substrates. This property allows aminocylases to be used in the industrial production of stereoisomers from racemic mixtures (Chibata et al. 1976). Several different types of aminoacylases have been identified and characterized including acylase I (EC 3.5.1.14, N-acylamino acid amidohydrolase); aspartoacylase or acylase II (EC 3.5.1.15, N-acyl-L-

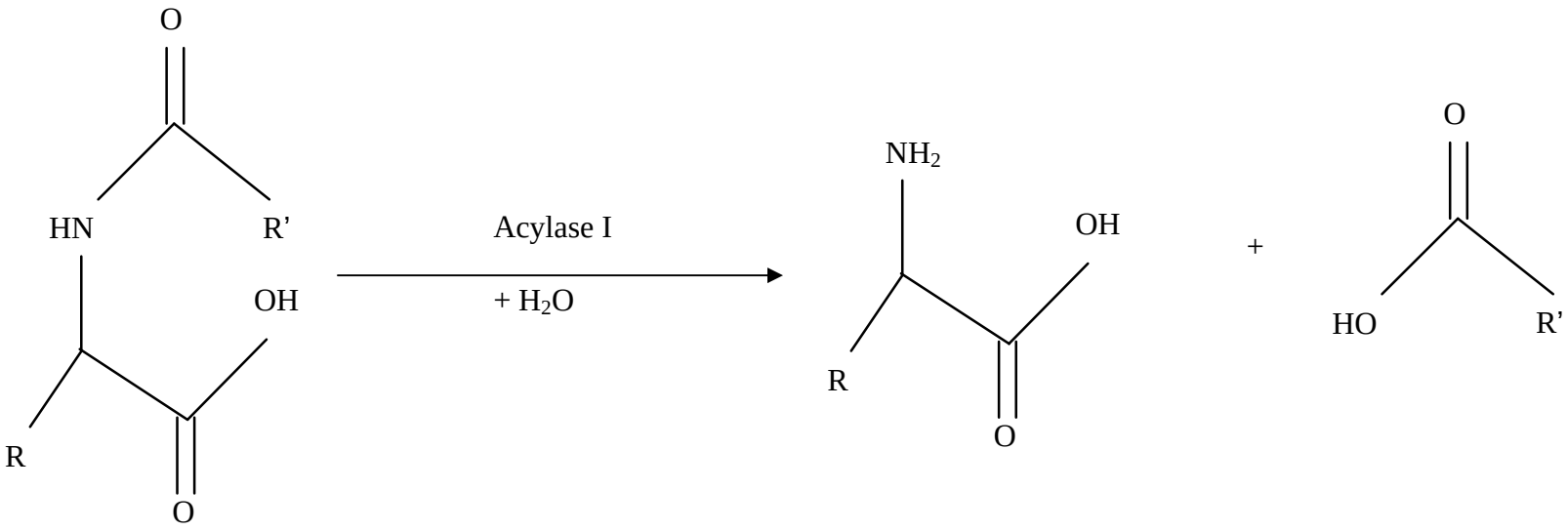
aspartate amidohydrolase); and acylase III, which prefers N-acyl aromatic acids as substrates.

7A. Acylase I

The first acylase I was purified from porcine kidney as early as 1881. In fact, this acylase was the first enzyme for which an intracellular localization was firmly established. It was found by the ability of crude kidney homogenates to hydrolyze N-benzoylglycine (hippurate) to benzoate and glycine (Lindner et al. 2000). Acylase I hydrolyzes a broad range of N-acyl-α-amino acids. A schematic of the reaction is shown in Fig. 1.7. The enzyme preferentially hydrolyzes neutral, aliphatic N-acylated amino acids, such as derivatives of alanine, valine, leucine, norleucine, 2-aminoheptanoid acid and methionine (Birnbaum 1952). The porcine renal enzyme is a homodimer of 90.6 kDa and contains one zinc ion per subunit (Kordel and Schneider 1977). Although the metal ion is necessary for activity, NMR studies using the Mn^{2+}-enzyme indicates that it is not directly involved in catalysis (Heese et al. 1990). Kinetic studies and isotope exchange experiments have shown that no covalent acyl enzyme intermediate is formed (Galaev and Svedas 1982; Rohm and Van Etten 1986). The zinc ion can be replaced by other divalent cations such as Co^{2+}, Mn^{2+}, Ni^{2+}, or Cd^{2+}. Although no crystal structure for the porcine aminoacylase has been determined, some structural information exists. It is known that each subunit is made up of a large trypsin-resistant domain containing the zinc site and a smaller domain that is trypsin-sensitive (Palm and Rohm 1995). The enzyme contains seven cysteine residues per subunit but only one is thought to be directly involved in catalysis (Heese and Rohm 1989).

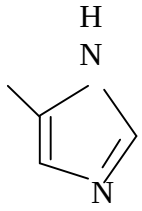
Fig. 1.7.

Acylase I-catalyzed hydrolysis of N-acetylated-a**-amino acids.** The preferred substrates are shown. Adapted from Anders, 1994.



R= -$CH_2(CH2)_nCH_3$,

R'= -CH_3, CH_2Cl, etc.



7B. Acylase II

Acylase II, also known as aspartoacylase, was found in hog kidney preparations by fractionation of kidney homogenates with ammonium sulfate and acetone. Unlike acylase I, acylase II is very specific preferring N-acylated aspartic acid. This enzyme has only been found in animals (Kaul et al. 1991; Kaul et al. 1993; Namboodiri et al. 2000). Acylase II is a monomeric protein with a molecular mass of approximatedly 60 kDa. The enzyme has been shown to play a significant role in brain biology. For instance, in humans, a defect (mutation) in acylase II results in a spongy degeneration of the brain called Canavan disease which is an autosomal recessive disorder involving increased levels of N-acetylaspartic acid (Matalon et al. 1995; Matalon 1997; Matalon and Michals-Matalon 2000).

7C. Acylase III

Proline acylase or acylase III is specific for hydrolyzing acetyl groups only from aromatic amino acid derivatives (Anders 1994) and more specifically N-acylated proline. It has been characterized from pig and rat kidney homogenates (Endo 1978). Acylase III consists of 8-12 subunits with a molecular mass in the range of 380,000-600,000. Similar to acylase I, the subunit molecular weight ranges from 45,000-55,000. A physiological role has not been established for acylase III.

8. **Aminopeptidases**

Aminopeptidases are exopeptidases that act at a free N-terminus of the polypeptide chain and either produces a single amino acid, a dipeptide, or a tripeptide. Aminopeptidases are classified in terms of their substrate specificities. They are

generally intracellular enzymes, however several extracellular aminopeptidases have been isolated from different microorganisms, some of these include an *Aspergillus oryzae* leucine aminopeptidase (Labbe 1974), *Streptococcus suis* arginine aminopeptidase (Jobin and Grenier 2003), *Streptococcus gordonii* arginine aminopeptidase (Goldstein et al. 2002), and a leucine aminopeptidase from *Aspergillus sojae* (Chien et al. 2002). Aminopeptidases have a broad substrate specificity (Taylor 1993) and can have a role in protein maturation, protein degradation, hormone level regulation, and cell-cycle control. In eukaryotes, cytosolic proteins are synthesized with an N-terminal methionine and maturation typically involves N-terminal processing in which aminopeptidases cleave the methionine residues (Ben-Bassat et al. 1987). Aminopeptidases can also have more specific functions, such as activation and inactivation of biologically active peptides and possibly in cleaving antigens for presentation by the major histocompatibility complex-1 system (Hersh et al. 1987; Cadel et al. 1995).

Unlike aminoacylases, aminopeptidases vary in their molecular subunit size, and metal composition from organism to organism (Rao et al. 1998). For example, leucine aminopeptidases are dizinc enzymes that preferentially remove leucine residues from the N-terminus of polypeptides and proteins, and these enzymes have a subunit molecular weight ranging from 55-60 kDa (Kim and Lipscomb 1994; Chien et al. 2002). In contrast, lysine aminopeptidases from fungi not only differ in subunit size and metal content from leucine aminopeptidases, but they also differ from each other. The *S. cerevisiae* aminopeptidase is a hexameric cobalt-dependent enzyme with a subunit size of approximately 48 kDa whereas the *A. niger* aminopeptidase has a subunit size of 95 kDa, however, metal content has not been reported. Of the aminopeptidases, the most

extensively studied enzymes are the methionine aminopeptidases and they have been purified from eukaryotic and prokaryotic sources. The recombinant forms of these enzymes indicate that they are cobalt-containing proteins that cleave the N-terminal methionine from newly translated polypeptide chains in both prokaryotes and eukaryotes (D'Souza V and Holz 1999). The methionine aminopeptidases purified from *Pyrococcus furiosus*, *Escherichia coli*, *Bacillus subtilus*, and *Salmonella typhimurium* have subunit sizes of approximately 30 kDa (Wingfield et al. 1989; Tsunasawa et al. 1997; D'Souza V and Holz 1999; Chung et al. 2002). On the other hand, the enzymes from yeast and porcine liver differ from the archaeal and bacterial aminopeptidases as well as each other, having subunit sizes of 43 kDa and 52 kDa, respectively (Chang et al. 1990; Kendall and Bradshaw 1992).

9. **Proposed Research**

The primary goal of this research project was to isolate and characterize three zinc metallopeptidases and determine their biochemical properties and possible function in *P. furiosus*. At the start of this project, there were no reports of an aminoacylase, lysine aminopeptidase, or alanine aminopeptidase isolated from a hyperthermophile or an archaeon. Little was known about peptidases and their functions in hyperthermophiles or archaea at the time. Chapters 2-4 will discuss the methods developed to isolate aminoacylase, lysine aminopeptidase, and alanine aminopeptidase from the same batch of *P. furiosus* cells. The possible physiological role of these enzymes is also addressed.

To date, complete genome sequences are available for numerous organisms as well as that of *P. furiosus*. Based on sequence homology to characterized enzymes, the

three zinc metallopeptidases isolated in this work were found to belong to two different families of metallopeptidases. Aminoacylase belongs to the M20 family of metallopeptidases of the MH clan which includes such enzymes as glutamate carboxypeptidase, acetylornithine deacetylase, and succinyl-diaminopimelate desuccinylase, which are involved in amino acid biosynthesis. On the other hand, lysine aminopeptidase and alanine aminopeptidase belong to the M18 family of aminopeptidases in which the only known member of this family is yeast aminopeptidase I. Therefore, it was of interest to determine if other members of these two families were present in *P. furiosus*. These enzymes will be discussed in Chapter 5.

CHAPTER 2

CHARACTERIZATION OF AN AMINOACYLASE FROM THE HYPERTHERMOPHILIC ARCHAEON *Pyrococcus furiosus*[1]

[1] Story, Sherry V., Amy M. Grunden, and Michael W. W. Adams. 2001. *J. Bacteriol.* **183,** 4259-4268**.** Reprinted here with permission of publisher.

ABSTRACT

Aminoacylase was identified in cell extracts of the hyperthermophilic archaeon *Pyrococcus furiosus* by its ability to hydrolyze N-acetyl-L-methionine and was purified by multistep chromatography. The enzyme is a homotetramer (42.06 kDa per subunit) and, as purified, contains 1.0 ± 0.48 g-atoms of zinc per subunit. EDTA caused a complete loss of activity. This was restored to 86% of the original value (200 U/mg) by treatment with $ZnCl_2$ (and to 74% by the addition of $CoCl_2$). After reconstitution with $ZnCl_2$, the enzyme contained 2.85 ± 0.48 g-atoms of zinc per subunit. Aminoacylase showed broad substrate specificity and hydrolyzed nonpolar N-acylated-L-amino acids (Met, Ala, Val, and Leu), as well as N-formyl-L-methionine. The high K_M values indicate that the enzyme plays a role in the metabolism of protein growth substrates and not degradation of cellular proteins. Maximal aminoacylase activity with N-acetyl-L-methionine as the substrate occurred at pH 6.5 and a temperature of 100°C. The N-terminal amino acid sequence of the purified aminoacylase was used to identify, in the *P. furiosus* genome database, a gene that encodes 383 amino acids. The gene was cloned and expressed in *Escherichia coli* by using two approaches. One involved the T7 *lac* promoter system, in which the recombinant protein was expressed as inclusion bodies. The second approach used the Trx fusion system that produced soluble but inactive recombinant protein. Renaturation and reconstitution experiments with Zn^{2+} ions failed to produce catalytically active protein. A survey of databases showed that, in general, organisms that contain a homolog of the *P. furiosus* aminoacylase ($\geq$50% sequence identity) utilize peptide growth substrates, whereas those that do not contain the enzyme are not known to be proteolytic, suggesting a role for the enzyme in primary catabolism.

INTRODUCTION

Aminoacylases (N-acylamino acid amidohydrolases: EC 3.5.1.14) catalyze the hydrolysis of N-acyl-amino acids to yield the corresponding organic acid and amino acid according to the following equation [2].

$$R'CO\text{-}NH.CH(R).COOH + H_2O \rightarrow NH_2.CH(R).COOH + R'COOH$$

They have been purified from various bacteria, including species of *Bacillus* and *Pseudomonas*, as well as from plants and animals [34, 37, 38, 41, 44, 52, 53], although the role of the enzyme is typically ill defined at best. In mammals, aminoacylases are thought to function in the detoxification of xenobiotic-derived amino acid derivatives and they are of great interest to the pharmaceutical industry (2, 13, and 50). Indeed, there is evidence that these enzymes may play a role in the development of lung cancer [17] and aminoacylase activity has been used as a monitor of hepatic dysfunction [35]. Moreover, based on the economic value of the products and their intrinsic industrial importance, aminoacylases are among the top ten enzymes used in biotechnology [47]. Due to their chiral specificity, chemically-synthesized mixtures of D- and L-forms of N-acyl-amino acids can be optically resolved with L- or D-aminoacylases to yield the L- or D-amino acids. Hence, they are used industrially in the synthesis of certain amino acids such as phenylalanine and alanine, as well as in the production of acylated amino acids (15, 44, 47).

The aminoacylases that have been characterized so far all consist of a single subunit of approximately 45,000 Da in size. Most are dimeric enzymes, although homotetrameric forms have been reported [14, 21, 40, 61]. Some have broad substrate

specificity whereas others are highly specific and hydrolyze, for example, only the acetylated form of aspartate, proline or one of the aromatic amino acids [2, 44]. One factor common to all of the enzymes is a requirement of a divalent cation for maximal activity. This is typically zinc, but cobalt, manganese, and nickel are also usually effective. The exact role of the metal is not clear although in some of these enzymes it is thought to have a structural role as well as a catalytic one [59]. Unfortunately, biochemical analyses of this group of enzymes have been limited mainly to kinetic studies [11, 55, 59, 60] and detailed structural information such as that obtained by crystallography is not yet available [46, 54]. Because of their biotechnological potential, there have been many attempts to stabilize various types of aminoacylases, either by immobilization or by obtaining the enzyme from thermophilic sources [5, 6, 7]. The most thermostable reported so far is the enzyme from *Bacillus stearothermophilus*. This has an optimal temperature for catalysis near 70 °C under the assay conditions used but the time required for a 50% loss of activity is less than 1 min at 80°C [56].

As yet, an enzyme that can hydrolyze N-acylated-amino acids has not been characterized from either an archaeon or a hyperthermophilic microorganism. Herein we report on the purification, biochemical properties and sequence of such an enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus*. This organism grows optimally at 100 °C utilizing proteins and peptides as substrates and it produces organic acids, CO_2, and H_2. Several enzymes involved in the catabolism of amino acids have been purified from *P. furiosus* [1, 22], including aminotransferases, glutamate dehydrogenase [3], 2-keto acid oxidoreductases [25] and acetyl CoA synthetases [36]. We have now found that cell-free extracts contain significant aminoacylase activity using N-acetyl-L-methionine

as the substrate. From the related organism, *P. horikoshii*, an acylamino acid-releasing enzyme (AARE) was recently characterized [26]. This enzyme catalyzes the hydrolysis of N-acylpeptides to release N-acylated amino acids and does not hydrolyze N-acylated amino acids like the aminoacylase of *P. furiosus*. The physiological role of these two enzymes in heterotrophic hyperthermophiles is also discussed herein.

MATERIALS AND METHODS

Growth of microorganisms. *Pyrococcus furiosus* (DSM 3638) was grown at 95 °C in a 600-liter fermentor with maltose as the carbon source as previously described [9]. *Escherichia coli* strains were grown in Luria Bertani media (LB) or M9 glucose media supplemented with 0.5% casamino acids. Ampicillin (100 μg/ml) and chloramphenicol 35 μg/ml) were added as needed for plasmid maintenance.

Enzyme assay. Aminoacylase activity was measured by the production of L-methionine from N-acetyl-L-methionine using the colorimetric ninhydrin method described by Rosen [43]. The assay mixture (500 μl) containing the enzyme sample in 50 mM MOPS (3-[N-morpholine] propane sulfonic acid) buffer (pH 7.0) and 30 mM N-acetyl-L-methionine (Sigma Chemical Co., St. Louis) was incubated at 100 °C for 5 min and 500 μl of trichloroacetic acid (TCA, 15%, w/v) was added to stop the reaction. Precipitated protein was removed by centrifugation and an aliquot (500 μl) of the supernatant solution was mixed with 250 μl of ninhydrin reagent (3% w/v ninhydrin in 2-methoxyethanol) and 250 μl of acetate-cyanide buffer (0.2 mM NaCN in 250 mM acetic acid) and incubated at 100

°C for 15 min. The mixture was cooled to ambient temperature by the addition of 1.5 ml isopropanol (50%, v/v) and the absorption was measured at 570 nm. The amount of methionine produced was determined from a standard curve. One unit of aminoacylase activity is defined as the amount of enzyme that liberates one μmole of L-methionine per minute under these assay conditions.

Purification of *P. furiosus* aminoacylase. Aminoacylase was purified from *P. furiosus* under anaerobic conditions at 23 °C. Frozen cells (200 g, wet weight) were thawed in 600 ml of 50 mM Tris-HCl buffer (pH 8.0) containing DNase I (10 μg/ml) and 2 mM sodium dithionite (DT), and were lysed by incubation at 37 °C for 2 h. A cell-free extract was obtained by ultracentrifugation at 18,000 x g for 2 h. The supernatant (600 ml) was loaded onto a column (10 by 14 cm) of Q-Sepharose Fast Flow (Pharmacia, Piscataway, NJ) equilibrated with 50 mM Tris (pH 8.0) containing 2 mM DT (Tris-DT buffer). The column was eluted at a flow rate of 12 ml/min with a 2.5-liter linear gradient of 0 to 1.0 M NaCl in the same Tris-DT buffer. Aminoacylase activity was eluted in fractions as 0.25 to 0.35 M NaCl. The active fractions were combined (300 ml), and solid sodium sulfate was added to a final concentration of 0.5 M. This solution was applied to a column (3.5 by 10 cm) of Phenyl Sepharose (Pharmacia) equilibrated with Tris-DT buffer containing 0.5 M sodium sulfate. The column was eluted with a gradient (1-liter) from 0.5 to 0-M sodium sulfate in the Tris-DT buffer at a flow rate of 7 ml/min. Aminoacylase eluted as 0.30 to 0.40-M sodium sulfate. The aminoacylase-containing fractions (200 ml) were applied to a column (3.5 by 10 cm) of Q-Sepharose High Performance (Pharmacia, Piscataway, N. J.) equilibrated with 25 mM Bis-Tris buffer (pH

6.5). The column was eluted with a gradient (1-liter) from 0 to 0.5 M NaCl in the same Tris-DT buffer at a flow rate of 8 ml/min. Aminoacylase activity was eluted as 0.23 to 0.28 M NaCl. The active fractions (186-ml) were applied to a column (1 by 10 cm) of hydroxyapatite (HAP; Pharmacia) equilibrated with 25 mM Bis-Tris buffer (pH 7.0). The column was eluted at a flow rate of 5 ml/min with a 100-ml linear gradient of 0 to 0.5-M potassium phosphate buffer. The active fractions (35 ml) from the HAP were applied to a column of HiTrap-Q (1.6 by 2.5 cm; Pharmacia) equilibrated with 25 mM Bis-Tris (pH 6.5), and the enzyme was eluted with a gradient (75 ml) from 0 to 0.5 M NaCl in the same buffer at a flow rate of 2 ml/min. Fractions containing aminoacylase activity (17 ml) eluted as 0.14 to 0.20 M NaCl was applied and were stored frozen as pellets in liquid nitrogen until required.

Characterization of recombinant aminoacylase. The recombinant form of *P. furiosus* aminoacylase was obtained by PCR amplification of the gene encoding the enzyme and its subsequent cloning into the T7-polymerase-driven expression vector pET-21b (Novagen, Milwaukee, WI). For amplification, the forward primer (GGATCCTTCGGAGGACCAAATGTT CAACCCCCTTGAGGAGG; Stratagene, La Jolla, CA) contained an engineered *BamH*I site and spanned from -19 to +22 on the coding strand, while the reverse primer (ATGCGGCCGCAATCCCAACTGTATAACCCATTACGAATATGA; Stratagene) had an engineered *Not*I site and corresponded to sequence ranging from +1406 to +1438 on the non-coding strand. PCR amplification was performed with native *P. furiosus* DNA polymerase and a Robocycler 40 (Stratagene) programmed for 1 cycle of denaturation at

95 °C for 5 min, 2 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 5 min, 39 cycles of denaturation at 95 °C for 1 min, annealing at 61°C for 1.5 min and extension at 72 °C for 5 min, and 1 cycle of extension at 72 °C for 7 min. The resultant 1.4-kb gene encoding aminoacylase was subcloned into the blunt end *Srf*I site of plasmid pCR-Script (Stratagene) to yield plasmid pAA. The insert DNA was then sequenced to ensure that no mutations were present in the gene. The gene was then excised from plasmid pAA by restriction digest with the enzymes *BamH*I and *Not*I (Stratagene) and cloned into the *BamH*I and *Not*I sites in the expression plasmid pET-21b, resulting in plasmid pET-AA2.

The gene encoding aminoacylase was initially expressed using *E. coli* BL21(λDE3)/pET-AA2 cultures (1L in 2.8L Fernbank flasks) grown in LB media with and without $ZnCl_2$ supplementation (100 μM) that were also coexpressing the rare Arg, Ile, and Leu tRNAs from plasmid pRIL (Stratagene). The cultures were incubated at 37 °C with shaking (250 RPM) until an OD of 0.6-0.8 was reached. Production of recombinant aminoacylase was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. The cultures were then maintained at 37 °C for 3h. This expression resulted in the production of insoluble, inactive protein. The expression using plasmid pET-AA2 was repeated with different media (glucose-M9 medium supplemented with casamino acids; 0.5%), with and without the addition of $ZnCl_2$, $NiCl_2$, or $FeSO_4$ (each at a final concentration of 100 μM). Expression of the gene was also attempted by induction over a range of temperatures, including 4 °C for 16 hr, 15 °C for 12 hours, 25 °C for 8 hours and 30 °C for 6 hours. None of these modifications yielded soluble, active protein.

In an attempt to generate active, soluble protein, the insoluble recombinant aminoacylase was dissolved in 8.0 M urea (in 50 mM Tris-HCl, pH 8.0) and renaturation buffer (50 mM Tris-HCl, pH 8.0; 1 mM $ZnCl_2$) was then slowly added with stirring. A second method involved dissolving the insoluble aminoacylase in 6.0 M guanidium-HCl (in 50 mM MOPS, pH 7.0, containing 20 mM DTT). In this case the solution was incubated with stirring for 2 h at room temperature and then spun at 5,000 x g for 10 min. The supernatant was diluted 1:100 with chilled renaturation solution (50 mM Tris-HCl, pH 8.0; 0.5 M L-arginine, 10 mM DTT, 100 µM $ZnCl_2$). The renaturation solution was stirred gently overnight at 4 °C. The renaturation solution (15-ml) was then dialyzed 12 h against 4 L of 50 mM MOPS buffer, pH 7.0 at 4 °C. In a third approach, insoluble aminoacylase (0.33 mg/ml, 86 ml) were initially suspended in 50 mM Tris-HCl, pH 8.0, containing 6 M guanidium-HCl, 10 mM DTT, and 20 mM EDTA. This solution was dialyzed overnight against 500 ml of acidic-denaturation solution (2 M guanidium-HCl, 10 mM DTT, 10 mM EDTA, 5% acetic acid, pH 2.7). The denatured protein solution was then separated into two samples and dialyzed overnight at 4 °C against 500 ml of renaturing solution I (50 mM Tris-HCl, pH 8.0, 100 µM $ZnCl_2$) or solution II (50 mM Tris-HCl, pH 8.0, 100 µM $CoCl_2$. The partially renatured protein solutions were then dialyzed overnight at 4 °C against 1 L of renaturation solution I or II, respectively.

The gene encoding aminoacylase was also cloned into the thioredoxin-fusion expression plasmid pET-32a (Novagen). The forward primer for PCR (CCATGGTCAACCCCCTTGAGGAGGCCATGA) contained an engineered *Nco*I site and spanned from +1 to +28 on the coding strand and the reverse primer (AAGAGGATCCACTGGCTAACCTCTAAAGTT) had an engineered *BamH*I site and

corresponded to +1134 to +1163 on the non-coding strand. The conditions of amplification were as described above except that an annealing temperature of 48 °C was employed. A resulting 1.2-kb gene was cloned into plasmid pCR-Script as described above to give plasmid pTrx-aa and was sequenced to ensure there were no PCR-induced errors. The gene was removed from plasmid pTrx-aa by digestion with the restriction enzymes *Nco*I and *BamH*I and cloned into the corresponding sites in the thioredoxin-fusion expression plasmid pET-32a, yielding plasmid pET-trx/aa. Recombinant aminoacylase produced from this system carries both a His-tag and an S protein-tag and is fused to thioredoxin. Using enterokinase, the thioredoxin and affinity tags can be cleaved from the aminoacylase protein, leaving no extra amino acids. For expression of the thioredoxin-aminoacylase fusion protein, 2-1 L cultures of BL21(λDE3)/pET-trx/aa were grown in LB at 37 °C with shaking (250 RPM). Protein expression was induced by the addition of IPTG (0.4 mM) once the O.D. of the cultures had reached 0.6-0.8. The cultures were then incubated at 28 °C for 2 h before harvesting the cells. Soluble thioredoxin-aminoacylase fusion protein was detected both by visualization of a Coomassie-stained SDS-12.5% polyacrylamide gel and by Western antibody detection according to manufacturer's instructions (Novagen).

To purify recombinant aminoacylase, 7 g (wet weight) of cell paste was resuspended in 20 ml of buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 % Triton X-100) and cells were broken by two passages of the suspension through a French pressure cell at 20,000 lb/in^2. The suspension was spun at 7,000 x g for 1.5 h and the supernatant was applied to a 6 ml S-protein affinity agarose column (Novagen). The column was washed with 30 ml of buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1

% Triton X-100; 1 mM DTT). Bound fusion protein was eluted from the column with 12 ml of elution buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 % Triton X-100; 1 mM DTT; 3 M $MgCl_2$). The eluted protein was concentrated to 1.3 ml using Centricon-30 concentrators (Amicon Inc, Beverly, MA). The concentrated protein solution was dialyzed against 2 L of enterokinase buffer (20 mM Tris-HCl, pH 8.0; 50 mM NaCl; 2 mM $CaCl_2$; 1 mM DTT). To remove the fusion part of the protein, 20 U of enterokinase (Stratagene) was added and the reaction mixture was incubated at room temperature for 15 h. The enterokinase and cleaved S-tag were sequentially removed from the recombinant aminoacylase protein by passage of the protein sample through enterokinase-capture STI-agarose (Stratagene) and S-protein affinity resin. The protein sample was applied to a 0.75 ml STI-agarose column equilibrated with enterokinase-binding buffer (50 mM Tris-HCl, pH 8.0; 200 mM NaCl). Recombinant aminoacylase was eluted with 4 ml of buffer and was applied to the 6 ml S-protein affinity agarose column from which it was eluted with 12 ml of wash buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 % Triton X-100; 1 mM DTT). Recombinant aminoacylase was concentrated to 0.9 ml (0.88 mg/ml) and stored as pellets in liquid nitrogen.

Other methods. Molecular weights were estimated by gel filtration with a column (1 by 27 cm) of Superdex 200 (Pharmacia LKB) with amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and carbonic anhydrase (29,000) as standard proteins. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed using 12% polyacrylamide by the method of Laemmli [33]. Molecular weights were estimated using a 10 kDa (10-120) standard molecular weight protein

ladder derived from T4 gene 32 (GIBCO BRL) also containing myosin (200). Protein concentrations were determined by the Bradford method [8] with bovine serum albumin as the standard. To determine metal content, exogenous metal ions were removed from the aminoacylase by gel filtration using a G-25 column equilibrated with 50 mM Tris-HCl, pH 8.0 containing 0.5 M NaCl. A complete metal analysis (31 elements) was obtained by plasma emission spectroscopy with a Jarrel Ash Plasma Comp 750 instrument at the Chemical Analysis Laboratory of the University of Georgia. Amino-terminal sequences were determined using an Applied Biosystems Model 477 sequencer in the Molecular Genetics Instrumentation Facility (MGIF) of the University of Georgia. Samples were electroblotted onto polyvinylidene difluoride protein-sequencing membranes (Stratagene) from SDS-electrophoresis gels by using a Bio-Rad electroblotting system. Electroblotting was carried out in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0, containing methanol (10% [vol/vol]) for 1 h at 50 V. DNA sequences were analyzed using the computer software programs Genetics Computer Group (University of Wisconsin, Madison, WI) and MacVector (International Biotechnologies, Inc., New Haven, CT).

RESULTS

Purification of *P. furiosus aminoacylase*. Extracts of *P. furiosus* cells grown with maltose as the primary carbon source contained a significant amount of aminoacylase activity (approximately 0.34 U/mg at 100 °C) using N-acetyl-L-methionine as the substrate. The enzyme appeared not to be regulated, as the specific activities of extracts of cells grown with yeast extract (5.0 g/l) and maltose (1.0 g/l) or with yeast extract (5.0

g/l), tryptone (5.0 g/l) and maltose (1.0 g/l) as the primary carbon sources, were similar. All cells used for purification were obtained from cell cultures grown on a 500L scale. Since maltose-grown cells are routinely used in this laboratory to purify various oxygen-sensitive, oxidoreductase-type enzymes from *P. furiosus*, such cells were also used for aminoacylase purification. In addition, the procedure was carried out under anaerobic conditions, not because the aminoacylase was sensitive to oxygen, but to allow for the purification of both aminoacylase and enzymes that are oxygen-sensitive from the same batch of *P. furiosus* cells.

Aminoacylase activity was not detected in the culture supernatant during the log phase of cell growth nor in the membrane fraction of the cell-free extract. The activity was found only in the soluble fraction, indicating that the enzyme is a cytoplasmic protein. The results of a typical purification are summarized in Table 1. The enzyme was purified 580-fold with a yield of 7% and a specific activity of approximately 200 U/mg. When the aminoacylase was treated with SDS sample buffer at 100 °C for 10 min prior to electrophoresis, it migrated as a single major band corresponding to a molecular mass of approximately 45 kDa (Fig. 2.1). The aminoacylase was eluted from a gel filtration column corresponding to a molecular mass of 190 $\pm$ 10 kDa. This result, taken together with the electrophoretic data, suggests that the enzyme is a homotetramer.

The N-terminal sequence of the purified aminoacylase is MFNPLEEAMKIKDEI-. This sequence was used to search the genomic sequence database of *P. furiosus* (http://comb5-156.umbi.umd.edu/). A gene was located whose translated N-terminal region matched exactly the sequence obtained from the purified enzyme. It consists of 1,149 base pairs (bp) and encodes a protein of 383 residues with a calculated molecular mass of 42.06 kDa

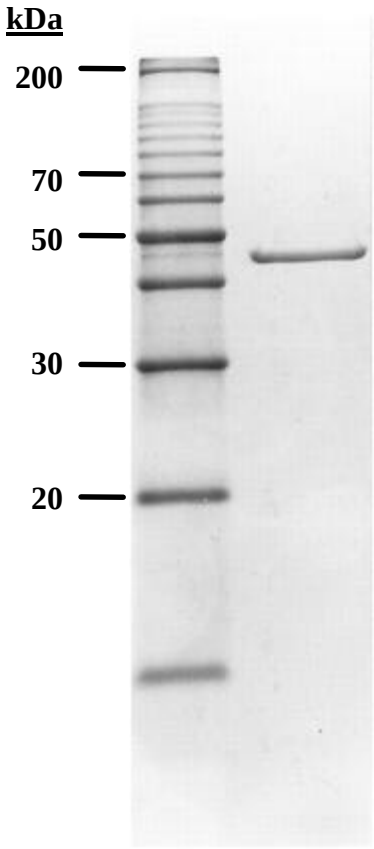
Table 2.1. Purification of aminoacylase from *P. furiosus*.

Step	Activity[a] (U)	Protein (mg)	Sp. Act. (U/mg)	Purification (fold)	Recovery (%)
Cell Extract	8,800	26,000	0.3	1	100
QFF Sepharose	5,800	1,600	3.7	11	65
Phenyl Sepharose	4,100	160	25.4	75	46
QHP Sepharose	1,050	27	39.0	115	12
Hydroxyapatite	700	7	100	290	8
HiTrap Q	660	3	200	580	7

[a]Activity was measured at 100 °C using N-acetyl-L-methionine (30 mM) as the substrate.

Fig. 2.1.

SDS-12% PAGE of the aminoacylase purified from *P. furiosus*. Lanes: 1, standard molecular size markers; 2, native aminoacylase (4μg).


kDa
200
70
50
30
20

(Fig. 2.2). The latter value is in good agreement with that (~45 kDa) obtained from the SDS-gel analysis. The enzyme appears to show non-ideal behavior when subjected to gel filtration, however, since the molecular weight estimated by that method (190 kDa) is higher than that expected (168 kDa) for a homotetrameric protein. A mass of 42.06 kDa for the aminoacylase subunit was used in all calculations.

Characterization of recombinant *P. furiosus* aminoacylase. The production of aminoacylase in recombinant *E. coli* cells was assessed by the appearance of a protein band corresponding to its subunit size (approx. 42 kDa) after SDS-gel analysis of cell-free extracts (data not shown). However, detectable levels of active aminoacylase were not produced after induction of the recombinant gene by IPTG. Analysis of the gene and translated amino acid sequence revealed that the gene contains 44 codons (17% of the total) that are rarely used in *E. coli* (a combination of the Arg codons, AGA and AGG; one Leu, CUA; and one Ile, AUA). This dramatically affected expression of the gene since when the same plasmid was expressed in conjunction with the genes encoding the relevant tRNAs (present on plasmid pRIL), the amount of recombinant aminoacylase produced was easily visible after SDS-PAGE analysis. However, aminoacylase activity (at 100 °C) could not be detected in the cell-free extract, and the recombinant protein appeared to be insoluble as it was removed by centrifugation. Attempts to produce a soluble recombinant protein by varying the induction temperature (4 ¯ 37 °C) or growth medium composition (LB media or casamino acid-supplemented M9-glucose medium with or without $ZnCl_2$, $NiCl_2$, or $FeSO_4$) proved unsuccessful as no soluble recombinant aminoacylase was detectable by SDS-PAGE analysis of the cytoplasmic fraction.

Furthermore, the aminoacylase present in the insoluble fraction could not be solubilized into a form with detectable activity using a variety of denaturation/renaturation strategies. Similarly, while a soluble form of recombinant aminoacylase was produced as part of an enterokinase-cleavable thioredoxin fusion protein, no activity could be detected either before or after cleavage of the thioredoxin fusion.

Physical properties of *P. furiosus* aminoacylase. Of the thirty-one metals analyzed, the aminoacylase purified from *P. furiosus* cells contained only zinc and magnesium in significant amounts (> 0.1 g-atoms/subunit). The values were 1.0 $\pm$ 0.48 g-atoms Zn^{2+}/subunit and 0.11 $\pm$ 0.3 g-atoms Mg^{2+}/subunit, respectively. When the enzyme (0.21 mg/ml in 50 mM Bis-Tris buffer, pH 6.5) was treated with EDTA (20 mM) for 1h at 23 °C, and then dialyzed against the same buffer (lacking EDTA), no activity could be detected using the routine assay at 100 °C. As shown in Fig. 3, activity could be restored by the addition of mM concentrations of Zn^{2+} ions, and to a lesser extent by Co^{2}ions. Other divalent (Mg^{2+}, Ni^{2+}, Fe^{2+}, Mn^{2+}, or Cu^{2+}) or monovalent (Na^{+} or K^{+}) cations were ineffective. Maximal recoveries of activity for the metal-reconstituted enzyme were obtained using 3.5 mM $ZnCl_2$ (86% of original activity recovered) and 4.6 mM $CoCl_2$ (74%) although both cations caused some inhibition when added above their optimal concentration (Fig. 2.3). When the zinc-reconstituted enzyme (using 3.5 mM $ZnCl_2$) was passed through a Superdex 200 column equilibrated with 50 mM Bis-Tris buffer, pH 6.5, or was dialyzed against 50 mM Bis-Tris buffer (1,000 volumes), pH 6.5, the enzyme preparations contained 3.0 $\pm$ 0.48 g-atoms and 2.7 $\pm$ 0.48 g-atoms Zn^{2+}/subunit, respectively. From Fig. 2.3, the apparent association constants (concentrations giving

Fig. 2.2.

The 1,178-bp gene encoding the P. furiosus aminoacylase and the deduced amino acid sequence (383 amino acids). A putative TATA box is indicated in bold print, and the ribosomal binding site is underlined.

→
ACCAGGTAA**TATA**TCCTTCGGTGGTCCAAATGTTCAACCCCCTTGAGGAGGCCATGAAGATTAAGGACGAAATCATCTCCTGGAGAAGAG
90
M F N P L E E A M K I K D E I I S W R R **20**
ACTTCCACATGTACCCAGAACTAGGTTACGAAGAGGAGAGAACTTCCAGAATAGTTGAGGAACACCTAAAAGAGTGGGGGTATAAGATAA
180
D F H M Y P E L G Y E E E R T S R I V E E H L K E W G Y K I **50**
AGAGAGTAGGAACGGGAATAATTGCTGACATTGGGAGTGGAGAAAAAACTGTGGCCTTAAGAGCAGATATGGATGCTCTCCCAATTCAAG
270
K R V G T G I I A D I G S G E K T V A L R A D M D A L P I Q **80**
AGGAGAACGAAGTCCCATACAAATCTAGAGTTCCTGGGAAAATGCATGCTTGTGGTCATGATGCTCACACCGCAATGCTTCTAGGAGCAG
360
E E N E V P Y K S R V P G K M H A C G H D A H T A M L L G A **110**
CGAAGATAATTGCGGAGCATGAAGAAGAGCTAAACAATAGGGTTAGGCTAATCTTCCAGCCTGCTGAAGAAGGGGGCAATGGAGCACTAA
450
A K I I A E H E E E L N N R V R L I F Q P A E E G G N G A L **140**
AAATGATAGAAGGAGGAGCATTAGAGGATGTAGACGCTATTTTCGGCCTTCACGTTTGGGCAGAGCTGGAGTCTGGTATAATTGGACTTA
540
K M I E G G A L E D V D A I F G L H V W A E L E S G I I G L **170**
GAAAGGGCCCATTCCTTGCAGGTGTTGGAAAGTTCAACGTGAAAATAATTGGGAAAGGAGGGCATGGGGCAGCTCCTCAATATGCAATAG
630
R K G P F L A G V G K F N V K I I G K G G H G A A P Q Y A I **200**
ACCCAGTTCCAGCGGTAGCGGAGGCAATTTTAGCTCTCCAAAGGATAGTTGCCAGGGAGATTGACCCGCTAGAAAGTGCAGTGGTTACGG
720
D P V P A V A E A I L A L Q R I V A R E I D P L E S A V V T **230**
TAGGGAAAGTTCAGGGTGGAACGGCTTTCAACGTAATTCCAGAAAGTGTGGAGTTTGAGGGGACTTTCAGGTTCTTCACGGAGGAACTTG
810
V G K V Q G G T A F N V I P E S V E F E G T F R F F T E E L **260**
GAGGGTTCATAAGAAAGAGAATTTCTGAAATTGTAAGTGAGGTTGCAAAGGCTCACAGGTGTAGAGCTGAAGTCAAGACTGAAATTCTAG
900
G G F I R K R I S E I V S E V A K A H R C R A E V K T E I L **290**
GCCCTCCCACAATAAACGATGACAGAATGGTTGAGTTCGTAAGGGAAGTTGCCCAGGGACTCGGGCTGAAAGTGGGAGAAGTTAAGAAGA
990
G P P T I N D D R M V E F V R E V A Q G L G L K V G E V K K **320**
CTTTAGGTGGAGAAGACTTTGCCTTCTATCTCCAGAGAGTACCTGGGGCATTTATAGCTTTAGGAATTAGAAATGAAAAGAAGGGGATAA
1080
T L G G E D F A F Y L Q R V P G A F I A L G I R N E K K G I **350**
TCTACCCACACCACAACCCAAGGTTTGACGTCGATGAGGACATTCTTCCCCTGGGAACTGCACTGGAAGTTGCCTTGGCCTTCAACTTTA
1170
I Y P H H N P R F D V D E D I L P L G T A L E V A L A F N F **380**
GAGGTTAG 1178
R G * **383**

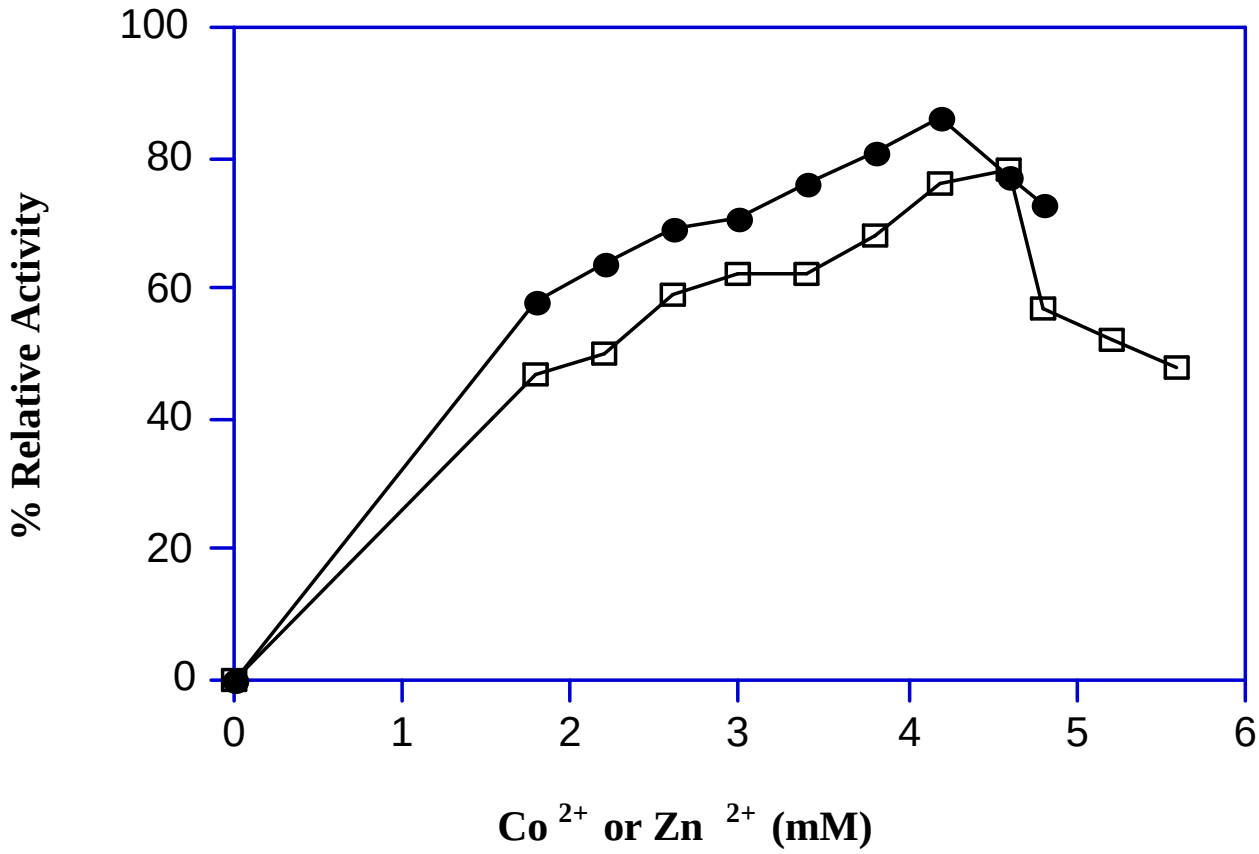
half-maximal activities)for Zn^{2+} and Co^{2+} were 1.7 mM and 1.1 mM, respectively. The aminoacylase as purified from *P. furiosus* was not very thermostable. When a sample (0.35 mg/ml in 50 mM Bis-Tris buffer containing 0.5 M NaCl, pH 6.5) was incubated at 100 °C, the time required for a 50% loss in activity ($t_{50\%}$) was 25 min. Under the same conditions, but in the presence of 100 µM $ZnCl_2$ (the concentration in the assay mixture giving the highest activity of the pure enzyme, Fig. 4) there was no detectable loss of activity after a 7 hr incubation at 100 °C.

Catalytic properties of *P. furiosus* aminoacylase. As shown in Fig. 2.5, the enzyme showed a sharp pH optimum at 6.5 (at 100 °C) and a temperature optimum of at least 100 °C (at pH 6.5). There was no detectable activity below 40 °C (at pH 6.5). From the temperature-dependent data, the calculated activation energy for aminoacylase is 12.1 kcal/mole. The standard assay mixture for aminoacylase did not contain any metal ions, but activity could be increased by addition of Zn^{2+}, Co^{2+}, Mg^{2+}, or Ni^{2+} ions, although other divalent (Ca^{2+}, Fe^{2+}, or Cu^{2+}) and monovalent (Na^{+} or K^{+}) cations had no effect. For Co^{2+}, Mg^{2+}, or Ni^{2+} ions, concentrations above 250 µM were required but for $ZnCl_2$, concentrations below 200 µM resulted in increased activity (Fig. 2.3).

Aminoacylase was identified in cell extracts of *P. furiosus* by detecting its ability to hydrolyze *N*-acetyl-L-methionine, and this substrate was used in all routine assays. The activity of the enzyme with other N-acetylated amino acids, N-chloroacetylated amino acids, and N-formylated amino acids is summarized in Table 2.2. No activity was detected with any of the following compounds: N-acetyl-DL-phenylglycine, N-acetylglycine, N-acetyl-D-methionine, N-acetyl-L-phenylalanine, N-acetyl-L-proline, N-

Fig. 2.3.

The effects of Co^{2+} and Zn^{2+} ions on the activity of *P. furiosus* aminoacylase. The enzyme (29 µg in 50 mM Bis-Tris, pH 6.5), was incubated at 80 °C for 30 min. with varying concentrations of either $ZnCl_2$ (solid symbols) or $CoCl_2$ (square symbols) and then assayed under standard conditions (in the absence of added metal ions). The assay mixture contained aminoacylase (2.9 µg), N-acetyl-L-methionine (30mM), and 50 mM Bis-Tris buffer, pH 6.5.


100
80
60
40
20
0
% Relative Activity
0
1
2
3
4
5
6
Co 2+ or Zn 2+ (mM)

acetyl-L-tryptophan, N-acetyl-L-tyrosine, N-formyl-DL-tryptophan, N-formyl-methionine-phenylalanine, N-acetylmethionine-alanine and N-acetylmethionine-leucine-phenylalanine. Notably, N-acetyl-D-methionine was not hydrolyzed, showing the stereospecificity of the aminoacylase, while the potential physiological substrate N-formyl-L-methionine was hydrolyzed, although N-formyl-Met-Phe was not. Table 2.3 shows the results from kinetic analyses with three substrates. All exhibited normal Michaelis-Menten-type kinetics over the range of 0.5 to 10 mM substrate concentrations, and the kinetic constants were calculated from linear double-reciprocal plots. The apparent K_M value for N-formyl-L-methionine was very high (13 mM) and about twice that of N-acetylmethionine. The best (nonphysiological) substrate was N-chloroacetyl-L-valine, where the k_{cat}/K_M value was almost threefold that measured with N-acetyl-L-methionine.

DISCUSSION

Although the gene encoding the enzyme was successfully expressed in *E. coli*, surprisingly, the recombinant form was not catalytically active, and the enzyme would not have been characterized by a cloning/expression approach. The production of what appears to be incorrectly-folded, recombinant apoprotein may be due to the inability of *E. coli* to insert the appropriate metal ion, as the enzyme, as purified from *P. furiosus*, contains 1 g-atom Zn/subunit. In this regard, the *P. furiosus* enzyme is similar to other members of the aminoacylase family, many of which have also been shown to contain Zn. For example, the enzymes from *Bacillus stearothermophilus* [56], *B. thermoglucosidius* [14], pig [23] and human [17] contain one zinc ion per catalytic

subunit, while the enzymes from *Alcaligenes denitrificans* DA181 [61] and *Aspergillus oryzae* [21] contain two and three zinc ions/subunit, respectively. Like the *P. furiosus* enzyme, some of these aminoacylases require the addition of a divalent cation (typically Zn, although Co, Mn and Mg are also effective in some cases) for maximal catalytic activity *in vitro* [14, 21, 61] but this is not true for all of them [23,56]. However, in all cases, incubation in the presence of EDTA results in complete loss of activity, and this can be restored by the addition of Zn^{2+} ions. The precise nature of the metal sites in these enzymes is not clear. Both the reconstituted *P. furiosus* enzyme and the aminoacylase from *A. oryzae* [21], contain three Zn^{2+} ions per/subunit, suggesting that this group of enzymes represents a novel class of zinc-containing protein that is distinct from members of the metallohydrolase family that contain binuclear metal sites [58].

All of the other aminoacylases that have been characterized have subunits of comparable size (37- 50 kDa) but the majority are dimers [21, 40, 52, 56, 61] and only one [14] is a homotetramer like the *P. furiosus* enzyme. The crystal structure of an aminoacylase has not yet been reported [46], although complete amino acid sequences are available for the enzymes from *B. stearothermophilus* [44], pig [37], and human [38], and sequences of putative aminoacylases can be identified in the genome sequences of *P. horikoshii* [29], *P. abysii* (http://www.genoscope.cns.fr/Pab), *B. subtilis* [31], *Deinococcus radiodurans* [57], *Lactococcus lactis* [18], *Synechocystis* sp. [28], and *Streptomyces coelicolor* [42]. The *P. furiosus* enzyme has 34, 15, 15, 80, 81, 26, 38, 32, 41 and 14% identity, respectively, with these enzymes (see Fig. 2.5), but shows no sequence similarity with that of the acylamino acid-releasing enzyme (AARE) found *in P. horikoshii* [26]. It does show high similarity with the sequences of *Sulfolobus*

Table 2.2. Substrate Specificity of *P. furiosus* aminoacylase.

Substrate[a]	% Relative Activity[b]
N-acetyl-L-methionine	100
N-chloroacetyl-L-valine	325
N-chloroacetyl-L-leucine	287
N-chloroacetylglycine	233
N-formyl-L-methionine	155
N-acetyl-L-valine	77
N-chloroacetyl-L-tyrosine	75
N-acetyl-DL-allylglycine	47
N-acetyl-L-alanine	36
N-acetyl-L-asparagine	33
N-acetyl-L-cysteine	15
N-acetyl-DL-serine	15
N-acetyl-L-glutamic acid	13
N-chloroacetyl-L-phenylalanine	13
N-acetyl-L-leucine	9
N-acetyl-DL-norleucine	6
N-chloroacetyl-L-tryptophan	5

N-formyl-L-leucine	4
N-acetyl-DL-phenylglycine	0
N-acetylglycine	0
N-acetyl-D-methionine	0
N-acetyl-L-phenylalanine	0
N-acetyl-L-proline	0
N-acetyl-L-tryptophan	0
N-acetyl-L-tyrosine	0
N-formyl-DL-tryptophan	0
N-formyl-methionine-phenylalanine	0

[a]All substrates were used at a final concentration of 30 mM.

[b]The rate of hydrolysis is expressed as a percentage of the activity compared to that obtained using N-acetyl-L-methionine as the substrate at 100°C where 100% activity corresponds to 200 U/mg.

Fig. 2.4.

The effects of metal ions on the catalytic activity of the *P. furiosus* aminoacylase under routine standard assay conditions. The assay mixtures contained aminoacylase (0.78 µg), N-acetyl-L-methionine (30 mM), and varying concentrations of either $ZnCl_2$ (solid circles), $CoCl_2$ (open squares), $MnCl_2$ (open circles), or $NiCl_2$ (solid squares).

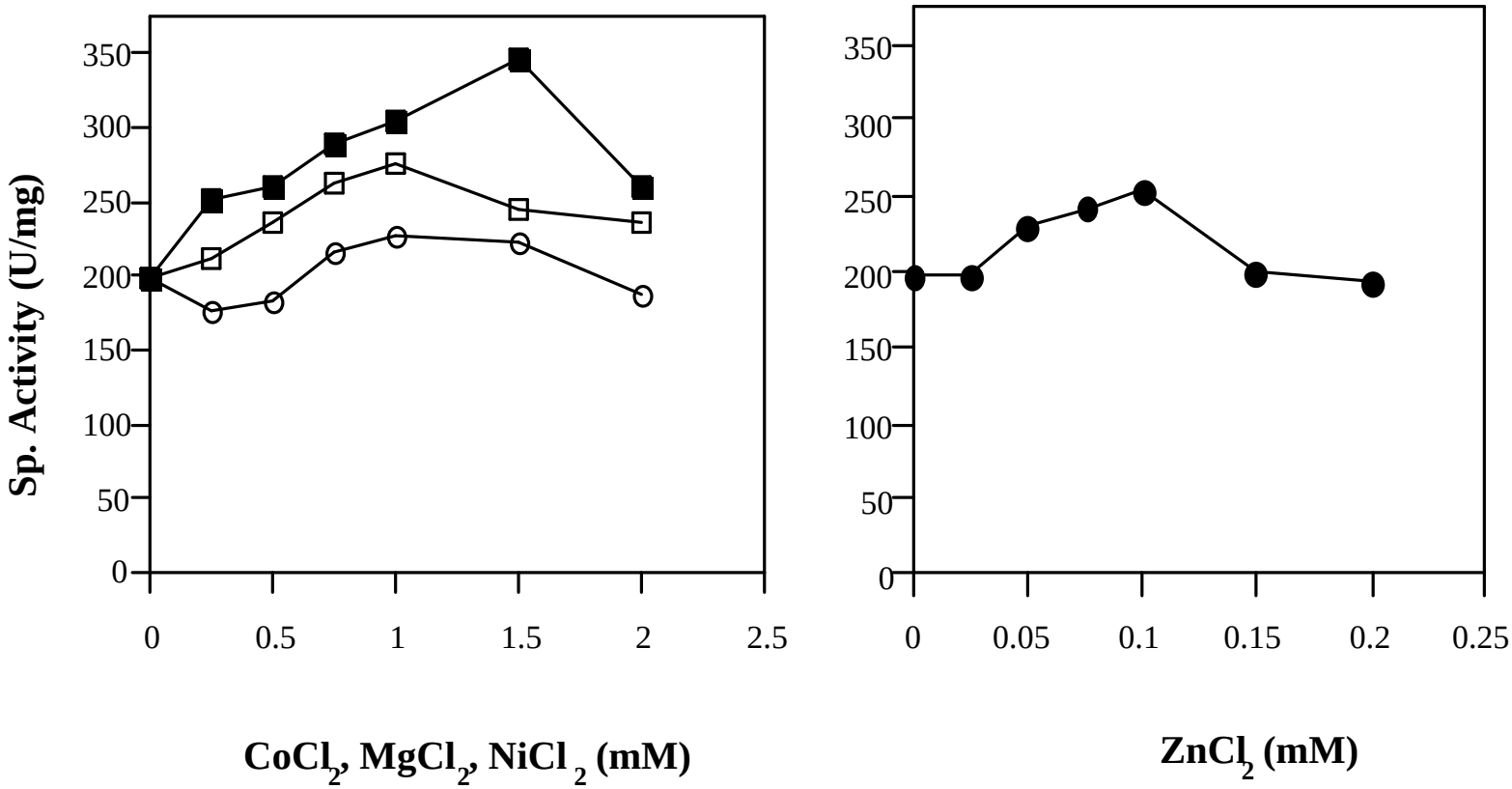

Sp. Activity (U/mg)
350
300
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
CoCl2, MgCl2, NiCl 2 (mM)
350
300
250
200
150
100
50
0
0
0.05
0.1
0.15
0.2
0.25
ZnCl2 (mM)

Table 2.3. Kinetic parameters for substrates of *P. furiosus* aminoacylase.

Substrate[a]	**K_M (mM)**	**V_{max} (μmoles/min/mg)**	**k_{cat}**[b] **(s^{-1})**	**k_{cat}/K_M ($mM^{-1}s^{-1}$)**
N-acetyl-L-methionine	6.6	500	2,720	412
N-formyl-L-methionine	13.0	99	538	41
N-chloroacetyl-L-valine	2.5	500	2,720	1,090

[a]All assays were carried out at 100 °C in 50 mM Bis-Tris HCl, pH 6.5.

[b]Based on a minimum molecular mass of 42.06 kDa.

solfataricus carboxypeptidase [16], *Thermotoga maritima* hippurate hydrolase [39], *Campylobacter jejuni* (sequence not shown) [24], *Arabidopsis thaliana* (ILR1) indole-3-acetic acid (IAA) amino acid hydrolase [4], and *Arabidopsis thaliana* JR3 protein (identities of 38, 28, 35, 38, and 40%, respectively, see Fig. 2.5). Most of these enzymes contain a conserved region near the N-terminus (MHACGHDXHTAMLLG-, residues 137-150 in the *P. furiosus* sequence) that have putative zinc-binding residues (Cys, Asp and three His). There are also other potential zinc-binding residues [51] (His-65; Glu-69; Glu-79; His-196; Glu-217; Glu-218; His-248 in the *P. furiosus* enzyme) that are conserved in all of these enzymes (Fig. 2.6). It therefore appears that these enzymes are part of a metallohydrolase family [58] that might contain three Zn atoms per mole, although the true nature of the metal sites will likely be apparent only from crystallographic analyses.

We now turn to the possible physiological role of *P. furiosus* aminoacylase. Unfortunately, as yet, none of the aminoacylases that have been characterized have well-defined functions. The *P. furiosus* enzyme hydrolyzes N-formyl-methionine as well as a broad range of N-acetylated-amino acids and is absolutely specific for the naturally-occurring L-isomers. The relatively high K_M values for these substrates, however, suggests that such amino acid derivatives must be present at significant intracellular concentrations *in vivo* if they are the physiological substrates. Such compounds could be generated either by cellular protein degradation or by the metabolism of protein growth substrates. The latter is a possible source, as *P. furiosus* grows well with peptides as the carbon source [20]. Similarly, other organisms from which aminoacylases have been purified, such as *B. stearothermophilus* [44], *A. denitrificans* [61], and *Pseudomonas*

Fig. 2.5.

Effects of temperature and pH on the activities of *P. furiosus* aminoacylase. The assay mixture contained aminoacylase (0.64 mg/ml) and *N*-acetyl-L-methionine (30 mM) in 50 mM bis-Tris, pH 6.5. For the effects of pH, the following buffers (each at 50 mM) were used at the indicated pHs: MES (morpholineethanesulfonic acid), pHs 5.5 and 6.0; bis-Tris, pH 6.5; MOPS, pH 7.0; EPPS (*N*-2-hydroxyethylpiperazine-*N*'-3-propanesulfonic acid), pH 8.4; CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid], pH 8.6; CAPS, pHs 10, 10.5, and 11.0. For effects of temperature, the buffer used was 50 mM bis-Tris, pH 6.5. A 100% activity level corresponds to 200 U/mg.

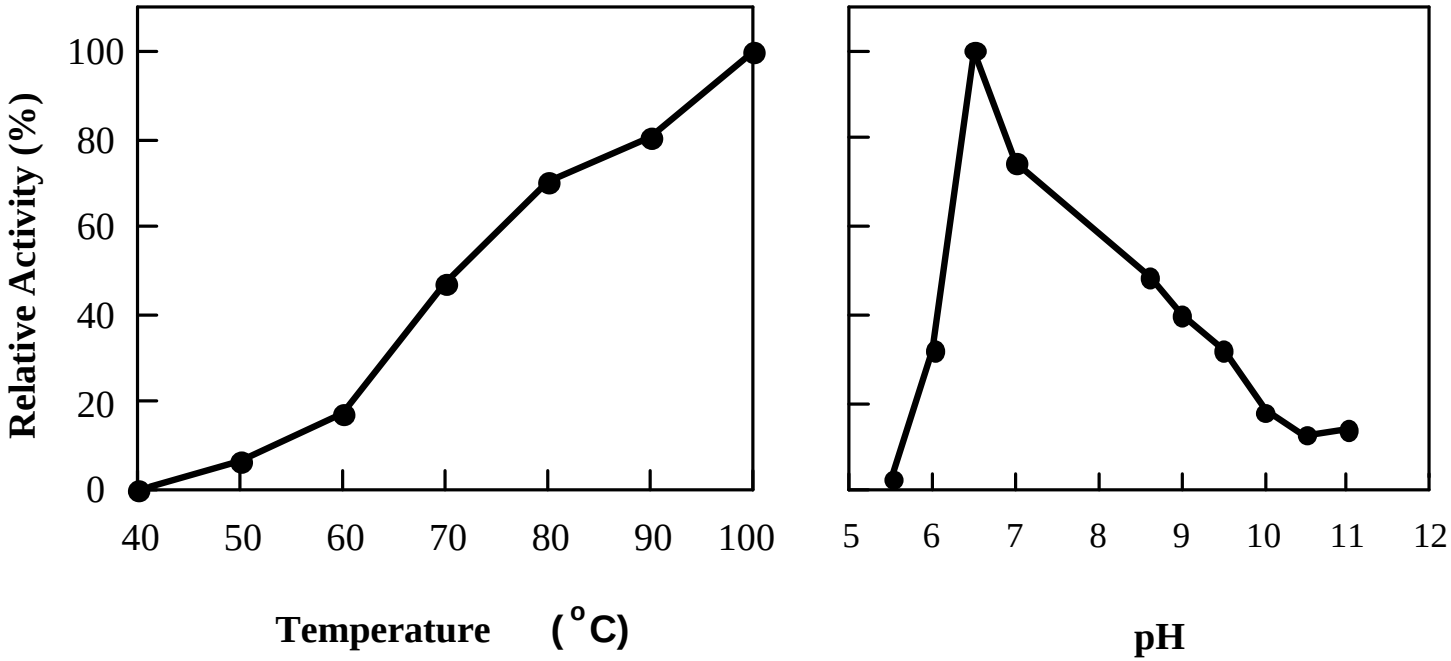
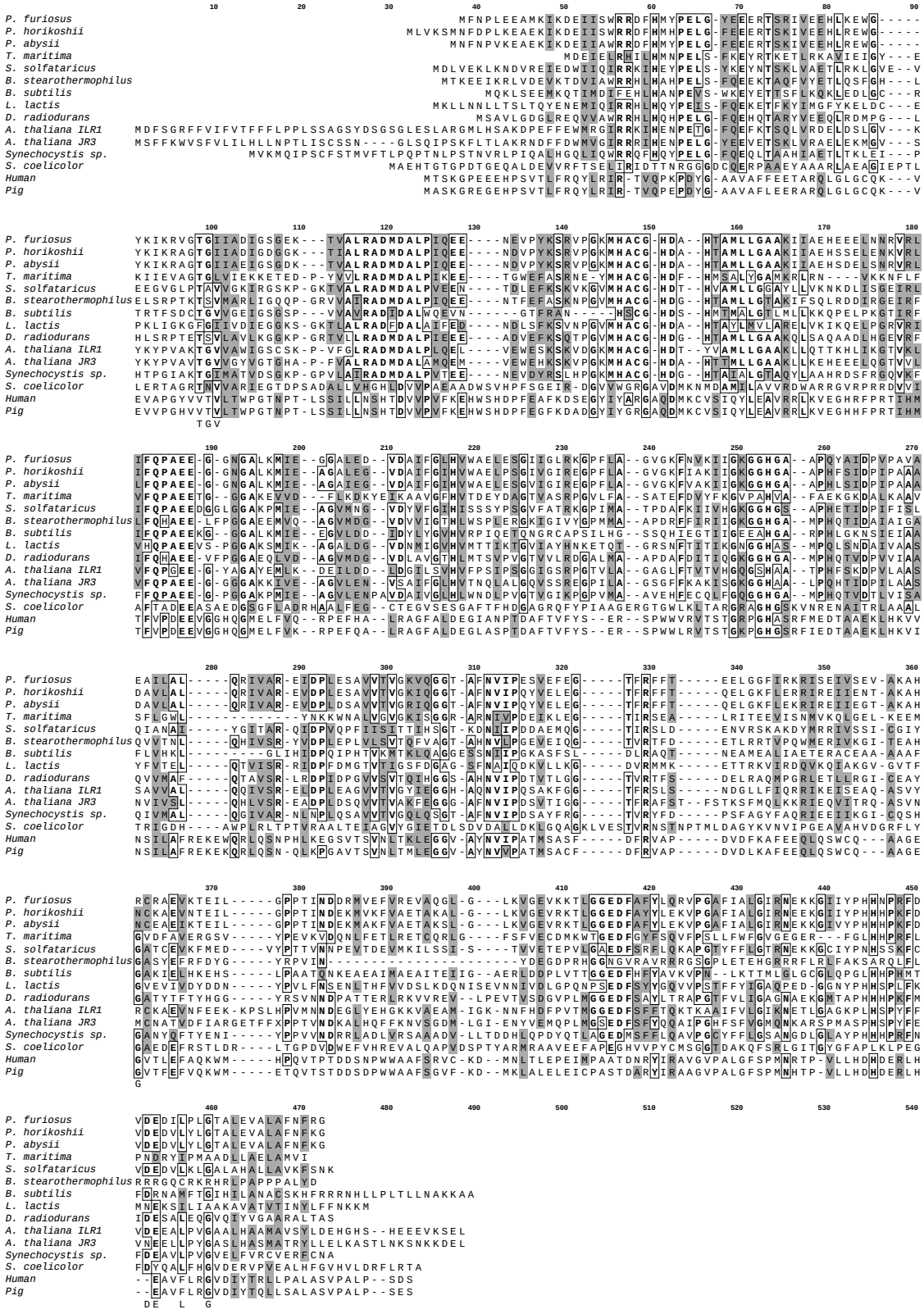

Relative Activity (%)
100
80
60
40
20
0
40
50
60
70
80
90
100
Temperature (°C)
5
6
7
8
9
10
11
12
pH

Fig. 2.6.

Alignment of the amino acid sequence of *P. furiosus* aminoacylase with those of the aminoacylases from various sources and with *Sulfolobus solfataricus* carboxypeptidase, and *Thermotaga maritima* hippurate hydrolase. The sources of the data are: *P. furiosus* aminoacylase (this work), *P. horikoshii* aminoacylase (BAA29813); *P. abysii* aminoacylase (CAB50230); *T. maritima* hippurate hydrolase (AAD36583); *S. solfataricus* carboxypeptidase (CAA88397); *B. stearothermophilus* aminoacylase (P37112); *B. subtilis* aminoacylase (P54983); *L. lactis* aminoacylase (AAF36227); *D. radiodurans* aminoacylase (AAF11266); *A. thaliana* ILR1 (P54968); *A.thaliana* JR3 (CAA73905); *Syncechocystis* sp. (BAA18770); *Streptomyces coelicolor* aminoacylase (T35974); human aminoacylase (A47488)pig aminoacylase (JN0584);. Only the name of the organisms are listed due to alignment formatting. Identical residues are outlined while similar residues are designated by gray shading.



maltophila [52], also are capable of growing on peptides. In fact, of the completed microbial genomes available, homologs of the *P. furiosus* enzyme (showing = 50% sequence similarity) are present in 21 organisms (including archaea and bacteria), and 19 of them are capable of peptidolytic growth. They include *P. horikoshii*, *P. abysii*, *B. stearothermophilus*, *B. subtilus*, *Clostridium acetabutylicum*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Deinococcus radiodurans*, *Staphylococcus aureus*, *Campylobacter jejuni* and *Treponema denticola*. The other two organisms are the photosynthetic bacteria, *Synechocystis* sp. and *Chlorobium tepidum*, which are not known to use peptides. Homologs of aminoacylase are not found in hyperthermophilic archaea that are not capable of peptidolytic growth, including *Archaeglobus fulgidus* [32], *Methanobacterium thermoautotrophicum* [45], and *Methanococcus jannaschii* [10] nor in the hyperthermophilic bacterium *Aquifex aeolicus* [19]. On the other hand, there is no obvious aminoacylase homolog in the hyperthermophilic archaeon *Aeropyrum pernix* [30], an organism that does grow on peptides.

The microbial genomic data, therefore, generally support the notion that aminoacylases represented by the *P. furiosus* enzyme are involved in metabolizing protein growth substrates rather than the cell's own proteins. Figure 2.7 shows a possible pathway for the initial steps of protein degradation in *P. furiosus*. It is proposed that the substrates for the aminoacylase are generated by the AARE. AARE generates N-acyl amino acids from short N-acyl peptides (four or fewer residues), but it does not hydrolyze *N*-acetyl- or *N*-formylmethionine [26]. The AARE of *P. horikoshii* has been characterized [26], and a homolog is present in *P. furiosus* (http://comb5-156.umbi.umd.edu/). *P. furiosus* also contains methionine aminopeptidase [48], but this

enzyme is not known to use N-acetylated proteins or peptides. The small N-acyl peptides used by AARE are presumably produced from larger N-acylated proteins by an acyl aminopeptidase. The nature of this enzyme is not clear, since *P. furiosus* contains an enzyme of this type, but it was reported to be extracellular [49]. It is also not known whether N-acylated proteins themselves serve as growth substrates for *P. furiosus* or if proteins and/or peptides are acylated intracellularly, perhaps as a signal for subsequent digestion (Fig. 2.7). *N*-Formylmethionine also serves as a substrate for *P. furiosus* aminoacylase (Table 2.3), although it remains to be seen if N-formylated rather than N-acetylated derivatives play a role in protein degradation. Growth studies with such substrates are currently underway.

While the present paper was under review, an article was published describing the cloning and expression of a gene encoding a bifunctional carboxypeptidase-aminoacylae from *P. horikoshii*, a close relative of *P. furiosus* [27]. As was mentioned above, this organism contains a gene that encodes a protein (a putative amidohydrolase; 29) with extremely high similarity (80% identity) to *P. furiosus* aminoacylase (Fig. 2.6), but this gene has not been expressed. The carboxypeptidase that was characterized from *P. horikoshii* [27] has a much lower sequence identity (57%) to the *P. furiosus* enzyme, although, like the carboxypeptidase from *S. solfataricus* [16] noted above, it is clearly a member of the aminoacylase family of enzymes (Fig. 2.6). From its substrate specificity [27], the *P. horikoshii* enzyme appears to have dipeptidase rather than carboxypeptidase activity, and it also hydrolyzes acetylated aromatic amino acids, unlike the *P. furiosus* enzyme described herein. The genome of *P. furiosus* does not contain any other gene analogous to that encoding the bifunctional carboxypeptidase of *P. horikoshii. P. furiosus*

does contain a conventional carboxypeptidase [12], as does *P. horikoshii* [29], but these enzymes show no significant sequence similarity to the sequences of the aminoacylase family (Fig. 2.6). Clearly, much remains to be understood about the diversity and physiological roles of this group of enzymes.

ACKNOWLEDGEMENTS

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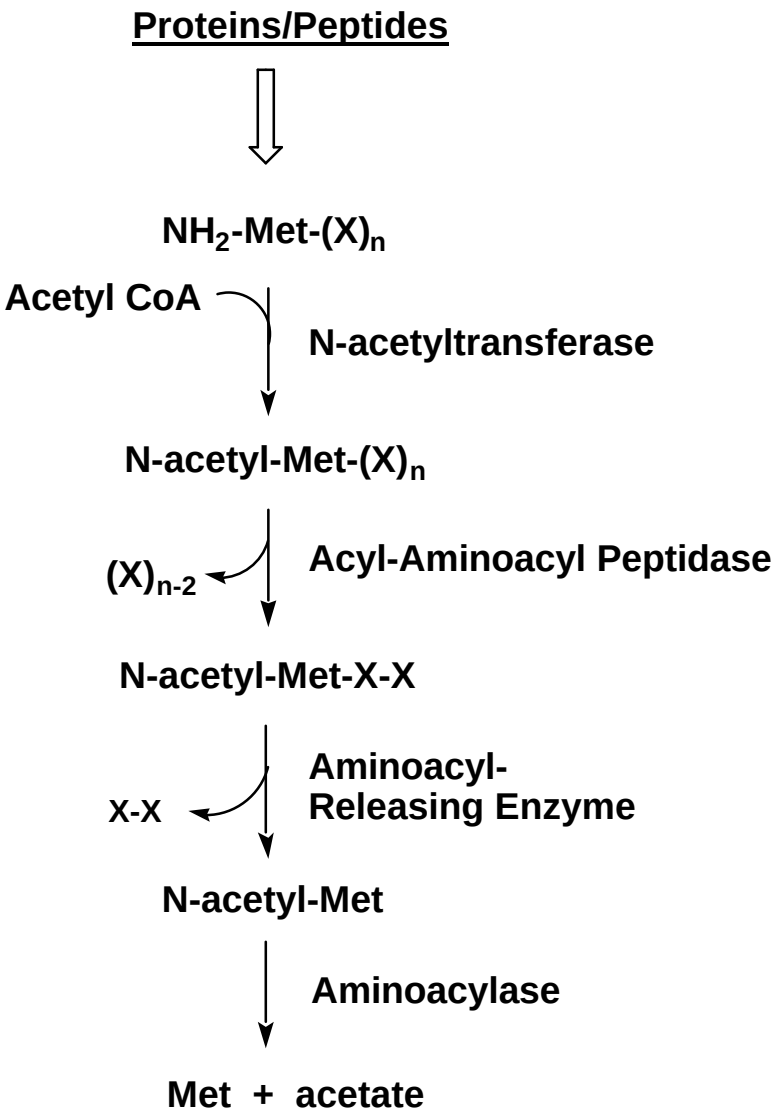
Fig. 2.7.

Proposed pathway of catabolism of N-acetylated proteins in *P. furiosus*. Enzymes enclosed in parentheses represent a putative gene found in the *P. furiosus* genome database.


Proteins/Peptides
NH2-Met-(X)n
Acetyl CoA
N-acetyltransferase
N-acetyl-Met-(X)n
(X)n-2
Acyl-Aminoacyl Peptidase
N-acetyl-Met-X-X
X-X
Aminoacyl-
Releasing Enzyme
N-acetyl-Met
Aminoacylase
Met + acetate

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CHAPTER 3

CHARACTERIZATION OF THE FIRST LYSINE AMINOPEPTIDASE FROM A PROKARYOTIC SOURCE: A NOVEL ZINC-CONTAINING ENZYME FROM THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*[2]

[2] Story, Sherry V. and Michael W. Adams. To be submitted to J. Bacteriol.

ABSTRACT

Lysine aminopeptidase (KAP) was purified by multistep chromatography from cell extracts of the proteolytic, hyperthermophilic archaeon *Pyrococcus furiosus*. Purified KAP is a homotetramer (38.2 kDa per subunit) and as purified contains 2.0 $\pm$ 0.48 zinc atoms per subunit. Surprisingly, its activity was stimulated four-fold by the addition of Co^{2+} ions (0.2 mM), indicating that the enzyme has a second metal ion binding site. Co^{2+} could not be replaced by any other divalent cation. KAP had a narrow substrate specificity and hydrolyzed only basic N-terminal residues (Lys- and Arg-pNA) with low, but measurable activity with nonpolar N-terminal residues (Ala, Leu, Phe). Optimal lysine aminopeptidase activity with Lys-pNA as the substrate occurred at pH 8.0 and a temperature of 100 ^{o}C. Mass spectroscopy analyses of the peptides derived from the purified enzyme was used to identify, in the *P. furiosus* genome database, a gene (PF 1861) that encodes a product corresponding to 346 amino acids. This gene was expressed in *Escherichia coli* and the recombinant protein was purified. Its properties, including molecular mass, metal ion dependence, pH and temperature optima were indistinguishable from those of the native form. However, the thermostability of the recombinant form ($t_{1/2}$ ~ 10 min at 100 ^{o}C) was dramatically lower than that of the native enzyme ($t_{1/2}$ ~ 6 hrs at 100 ^{o}C). Based on its amino acid sequence, KAP is part of the M18 family of peptidases which includes yeast aminopeptidase I. KAP is the first lysine aminopeptidase of the M18 family to be purified from a prokaryotic source.

INTRODUCTION

Aminopeptidases are exopeptidases that catalyze the removal of amino acid residues at the N-termini of peptides and proteins. Intracellular proteolytic degradation by aminopeptidases is necessary for modulation of protein concentrations, maintenance of amino acid pools, and removal of damaged proteins (1). In addition, aminopeptidases have more specific functions including activation (9) and inactivation (19) of biologically active peptides, and the removal of N-terminal methionyl residues of newly synthesized proteins. Proteinaceous substrates, whether imported into the cell or generated from damaged proteins, are further degraded by di-, tri-, and carboxy-, as well as aminopeptidases (15). Classification of aminopeptidases is generally based upon their substrate specificities, such as preference for a neutral, acidic, or basic amino acid in the P1 position of the amino terminus of peptides (38). These enzymes are widely distributed amongst eukaryotes and prokaryotes although only a few have been isolated from archaea (36). About two-thirds of all aminopeptidases are the metal-dependent or metallopeptidases in which zinc is the most frequently associated metal (14).

Hyperthermophilic archaea are potentially rich sources of peptidase-type enzymes since most of them are capable of using protein-based substrates as their sole carbon source (1). For example, *Pyrococcus furiosus* grows optimally at 100 ^{0}C utilizing proteins and peptides as substrates and it produces organic acids, CO_2, and H_2. Several enzymes involved in the catabolism of peptides have been purified from *P. furiosus* including aminotransferases (4), 2-keto acid oxidoreductases (2), glutamate dehydrogenase (17), prolidase (14), acetyl CoA synthetases (26), aminoacylase (33), a cobalt-activated carboxypeptidase (10), and pyrollidone carboxypeptidase (36). This list

also includes a methionine aminopeptidase (35) and a deblocking aminopeptidase (DAP) (34) as well as a deblocking aminopeptidase from the related archaeon *P. horikoshii* (3). The *P. furiosus* genome contains a gene encoding a homolog of the latter enzyme (PF0369) which also has 85% identity to the *P. horikoshii* DAP, but there are no reports on its biochemical and kinetic properties so a comparison between these two enzymes cannot be made.

Surprisingly, an aminopeptidase that can catalyze the release of lysyl residues from the N-terminus of peptides has yet to be characterized from any prokaryote. In fact, only two enzymes of this type are known and both are from fungi: the native form of a cobalt-dependent enzyme, yscCo-II, from the unicellular eukaryote *Saccharomyces cerevisiae* (18), and a recombinant form of a zinc-dependent enzyme from the filamentous fungus *Aspergillus niger* (5). The majority of aminopeptidases characterized so far, including these two enzymes, belong to the M1 family of metallopeptidases. These typically require zinc for activity and their sequences contain the zinc-binding motif HEXXH (5). Sequence analyses of the M1 metallo-aminopeptidases shows that it consists of three main groups (30). These include the aminopeptidase N and the leukotriene A4 hydrolase groups, members of which have been extensively characterized. The third group consists of a variety of aminopeptidases that share a high sequence similarity, but differ considerably in their catalytic properties and other characteristics (30).

The object of the present study was to characterize the lysine aminopeptidase (KAP) from the hyperthermophilic archaeon, *P. furiosus*. It is shown that the *P. furiosus* enzyme, while having the same substrate specificity as the KAPs from yeast and *A. niger*,

contains conserved sequences that are homologous to members of the M18 rather than M1 family of peptidases. In contrast to the large M1 family, only two members of this family have been characterized. These are a leucyl aminopeptidase from yeast (27) and an aspartyl aminopeptidase from rabbit (38). Herein we report the isolation of the first prokaryotic member of the M18 family and the first prokaryotic KAP in the form of the *P. furiosus* enzyme. The native and recombinant forms of it were purified and their biochemical properties were determined.

MATERIALS AND METHODS

Growth of microorganisms. *Pyrococcus furiosus* (DSM 3638) was grown at 95 °C in a 600-liter fermentor with maltose as the carbon source as previously described (7). *Escherichia coli* strains were grown at 37 °C in Luria Bertani media (LB). Kanamycin (50 μg/ml) and chloramphenicol (35 μg/ml) were added as needed for plasmid maintenance.

Enzyme assay. Lysine aminopeptidase activity was determined with the chromogenic substrate Lys-pNA. The assay mixture (350 μl) containing the enzyme sample in 50 mM MOPS (3-[N-morpholine] propane sulfonic acid) buffer (pH 8.0) and 15 mM Lys-pNA (Bachem Co., King of Prussia, PA) was incubated at 100°C for 5 min and 70 μl of sodium dodecyl sulfate (SDS, 10%, w/v) was added to stop the reaction. The final volume was made up to 820 ml using deionized H_2O and the absorption was measured at 405 nm (24). The amount of p-nitroaniline produced was determined from a standard

curve. One unit of enzyme activity is defined as the amount of enzyme that liberates one µmole of p-nitroaniline per min under these assay conditions.

Purification of *P. furiosus* lysine aminopeptidase. Lysine aminopeptidase was purified from *P. furiosus* under anaerobic conditions at 23°C. Frozen cells (100 g, wet weight) were thawed in 300 ml of 50 mM Tris-HCl buffer (pH 8.0) containing DNase I 37 °C for 2 h. A cell-free extract was obtained by ultracentrifugation at 18,000 x g for 2 h. The supernatant (300 ml) was applied to a column (10 by 14 cm) of DEAE-Sepharose Fast Flow (Pharmacia, Piscataway, N.J.) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 2 mM dithionite (DT) (Tris-DT buffer). The column was eluted at a flow rate of 10 ml/min with a 2.5-liter linear gradient from 0 to 1.0 M NaCl in the same Tris-DT buffer. Lysine aminopeptidase activity was eluted from 0.29 to 0.39 M NaCl. The active fractions were combined (300 ml), and sodium sulfate was added to a final concentration of 1.0 M. This solution was applied to a column (3.5 by 10 cm) of Phenyl Sepharose (Pharmacia) equilibrated with Tris-DT buffer containing 1 M sodium sulfate. The column was eluted with a gradient (1 liter) from 1.0 to 0 M sodium sulfate in the Tris-DT buffer at a flow rate of 7 ml/min. Lysine aminopeptidase activity eluted at 0.50 to 0.57 M sodium sulfate. The lysine aminopeptidase-containing fractions (120 ml) were applied to a column (1 x 10 cm) of hydroxyapatite (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0). The column was eluted at a flow rate of 5 ml/min with a 100 ml linear gradient of 0 to 0.5 M potassium phosphate buffer (pH 8.0). The active fractions from the hydroxyapatite were applied to a column (1.6 by 60 cm) of Superdex-200 (Pharmacia) equilibrated with 50 mM Tris-DT buffer (pH 8.0) containing 0.5 M NaCl at a flow rate of

0.6 ml/min. Fractions containing lysine aminopeptidase activity were concentrated using an ultrafiltration were stored frozen as pellets in liquid nitrogen until required.

Cloning and expression of the lysine aminopeptidase-encoding gene. The gene encoding *P. furiosus* lysine aminopeptidase was obtained by PCR amplification using genomic DNA and was subsequently cloned into the modified T7 polymerase expression vector pET-24d (Novagen, Milwaukee, WI.). For amplification, the forward primer (CACCGGATCCGTAGATTGGGAACTAATGAAA; MWG, High Point, NC), contained an engineered BamHI site, while the reverse primer (AAGCTCGAGCGGCCGCTCACGGTGTAAAGTCCATTGGCTTTA) had an engineered NotI site. PCR amplification was performed with cloned *P. furiosus* DNA polymerase (Stratagene, La Jolla, CA) and a Robocycler 40 (Stratagene) programmed with an initial denaturation of 4 min then 30 cycles, consisting of denaturation at 94 ^{o}C for 1 min, annealing at 50 ^{o}C for 2 min, and extension at 72 ^{o}C for 3 min. The PCR fragment (1.06-kb) was isolated from the reaction mixture using a PCR clean up kit (TeleChem, Sunnyvale, CA) and digested with Bam HI and Not I, and cloned into the modified pET-24d vector. The ligation mixture (3 ul) was used to transform *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA). After screening for the presence of the gene by restriction digestion, the plasmid was transformed into the expression host BL21(DE3)Star (Invitrogen) containing the pRIL vector (Stratagene). The expression of the gene encoding lysine aminopeptidase was induced with IPTG (0.4 mM) when the culture, reached an optical density of 0.6. The induced culture was incubated for 3 h prior to harvesting of the cells

Purification of recombinant lysine aminopeptidase. Recombinant lysine aminopeptidase was purified in two steps. IPTG-induced BL21 (λDE3) Star/pRIL cells that had been harvested from a 1-L culture were suspended in 30 ml of 10 mM imidazole buffer (pH 8.0), containing 10 mM sodium phosphate and 0.5 mM NaCl. The cells were placed on ice and lysed by sonication for 5 min. The lysed extract was centrifuged at 16,000 × *g* for 20 min to remove any intact cells and large cellular debris. The cell suspension was centrifuged at 5,000 x *g* for 15 min to remove precipitate and the supernatant was applied to a column (1.6 x 3 cm) of Co^{2+}-Talon affinity (Clontech, Palo Alto, CA) that was equilibrated with 10 mM imidazole buffer (pH 8.0). The lysine aminopeptidase was eluted with 300 mM imidazole buffer (pH 8.0) and was heat treated for 5 min at 70 ^{o}C in the presence of 0.2mM $CoCl_2$. The precipitate was removed by centrifugation (14,000 x g for 10 min) and the supernatant was concentrated by ultrafiltration. The purified enzyme was stored in liquid nitrogen until required.

Other methods. Molecular weights were estimated by gel filtration with a column (1 by 27 cm) of Superdex 200 (Pharmacia, Piscataway, NJ) with amylase (200,000), alcohol dehydrogenase (150,000), and bovine serum albumin (66,000) as standard proteins. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed using 12.5% polyacrylamide by the method of Laemmli (25). Protein concentrations were determined by the Bradford method (6) with bovine serum albumin as the standard. To determine metal content, exogenous metal ions were removed from the lysine aminopeptidase by gel filtration with a G-25 column equilibrated with 50 mM MOPS (3-[N-morpholine] propane sulfonic acid) buffer (pH 8.0). A metal analysis (20 elements) was obtained by plasma emission spectroscopy with a Thermo Jarrell-Ash Enviro 36 Inductively Coupled

Argon Plasma instrument at the Chemical Analysis Laboratory of the University of Georgia. Subunit molecular weight analysis of the native and recombinant lysine aminopeptidase were determined by liquid chromatography-mass spectrometry (LC-MS) using an Applied Biosystems syringe pump HPLC system and the Perkin Elmer Sciex API I Plus Quadrupole Mass Spectrometer at the Chemical and Biological Sciences Mass Spectrometry Facility of the University of Georgia.

RESULTS

Purification of the native *P. furiosus* lysine aminopeptidase. Cell extracts contained a significant amount of lysine aminopeptidase activity (approximately 11 U/mg at 100 ^{o}C) using Lys-pNA as the substrate. Lysine aminopeptidase was purified to apparent homogeneity by four chromatography steps. All cells used for purification were obtained from cell cultures grown on a 500-liter scale. The procedure was carried out under anaerobic conditions, not because the lysine aminopeptidase was oxygen-sensitive, but to allow the purification of other enzymes that are oxygen sensitive from the same batch of *P. furiosus* cells.

Lysine aminopeptidase activity was only found in the soluble fraction, indicating that the enzyme is a cytoplasmic protein. The enzyme was purified 170-fold with a yield of 28% and a specific activity of approximately 1,900 U/mg (Table I). On a denaturing electrophoresis gel, the purified enzyme migrated as a single major band corresponding to a molecular mass of approximately 38 kDa (Fig. 3.1). Tryptic digestion of the purified protein was used to identify a gene (PF1861) in the *P. furiosus* genome (http://comb5-156.umbi.umd.edu/genemate) that is annotated as an endoglucanase. Analyses of a sample of the purified enzyme by LC-MS indicated a molecular weight of 38,210. This

value is in good agreement with the lysine aminopeptidase gene that encodes a protein of 390 residues with a calculated molecular weight of 38, 214 kDa. Lysine aminopeptidase was eluted from a gel filtration column, corresponding to a molecular mass of 160 $\pm$ 10 kDa. This result, together with the electrophoretic and LC-MS data, suggests that the enzyme is a homotetramer. A mass of 38.2 kDa for the lysine aminopeptidase subunit was used in all calculations.

Purification of recombinant *P. furiosus* lysine aminopeptidase. The gene encoding lysine aminopeptidase protein was successfully expressed in *E. coli* cells by the addition of IPTG after a 4-h period of induction at 37°C. The presence of recombinant enzyme in cell-free extracts of *E. coli* was evident by both by high-temperature (100°C) enzyme assays as well as by the appearance of a protein band corresponding to a mass 38 kDa after SDS-gel analysis of cell-free extracts (Fig. 3.1). The specific activity of the lysine aminopeptidase in the recombinant *E. coli* cells was approximately 207 U/mg, which is approximately 20-fold greater than the activity in cell-free extracts of *P. furiosus* when measured under the same conditions at 100°C. The results of a typical purification of the recombinant enzyme are summarized in Table 3.2. It was purified in two steps by affinity chromatography and by a heat treatment step. The recombinant KAP was indistinguishable from the native protein from *P. furiosus* when analyzed by SDS-gel electrophoresis, and LC-MS analysis indicated a subunit molecular weight of 38.2 kDa.

Catalytic properties of native and recombinant lysine aminopeptidases. Both the native (n-KAP) and the recombinant forms (r-KAP) were catalytically active as purified

Fig. 3.1.

SDS-PAGE (12%) of the lysine aminopeptidase purified from *P. furiosus*. Lane 1: standard molecular weight markers; Lane 2: native lysine aminopeptidase (4μg); Lane 3: recombinant lysine aminopeptidase (4μg).

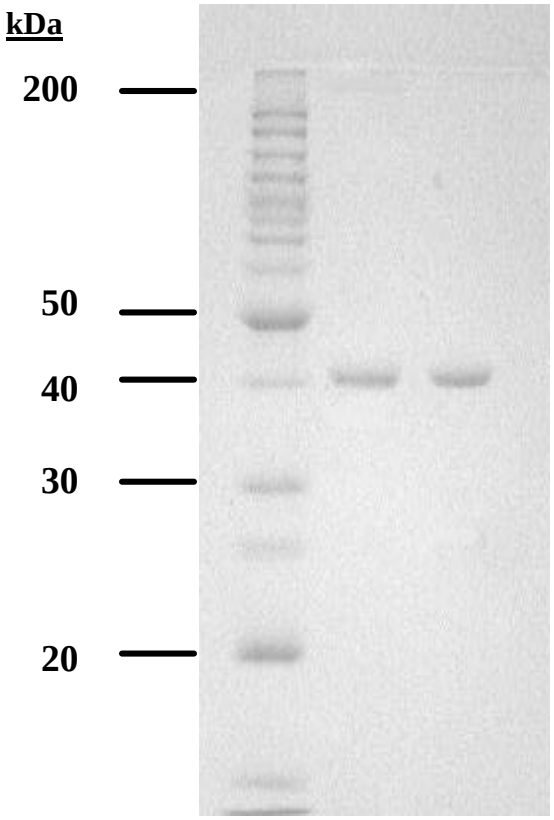

kDa
200
50
40
30
20

Table 3.1. Purification Table for Lysine Aminopeptidase from *P. furiosus*.

Step	Activity[a] (U)	Protein (mg)	Sp. Act. (U/mg)	Purification (fold)	Recovery %
Cell-free extract	28,700	2,600	11	1	100
DEAE	14,500	495	29	2.7	51
Phenyl Sepharose	11,500	27	419	38	40
Hydroxyapatite	7,051	8.7	810	74	25
Superdex 200	8,045	4.3	1,900	170	28

[a]Activity was measured at 100 ^{o}C by using Lys-pNA (10 mM) as the substrate.

(470 U/mg and 525 U/mg, respectively). However, both were stimulated by approximately 4-fold to 1,900 U/mg and 2,100 U/mg, respectively, by the presence of 0.2 mM Co^{2+} ions and these were included in all assays unless otherwise noted. The Co^{2+} ions could not be replaced with other divalent (Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}) or monovalent (Na^{+} or K^{+}). n-KAP and r-KAP showed the same responses to temperature and pH (Fig. 3.2). Each had a pH optimum at 8.0 and a temperature optimum of $\geq$ 100 ^{o}C. Surprisingly, both forms exhibited at 25 ^{o}C approximately 15% of their activity at 100 ^{o}C. Lys-pNA was used as the substrate was used in all routine assays and the activities of the two enzyme forms were investigated. As shown in Table 3.3, the enzymes were active only with basic and, to a lesser extent, nonpolar residues at the N-terminus. Kinetic analyses were carried out for both enzyme forms using Lys-pNA and Lys-Gly-Gly as substrates. All showed normal Michaelis-Menten-type kinetics, and the kinetic constants shown in Table 3.4 were calculated from the linear double-reciprocal plots. The results are comparable for both enzymes and both substrates with K_M values in the range of 2-4 mM and V_{max} values approaching 3,000 U/mg. r-KAP had a slightly higher affinity for Arg-pNA, but exhibited slightly lower activity (Table 3.4).

Physical properties of native and recombinant lysine aminopeptidases. The metal contents of both the n-KAP and r-KAP were determined by plasma emission spectroscopy. Surprisingly, of the 20 elements that were analyzed, zinc was the only one present in significant amounts. n-KAP contained 1.8 $\pm$ 0.48 g-atom/subunit whereas the r-KAP contained 2.3 $\pm$ 0.48 g-atom/subunit. When the n-KAP and r-KAP (0.000047

Table 3.2. Purification Table for Recombinant Lysine Aminopeptidase.

Step	Activity[a] (U)	Protein (mg)	Sp. Act. (U/mg)	Purification (fold)	Recovery %
Cell-free extract	43,800	202	217	1	100
Co^{2+}-Talon Affinity	30,700	28	1,100	5	70
Heat Treatment	53,100	24	2,200	10	120

[a]Activity was measured at 100 ^{o}C by using Lys-pNA (10 mM) as the substrate.

mg/ml in 50 mM MOPS, pH 8.0) were treated with EDTA (20 mM) for 1 h at 23 ^{o}C and then subjected to gel filtration, there was no significant loss in activity with either form of the enzyme. When either form (0.000047 mg/ml 50 mM MOPS, pH 8.0) was heated at 100 ^{o}C for 5 min with EDTA (20 mM), both lost apparently 40% loss of their activity. None of any of the following divalent (Ca^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}) or monovalent (Na^{+} or K^{+}) cations restored activity. When the enzyme forms (0.047 μg/ml 50 mM MOPS, pH 8.0) were pre-incubated with o-phenanthroline (20 mM) at 100 ^{o}C both forms completely lost activity and when they were incubated with 0.4 mM $CoCl_2$ in the standard assay, only 40% of the total activity was restored. The native lysine aminopeptidase was very thermostable. When a sample (0.047 μg/ml in 50 mM MOPS, pH 8.0) was incubated at 100 ^{o}C, the time required for a 50% loss of activity was 6 h. However, r-KAP (0.047 μg/ml in 50 mM MOPS, pH 8.0) lost 50% of its activity after a 10 min incubation under the same conditions. Interestingly, only the addition of 0.2 mM $CoCl_2$ to the r-KAP, increased its thermostability dramatically and its $t_{1/2}$ value was comparable to that of the native enzyme. However, incubation of n-KAP with 0.2 mM $CoCl_2$ for 10 min at 100 ^{o}C resulted in a dramatic loss of activity (< 50%).

DISCUSSION

P. furiosus contains significant lysine aminopeptidase activity in its cytoplasm. This reaction is catalyzed by a single enzyme which has a strong preference for peptides containing lysyl or arginyl residue at the N-terminus of di- and tri-peptides with dramatically lower activity with nonpolar residues (Ala, Leu, Phe). The relatively high

Table 3.3. Substrate specificity of native and recombinant *P. furiosus* Lysine Aminopeptidase.

Substrate[a]	% Relative Activity[b]	
	n-KAP	r-KAP
p-nitroanilide amino acids		
Lys-pNA	100	100
Arg-pNA	34	47
Leu-pNA	0	7
Pro-pNA	0	6
Ala-pNA	0	3
Gly-pNA	4	0.7
His-pNA	5	0
Phe-pNA	14	0
Ac-Phe-pNA	4	0

[a]All substrates were used at a final concentration of 10 mM.
[b]The rate of hydrolysis is expressed as a percentage of the activity compared to that obtained by using Lys-pNA as the substrate at 100 ^{o}C, where 100% activity corresponds to 1,900 U/mg and 2,200 U/mg for native and recombinant KAP, respectively.

Figure 3.2.

The effects of temperature and pH on the *P. furiosus* lysine aminopeptidase activity. The assay mixture contained lysine aminopeptidase (0.047 mg/ml), lysine-pNA (10mM) in 50 mM MOPS, pH (8.0). For the effects of pH, the following buffers (each 50 mM) were used at the indicated pH: Bis-Tris, pH 6.0, pH 6.5, and pH 6.8; MOPS, pH 7.0, pH 7.2, and pH 8.0; CHES, pH 8.6 and 9.0. For effects of temperature, the buffer used was 50 mM MOPS (pH 8.0). 100 % activity corresponds to 1,900 U/mg and 2,200 U/mg for native and recombinant KAP, respectively. The closed squares represent native KAP and the closed circles represent recombinant KAP.

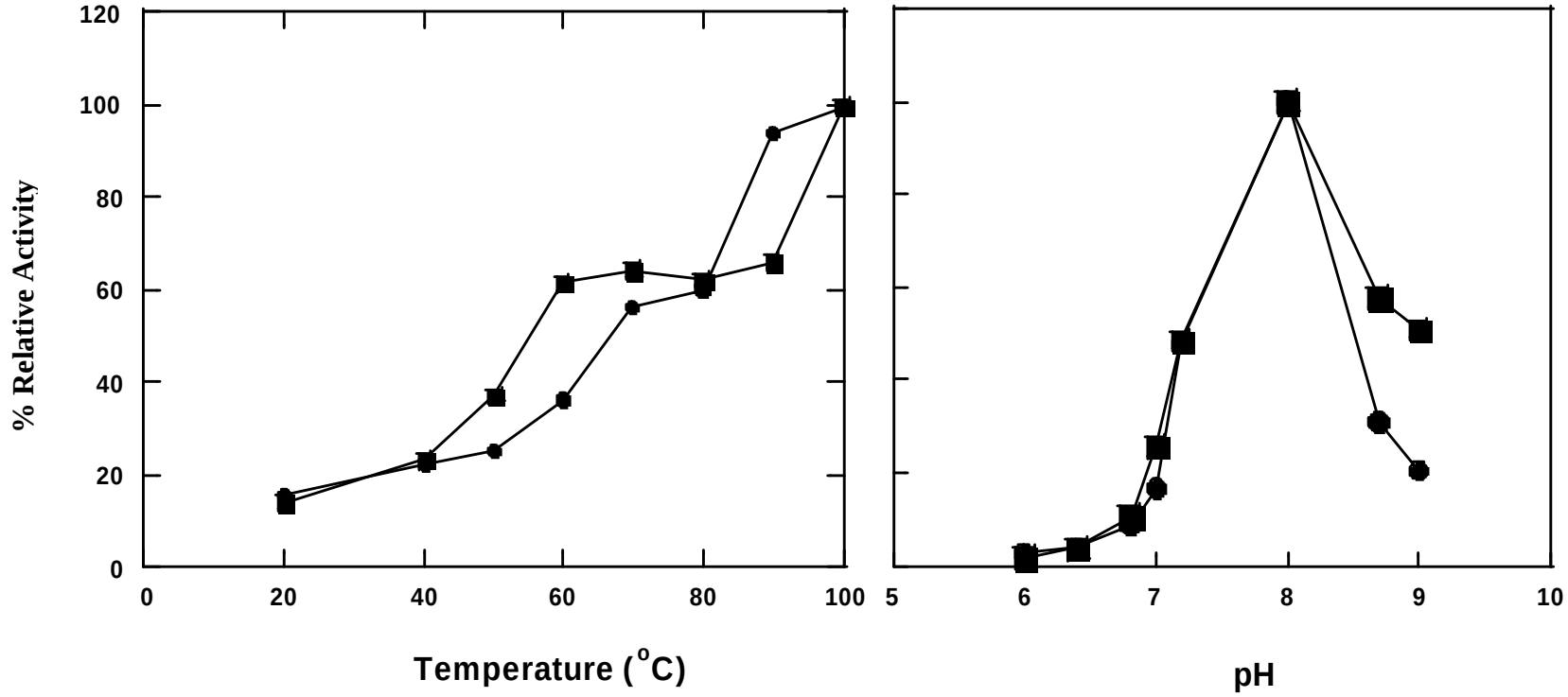

% Relative Activity
120
100
80
60
40
20
0
0
20
40
60
80
100
Temperature (°C)
5
6
7
8
9
10
pH

Table 3.4. Kinetic parameters for substrates of *P. furiosus* lysine aminopeptidase.

KAP	Substrate[a]	K_M (mM)	V_{max} (mmoles/min/mg)	k_{cat}[b] (s^{-1})	k_{cat}/K_M $mM^{-1}s^{-1}$
n-KAP	Lys-pNA	4.6	2,900	1,260	274
	Lys-Gly-Gly	1.8	677	305	169
r-KAP	Lys-pNA	4.0	2,870	1,840	460
	Lys-Gly-Gly	2.7	2,300	1,460	540
	Arg-pNA	1.4	1,200	769	550

[a]All assays were carried out at 100°C in 50 mM MOPS, pH 8.0.
[b]Based on a molecular mass of 38.2 kDa.

K_M values (2.0 mM) for such basic substrates indicate that such peptides may be present at significant intracellular levels in vivo. The gene (PF1861) encoding the enzyme was expressed in *E. coli* and the properties of the recombinant form, including temperature, pH dependence, and substrate specificities were indistinguishable from that of the native enzyme. However, the recombinant form was considerably less thermostable than the native enzyme suggesting that it may not be completely folded, although its catalytic properties were the comparable. The recombinant form was stabilized by Co^{2+} ions, but these destabilized the native form, the reasons for which are unclear at this point. The native and recombinant forms of lysine aminopeptidase each contain two zinc ions per subunit. These are essential for activity as they are lost upon incubation with chelators such as EDTA and o-phenanthroline. Activity could only be restored upon addition of Co^{2+} ions whereas Zn^{2+} ions had no effect. Addition of Co^{2+} ions to the purified enzymes resulted in a significant stimulation (4-fold) of enzymatic activity, but the role of cobalt has yet to be determined.

Two lysine aminopeptidases with substrate specificities similar to that of the *P. furiosus* enzyme have been reported only from two eukaryotes, from the fungi *A. niger* (5) and *S. cerevisiae* (18). The yeast enzyme, like that of *P. furiosus*, is also stimulated by Co^{2+} ions (0.5 mM), however, the *A. niger* enzyme does not require the presence of any metal ions. The two eukaryotic enzymes have 68% sequence similarity with each other and belong to the M1 family of metallo-aminopeptidases. This family also includes two other yeast enzymes leucine aminopeptidase I and alanyl aminopeptidase I of the same family, however, the sequence of the *P. furiosus* enzyme shows very little sequence similarity to any of these enzymes. Rather, it shows low similarity (27% and 22%) to

two members of the M18 family of proteases, two eukaryotic metallo-peptidases, which also include rabbit aspartyl aminopeptidase (38) and yeast aminopeptidase I (27). These two eukaryotic enzymes show approximately 43% sequence similarity with each other and are the only two members of this family that have been characterized. The nature of the active site of the aminopeptidases of the M18 family are not known since these enzymes lack the classical signature sequence (HEXXH) for zinc metallopeptidases. Their catalytic sites are thought to contain four conserved histidine residues (37) and three of them are also found in the sequence of the *P. furiosus* enzyme (Fig. 2). There are other residues conserved in all three enzymes, including (using the *P. furiosus* notation) Glu/Asp88, Asp135, Asp305, Glu342, Glu343, and Asp388, and these may also be involved in catalysis. Clearly, structural analyses of the *P. furiosus* enzyme are required to determine the role of these residues. Such analyses would reveal a class of metallopeptidases which contain a novel signature sequence for binding zinc. To this end, samples of the P. furiosus enzyme have been crystallized and the crystals diffract at 2.2 angstroms (Wang, B. B., unpublished data). The crystal structure of the *P. furiosus* lysine aminopeptidase will prove to be novel in relevance to metal-binding ligands and also with regard to overall structure as there are no analogs of this enzyme in the protein database (PDB).

Metal analyses indicated that neither form of the *P. furiosus* lysine aminopeptidase contain Co^{2+} as purified, however, the ability of the enzyme to be activated by Co^{2+} ions suggests that an active form containing a Co-Zn binuclear center may be possible. It will be interesting to see what the structural analyses reveal about this site. Such exchanging of divalent cations has been observed in leucine

aminopeptidases (LAPs) (21, 28). LAPs (EC 3.4.11.1) hydrolyze amino acid residues from the N-terminus of peptides with a preference for leucine residues. These enzymes are hexameric and contain two zinc-binding sites per subunit (16) in which the first site, site 1, readily exchanges Zn^{2+} for other divalent cations including Mn^{2+}, Mg^{2+}, and Co^{2+}. On the other hand, site 2 binds the second Zn^{2+} more strongly thereby allowing the exchange of Zn^{2+} in site 1 and the second site is more specific for zinc than the first site (28). Hence, such an exchange of metals may occur in the *P. furiosus* KAP since it also contains a dinuclear zinc site, but further metal studies need to be carried out to address this, in combination with structural analyses.

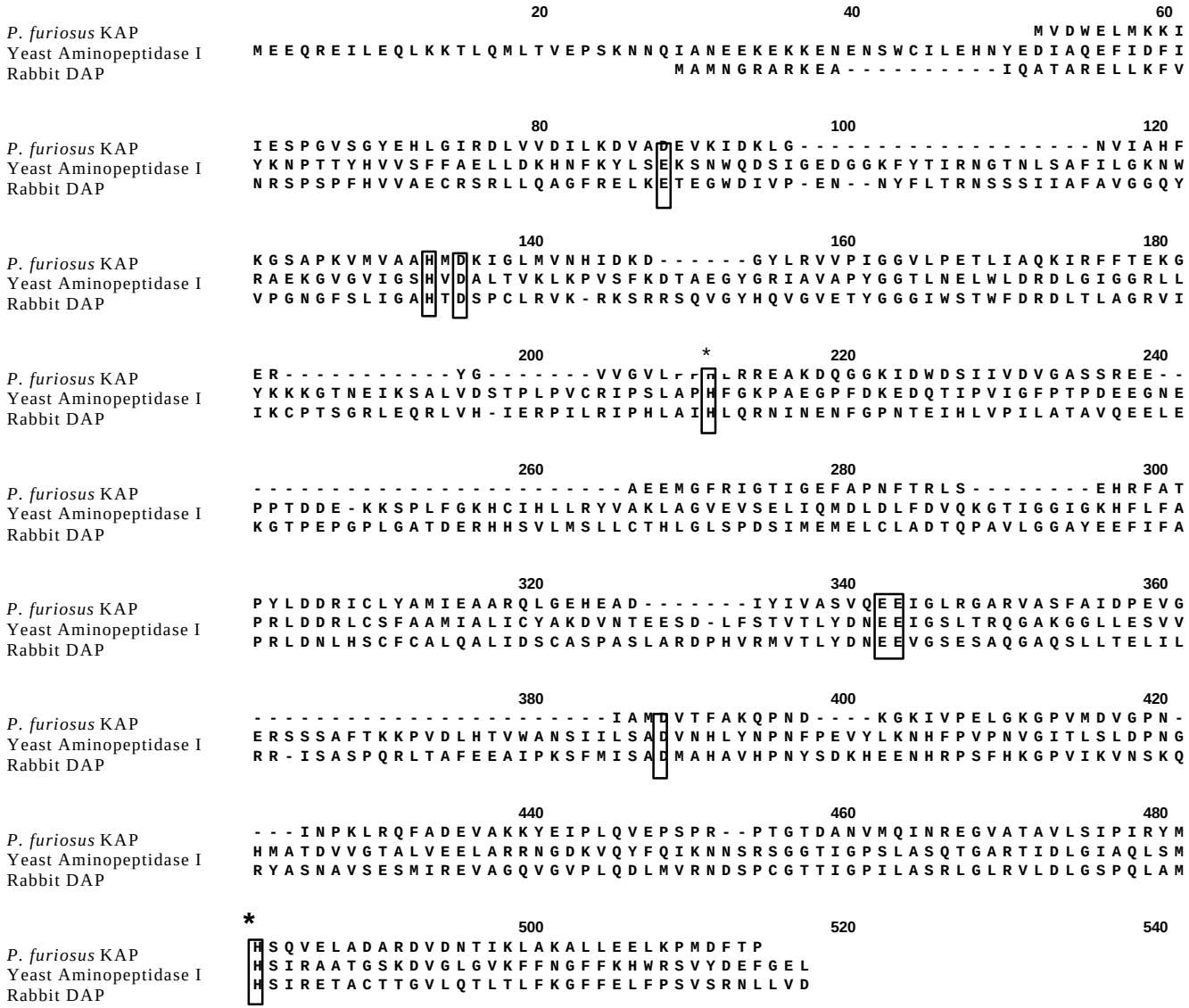
The lysine aminopeptidase from *P. furiosus* is the first such enzyme of the M18 family to be purified from a prokaryote, a hyperthermophile, or an archaeon. As expected, the *P. furiosus* enzyme is very thermostable, with a temperature optimum above 100 ^{o}C and no loss of activity after 6 h. The thermostability of the other members of the M18 family, yeast aminopeptidase I and rabbit aspartyl aminopeptidase, were not reported. Although the sizes of their subunits are similar to that of the *P. furiosus* enzyme, they form much larger complexes. These are 640 kDa and 440 kDa, respectively, (38) compared to 160 kDa for *P. furiosus* lysine aminopeptidase. Yeast aminopeptidase I has been characterized as a dodecameric protein whereas the rabbit aspartyl aminopeptidase appears to be octameric (27, 38).

The sequence of *P. furiosus* lysine aminopeptidase shows similarity to genes encoding two putative proteins in the genome sequences of *P. horikoshii* and *P. abysii*, although these proteins have not yet been characterized. These proteins are likely to also be members of the M18 family of metallopeptidases, although this remains unclear.

Homologs of *P. furiosus* lysine aminopeptidase amino acid sequence are also present in the genome sequences of other archaea. These include those of *Methanococcus jannaschii* (8), *Methanobacterium thermoautotrophicus* (32), *Methanosarcina mazei* (11), *Methanosarcina acetivorans* (13), *Pyrobaculum aerophilum* (12), *Methanococcus maripaludis* (22), *Archaeoglobus fulgidus* (23), *Methanopyrus kandeleri* (31), *Thermotoga maritima* (29), and *Aeropyrum pernix* (20), all of which have 50-60% overall sequence similarity to the *P. furiosus* lysine aminopeptidase. Aminopeptidases are known to be involved in degradation of cellular proteins for controlling protein concentrations, maintaining amino acid pools, and removing damaged proteins (1). In addition, they may also be involved in the breakdown of proteinaceous growth substrates. Interestingly, homologs of the *P. furiosus* lysine aminopeptidase are found in all heterotrophic archaea capable of utilizing peptides as a carbon source (*P. furiosus*, *P. horikoshii*, *P. abysii*, *P. aerophilum* (also autotrophic), *T. maritima*, and *A. pernix*) as well as autotrophic archaea (*M. jannaschii*, *M. thermoautotrophicus*, *M. mazei*, *M. acetivorans*, *M. maripaludis*, and *M. kandleri*). These findings indicate that the enzyme may either function intracellularly to breakdown proteinaceous growth substrates or carries out proteolytic degradation of the organisms' own cellular proteins. However, more studies on the physiological significance of these enzymes in *P. furiosus* and related organisms will have to be conducted in order to determine whether or not such enzymes play dual roles.

Figure 3.3.

Alignment of the amino acid sequence of *P. furiosus* lysine aminopeptidase with other aminopeptidases of the M18 family, yeast aminopeptidase I (AAA34738) and mouse aspartyl aminopeptidase (NP_058574). Conserved histidine, aspartate, and glutamate residues are boxed. Residues that are believed to play a role in metal binding are denoted by asterisks.


20
40
60
P. furiosus KAP
Yeast Aminopeptidase I
Rabbit DAP
80
100
120
140
160
180
200
260
280
300
320
340
360
380
400
420
440
460
480
500
520
540

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CHAPTER 4

CHARACTERIZATION OF A NOVEL ZINC-CONTAINING ALANINE AMINOPEPTIDASE (AAP) FROM THE HYPERTHERMOPHILIC ARCHAEON *PYROCOCCUS FURIOSUS*[3]

[3] Story, Sherry V. and Michael W. Adams. To be submitted to J. Bacteriol.

ABSTRACT

A gene annotated in the genome of *Pyrococcus furiosus* as encoding an endoglucanase has been cloned and expressed in *Escherichia coli*. The gene is predicted to encode a protein of 332 amino acid residues with a predicted mass of 36.7 kDa. The recombinant protein was produced with a polyhistidine tag at the N-terminus and was purified in a single-step by Ni^{2+}-NTA affinity chromatography. After gel electrophoresis, the purified enzyme gave rise to a single band corresponding to a mass of 36.7 kDa. Surprisingly, the enzyme lacked endoglucanase activity. Rather it hydrolyzed tri- and tetrapeptides containing an Ala residue at the N-terminus and appear to be an alanine aminopeptidase. It has an optimal temperature of 100 ^{o}C at pH 8.0, using Ala-Ala-Ala-Ala [2mM] as the substrate. The enzyme contains 2.0 g-atoms Zn^{2+} per subunit as purified. Although metal analyses indicated that the enzyme contained two Zn^{2+} ions, activity was strictly dependent on the addition of Co^{2+} ions ($CoCl_2$) and these could not be replaced by Zn^{2+} or any other divalent cation (Ca^{2+}, Cd^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+}).

INTRODUCTION

Aminopeptidases catalyze the sequential removal of amino acids from the unblocked N-terminus of peptides and proteins (35). These enzymes are widespread in eukaryotes and prokaryotes as either integral membrane proteins or cytosolic proteins (14, 31). Generally, aminopeptidases are classified on their substrate specificities, according to whether they have a preference for a neutral, acidic, or basic amino acid in the P1 position. Although some contain cysteine and serine residues as part of their catalytic sites (14), most aminopeptidases are metalloenzymes (35). Aminopeptidases not only play a general role in protein and peptide metabolism, but they also have more specific functions which include the activation (7) or inactivation (15) of biologically active peptides, and removal of the N-terminal methionine of newly synthesized proteins (17).

Pyrococcus furiosus is a strict anaerobic heterotroph that grows optimally at 100 ^{0}C, utilizing proteins and peptides as substrates and it produces organic acids, CO_2, and H_2. Several enzymes involved in the catabolism of peptides have been purified from *P. furiosus* including aminotransferases (3), 2-keto acid oxidoreductases (1), glutamate dehydrogenase (20), prolidase (13), acetyl CoA synthetases (23), aminoacylase (30), cobalt-activated carboxypeptidase (8), thiol protease (24), and pyrollidone carboxypeptidase (34). In addition, two aminopeptidases have been isolated from *P. furiosus*, a methionine aminopeptidase (33) and a deblocking aminopeptidase (32) and a deblocking aminopeptidase has been characterized from the related organism, *P. horikoshii* (2, 26). Based on sequence similarity to known enzymes, a blast search of the genome revealed that *P. furiosus* contains nine families of peptidases, including serine,

thiol, and metalloproteases. Of the metalloproteases, one zinc containing enzyme and three cobalt-activated enzymes have been characterized from *P. furiosus* (8, 13, 30, 33). These include aminoacylase, prolidase, methionine aminopeptidase, and a cobalt-activated carboxypeptidase, respectively. Recently, cobalt-dependent enzymes have become an increasingly studied research field in bioinorganic chemistry as well as biotechnology. This interest has lead to the description of new types of proteolytic enzymes from both prokaryotes and eukaryotes (4, 27). The availability and chemical versatility makes cobalt an invaluable biocatalyst (21).

The genome of P. furiosus contains an ORF (PF0369) that appears to encode a homolog of the deblocking aminopeptidase isolated from *P.* horikoshii (85% identity), although it is very similar (51% identity) to the deblocking enzyme already characterized from *P. furiosus* (32), as well as to a lysine aminopeptidase from *P. furiosus* (50% identity) ((2, 32). The *P. horikoshii* enzyme catalyzes the removal of acyl groups from the N-termini of peptides and its activity is dependent upon the presence of cobalt ions, as the activity is stimulated 6-fold by the addition of $CoCl_2$. In contrast, the product of PF0369 lacks deacetylating activity and catalyzes the hydrolysis of alanyl peptides in a cobalt-dependent reaction. It was of some interest to determine the properties of this enzyme in comparison to the deacetylating enzyme of the closely related organism, *P. horikoshii.*

MATERIALS AND METHODS

Growth of Microorganisms. *Escherichia coli* strains (BL21 (λDE3) Star/pRIL) were grown in Luria Bertani media (LB). Kanamycin (50 μg/ml) and chloramphenicol (35

μg/ml) were added as needed for plasmid maintenance.

Cloning and expression of the alanine aminopeptidase-encoding gene. The gene corresponding to PF0369 was cloned by PCR amplification of the alanine aminopeptidase gene and subsequent cloning into the modified T7 polymerase expression vector pET-24d (Novagen, Milwaukee, Wis.). For amplification, the forward primer (CACCGGATCCAAGCTAATTGAAATGCTAAAG; MWG, High Point, NC), contained an engineered BamHI site, while the reverse primer (AAGCTCGAGCGGCCGCCTAGATCTCAAATGTTATTGCTTCTA ;MWG) had an engineered NotI site. PCR amplification was performed with cloned *P. furiosus* DNA polymerase (Stratagene, La Jolla, CA) and a Robocycler 40 (Stratagene) programmed with an initial denaturation of 4 min then 30 cycles, consisting of denaturation at 94 ^{o}C for 1 min, annealing at 50 ^{o}C for 2 min, and extension at 72 ^{o}C for 3 min. The PCR fragment (1.06-kb) was purified using a PCR clean up kit (TeleChem), digested with Bam HI and Not I, and cloned into the modified pET-24d vector. The ligation mixture (3 ul) was used to transform *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA). After screening for the presence of the gene by restriction digestion, the plasmid was transformed into the expression host BL21 (DE3) Star (Invitrogen) containing the pRIL vector (Stratagene). Expression of PF0369 was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.4 mM) when the culture reached an optical density of 0.6 at A_{600}. The induced culture was incubated for 3 h prior to harvesting of the cells.

Purification of the recombinant alanine aminopeptidase. The recombinant protein was purified in one step by His-tag affinity chromatography. IPTG-induced BL21(λDE3)

Star/pRIL cells grown in a 1-L culture were suspended in 30 ml of 10 mM imidazole buffer (pH 8.0), containing 10 mM sodium phosphate and 0.5 mM NaCl. The cells were harvested, placed on ice and lysed by sonication for 5 min. The lysed extract was centrifuged at 16,000 × *g* for 20 min to remove cellular debris and the supernatant was collected. The supernatant was applied to a column (1.6 x 3 cm) of Ni^{2+}-NTA (Clontech, Palo Alto, CA) that was equilibrated with 10 mM imidazole buffer (pH 8.0). The protein was eluted with 300 mM imidazole buffer (pH 8.0), concentrated by ultrafiltration and stored in liquid nitrogen until required.

Enzyme Assay. Alanine aminopeptidase activity was determined using the cadmium-ninhydrin colorimetric method (10) with Ala-Ala-Ala-Ala as the substrate. The assay mixture (200 µl) containing the enzyme sample in 50 mM MOPS (3-[N-morpholine] propane sulfonic acid) buffer (pH 8.0) and 2 mM Ala-Ala-Ala-Ala (Bachem Co.) and 0.2 mM $CoCl_2$ was incubated at 100°C for 5 min. Then, 1.5 ml of cadmium-ninhydrin reagent was added and the mixture incubated at 84 ^{o}C for 5 min. The absorption was measured at 505 nm. The amount of L-Ala produced was determined from a standard curve. One unit of enzyme activity is defined as the amount of enzyme that liberates one µmole Ala-Ala-Ala-Ala per minute under these assay conditions. This assay was also used for testing for activity with N-aylated peptides and non-acylated peptides.

Other methods. Molecular weights were estimated by gel filtration with a column (1 by 27 cm) of Superdex 200 (Pharmacia, Piscataway, NJ) with amylase (200,000), alcohol dehydrogenase (150,000), and bovine serum albumin (66,000) as standard proteins. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed using 12.5%

polyacrylamide by the method of Laemmli (22). Protein concentrations were determined by the Bradford method (5) with bovine serum albumin as the standard. To determine metal content, exogenous metal ions were removed from the alanine aminopeptidase by gel filtration with a G-25 column equilibrated with 50 mM MOPS (3-[N-morpholine] propane sulfonic acid) buffer (pH 8.0). A metal analysis (20 elements) was obtained by plasma emission spectroscopy with a Thermo Jarrell-Ash Enviro 36 Inductively Coupled Argon Plasma instrument at the Chemical Analysis Laboratory of the University of Georgia.

RESULTS

Purification of recombinant *P. furiosus* alanine aminopeptidase. The gene (PF0369) encoding alanine aminopeptidase was successfully expressed in *E. coli* cells by the addition of IPTG after a 4-h period of induction at 37°C. The purified enzyme was assayed for activity using tri-, tetra-, and pentapeptides with nonpolar and polar amino acid residues at the N-terminus as well as N-acylated tripeptides. The enzyme showed a preference for substrates with an alanyl residue at the N-terminus. Alanine activity could be identified in cell extracts of *E. coli* both by high-temperature (100°C) enzyme assays as well as by the appearance of a protein band corresponding to the predicted size of the enzyme (38 kDa) after SDS-gel analysis of cell extracts (Fig. 1). The specific activity of the alanine aminopeptidase in the recombinant *E. coli* cells was approximately 12 U/mg. The results of a typical purification of the recombinant alanine aminopeptidase are summarized in Table 4.1. The enzyme was purified in one step by metal affinity

Fig. 4.1.

SDS-PAGE (12%) of the recombinant alanine aminopeptidase purified from *E. coli*.

Lane 1: standard molecular weight markers; Lane 2: recombinant alanine aminopeptidase (4μg).

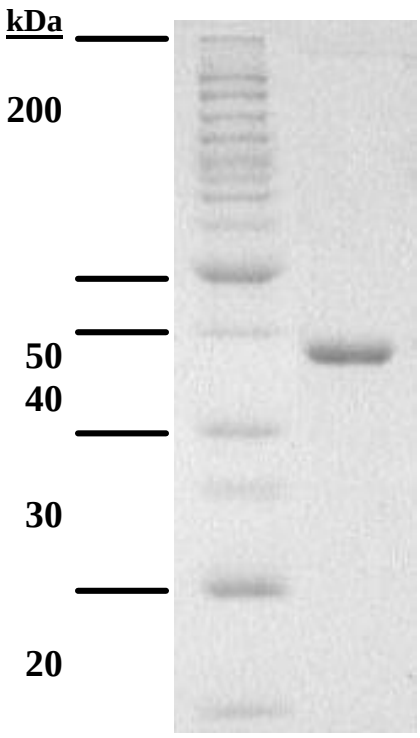

kDa
200
50
40
30
20

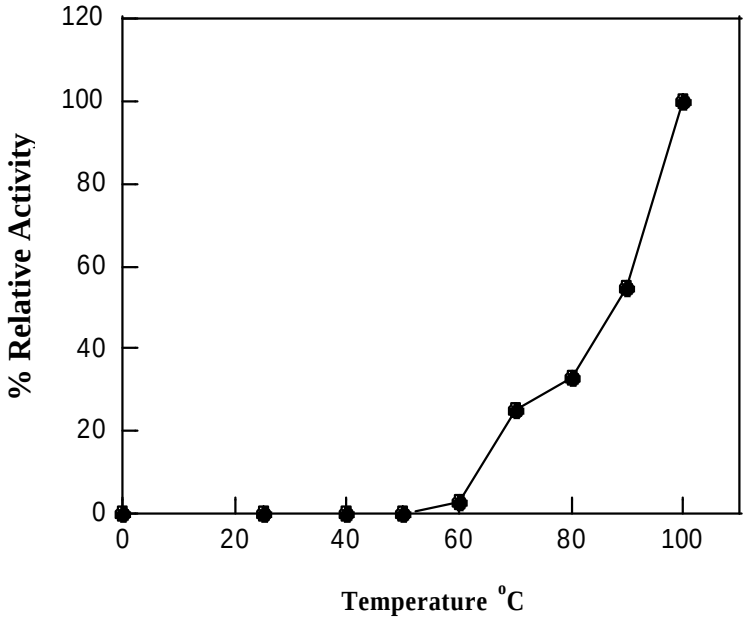
Table 4.1. Purification of Recombinant Alanine Aminopeptidase from *E.coli*.

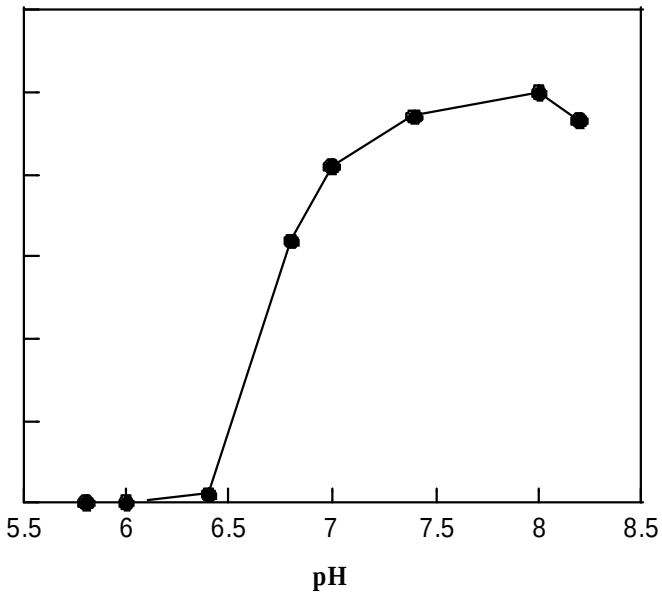
Step	Activity[a] (U)	Protein (mg)	Sp. Act. (U/mg)	Purification (fold)	Recovery %
Cell-free Extract	254	424	0.6	1	100
Ni^{+}-NTA Affinity	192	16	12	20	75

[a]Activity was measured at 100 ^{o}C by using Ala-Ala-Ala-Ala (10 mM) as the substrate

Fig. 4.2.

The effects of temperature and pH on the *P. furiosus* alanine aminopeptidase activity. The assay mixture contained alanine aminopeptidase (0.0028 mg/ml), Ala-Ala-Ala-Ala (2mM) in 50 mM MOPS, pH (8.0). For the effects of pH, the following buffers (each 50 mM) were used at the indicated pH: Bis-Tris, pH 6.0, pH 6.5, and pH 6.8; MOPS, pH 7.0, pH 7.2, and pH 8.0; CHES, pH 8.6 and 9.0. For effects of temperature, the buffer used was 50 mM MOPS (pH 8.0). 100 % activity corresponds to 12 U/mg.


% Relative Activity
120
100
80
60
40
20
0
0
20
40
60
80
100
Temperature °C


5.5
6
6.5
7
7.5
8
8.5
pH

chromatography. Analyses by SDS-gel electrophoresis and LC-MS indicated a subunit molecular weight of 36.7 kDa. These agree well with the calculated mass of 38.7 kDa based on the amino acid sequence of PF0369. Gel filtration analyses indicated that the enzyme was hexameric with a molecular mass of 280,000 $\pm$ 10,000 Da.

The metal content of alanine aminopeptidase (hereafter referred to as AAP) were determined by plasma emission spectroscopy. Of the 20 elements that were analyzed, the only metal present in significant amounts was zinc. The AAP contained 2.0 $\pm$ 0.48 g-atom/subunit, however, enzyme activity could only be detected in the presence of 0.2 mM $CoCl_2$. No other divalent (Ca^{2+}, Cd^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+}) or monovalent cations (K^+ or Na^+) were effective. All routine assays were therefore carried out in the presence of 0.2 mM $CoCl_2$. When a sample of the enzyme (0.0028 mg/ml in 50 mM MOPS, pH 8.0) was treated with EDTA (20 mM) for 2 h at room temperature, no significant loss in activity was observed. When EDTA was replaced by o-phenanthroline (20 mM) under the same conditions it still maintained 90% of its activity. Incubation of the enzyme (0.0028 mg/ml in 50 mM MOPS, pH 8.0) with o-phenanthroline (20 mM) for 5 min at 100 ^oC resulted in complete loss of activity whereas EDTA (20 mM) had no effect under these conditions. On the other hand, incubation with phenylmethylsufonylfluoride (PMSF) (20mM) for 5 min at 100 ^oC caused 70% inhibition of enzymatic activity. The alanine aminopeptidase as purified was very thermostable. When a sample (0.0028 mg in 50 mM MOPS, pH 8.0) was incubated at 100 ^oC, the time required for 50% of its activity was 4 h. Addition of cobalt ions (0.2 mM $CoCl_2$) did not have any effect on its thermostability.

Table 4.2. Substrate specificity of *P. furiosus* alanine aminopeptidase.

Substrate[a]	% Relative Activity[b]
Ala-Ala-Ala-Ala	100
Ala-Ala-Ala	97
Ala-Ala-Ala-Ala-Ala	68
Ala-Ala-Pro	24
Ala-Ala-Lys	5
D-Ala-Ala-Ala	0
Ala-Pro-Ala	0
Ala-D-Ala-Ala	0
Leu-Gly-Gly	0
Lys-Gly-Gly	0
N-acetyl-Ala-Ala-Ala	0
Ac-Ala-pNA	0
Ala-pNA	0
Lys-pNA	0

[a]All substrates were used at a final concentration of 2 mM.
[b]The rate of hydrolysis is expressed as a percentage of the activity compared to that obtained by using Ala-Ala-Ala-Ala as the substrate at 100 ^{o}C, where 100% activity corresponds to 12 U/mg.

Table 4.3. Kinetic parameters for substrates of *P. furiosus* alanine aminopeptidase.

Substrate[a]	K_M (mM)	V_{max} (mmoles/min/mg)	k_{cat}[b] (s^{-1})	k_{cat}/K_M $mM^{-1}s^{-1}$
Ala-Ala-Ala-Ala	1.1	21	13	12
Ala-Ala-Ala	1	20	12	11
Ala-Ala-Pro	1.8	10	6.1	3.4

[a]All assays were carried out at 100°C in 50 mM MOPS, pH 8.0.
[b]Based on a minimum molecular mass of 36.7 kDa.

Catalytic properties of *P. furiosus* alanine aminopeptidases. The *P. furiosus* enzyme was catalytically inactive as purified and could only be activated by the addition of Co^{2+} ions and these were used in all routine assays unless otherwise noted. Under such conditions, the enzyme had a pH optimum at 8.0 and a temperature optimum of $\geq$ 100 ^{o}C (Fig. 4.2) using the tetrapeptide, Ala-Ala-Ala-Ala as the substrate. The specific activity of the enzyme was 12 U/mg under standard assay conditions. However, it showed no detectable activity with N-acylated peptides. The activity of the enzyme with other potential substrates is shown in Table 4.2. Clearly, it is a true alanine aminopeptidase since it only hydrolyzes peptides with an alanyl residue at the N-terminus. The kinetic parameters obtained were using alanine tri- and tetra- peptides all showed normal Michaelis-Menten-type kinetics. The kinetic constants are listed in Table 4.3.

DISCUSSION

P. furiosus alanine aminopeptidase activity is strictly dependent on Co^{2+} ions and these could not be replaced by any of the other divalent cations tested. The enzyme appears to be specific for peptides containing an alanine residue at the N-terminus. The recombinant enzyme was purified to homogeneity from the cell extract *of E. coli* cells in one step using affinity chromatography. The enzyme was purified approximately 20-fold with a yield of 75%. It contained approximately 2 atoms of zinc per subunit. At room temperature, EDTA and o-phenanthroline had little effect upon *P. furiosus* alanine aminopeptidase activity after 2 h. However, when the enzyme was incubated with o-phenanthroline at 100 ^{o}C virtually all activity was lost (90%) although surprisingly, EDTA had no effect. The strong inhibition of enzymatic activity observed by using o-

phenanthroline, in contrast to that of EDTA, has been observed in the rabbit aspartyl aminopeptidase (35). EDTA (20mM) was ineffective in inhibiting rabbit aspartyl aminopeptidase activity whereas o-phenanthroline (2 mM) resulted in a complete loss of activity. In these enzymes it appears that the zinc ions are tightly bound to the enzyme and are not accessible to EDTA, but are bound by the more hydrophobic chelators, o-phenanthroline. The serine protease inhibitor, PMSF, caused 70% inhibition of the *P. furiosus* AAP suggesting that serine, tyrosine, or threonine residues may be involved in enzyme catalysis.

The molecular weight of the *P. furiosus* enzyme as calculated by gel filtration was approximately 280 kDa indicating that it is a hexameric protein. In contrast, the homologous deblocking enzyme from *P. horikoshii* has a molecular weight of 440 kDa, even though the two enzymes have highly similar sequences (85%) and have subunits of comparable size (36.0 and 36.7 kDa, respectively). The two enzymes also differ in their substrate specificities as the *P. furiosus* enzyme does not hydrolyze N-acetylated peptides or amino acids, such as N-acetyl Alal-Ala-Ala or N-acetyl-Ala. Furthermore, *P. furiosus* alanine aminopeptidase only has specificity for peptides consisting of three or more residues which contain an alanine residue at the N-terminus. Additionally, *P. furiosus* alanine aminopeptidase contains zinc and no other metal ion (including Ca^{2+}) could be detected. It has been reported that the *P. horikoshii* enzyme contains Ca^{2+} (2, 26), so inspite of the high sequence similarities, these may not be the same enzyme with regards to function.

Not only does *P. furiosus* alanine aminopeptidase have 85% sequence similarity to the *P. horikoshii* deblocking aminopeptidase, it also has 87% sequence similarity to a

putative protein in *P.* abysii (PAB2375). The *P. furiosus* enzyme also has homologs in the genomes of other archaea, including *Methanococcus jannaschii* (6), *Methanobacterium thermoautotrophicus* (29), *Methanosarcina mazei* (9), *Methanosarcina acetivorans* (12), *Pyrobaculum aerophilum* (11), *Methanococcus maripaludis* (18), *Archaeoglobus fulgidus* (19), *Methanopyrus kandeleri* (28), *Thermotoga maritima* (25), and *Aeropyrum pernix* (16), all of which have 50-60% overall sequence similarity to the *P. furiosus* alanine aminopeptidase. Except for the autotrophic methanogens and *Archaeoglobus* which uses carbohydrates, these archaea are heterotrophs and use peptides as a carbon source. Since aminopeptidases are involved in the breakdown both intracellular proteins and proteinaceous growth substrates, it was of interest to understand the physiological role of the *P. furiosus* enzyme.

DNA microarray analyses (Schut 2003, submitted) indicate a dramatic up-regulation of the expression of the alanine aminopeptidase gene (PF0369) at the transcriptional level by five-fold when *P. furiosus* cells are grown on peptides only. However, these results have not been confirmed by enzymatic methods. To confirm the results from DNA microarray analyses, *P. furiosus* cells will have to be grown using peptides as the carbon source in order to have detectable alanine aminopeptidase activity. These preliminary data suggests a possible role of the alanine aminopeptidase in peptide metabolism in *P. furiosus*. More studies including purification of the native alanine aminopeptidase, growth experiments, and DNA microarray analyses will have to be conducted in order to determine whether or not the *P. furiosus* enzyme plays a role in breakdown of proteinaceous growth substrates or functions intracellularly to degrade the cell's own proteins. In the *P. furiosus* genome, PF0369 encoding alanine aminopeptidase

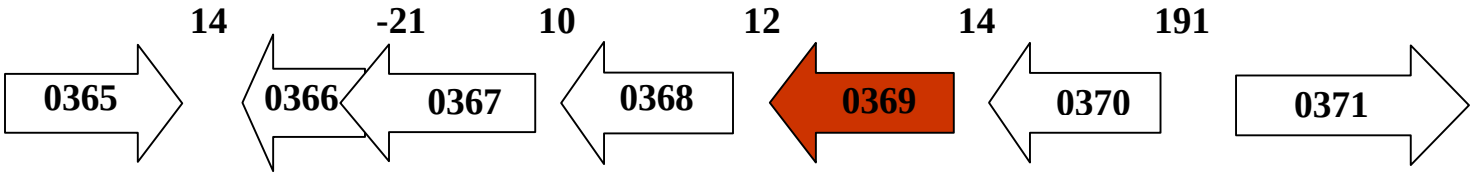
appears to be part of an operon (Fig. 3) with four other genes encoding a phosphoglycerate dehydrogenase and two conserved hypothetical proteins. Phosphoglycerate dehydrogenase is involved in the first step in the biosynthesis of serine by converting the glycolytic intermediate, 3-phosphoglycerate, to 3-phosphohydroxypyruvate. All four genes are also up-regulated (4-5 fold) at the transcriptional level according to DNA microarray results (Table 4.4).

Table 4.4. Up-regulation of *P. furiosus* gene encoding alanine aminopeptidase and surrounding genes.

PF#	Peptides + S^o vs Maltose + S^o (Fold of Regulation)
PF0369	5.5
PF0366	4.3
PF0367	4.8
PF0368	4.4
PF0370	5.2

Fig. 4.3.

Schematic representation of the gene encoding *P. furiosus* alanine aminopeptidase and its surrounding genes. PF0369 (red) is the alanine aminopeptidase gene. PF0365, PF0366, PF0367, and PF0368 are conserved hypothetical genes. PF0370 encodes a phosphoglycerate dehydrogenase. PF0371 encodes a putative transporter.


14
-21
10
12
14
191
0365
0366
0367
0368
0369
0370
0371

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Zinc is the most widely used transition metal in biology, although it is not the most abundantly available in nature (Vallee, 1993). Zinc metalloenzymes are found in eukaryotes and prokaryotes, including archaea (Coleman 1998). In bacteria and eukaryotes, more than 300 enzymes have been discovered representing over 50 different types that are known to require zinc for their function (Vallee, 1993). On the other hand, there are not many different iron metalloproteins or enzymes, and even fewer enzymes that contain copper, molybdenum, selenium, nickel, manganese, or cobalt. Zinc enzymes include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases, and therefore, are found in all six enzyme classes that have been established (Vallee, 1993). Based on genome sequence and homology searches based on characterized enzymes, *Pyrococcus furiosus* contains nine putative zinc-containing enzymes. The primary focus of this research project described herein was to determine the biochemical and kinetic properties of some of these enzymes and attempt to understand their physiological role in *P. furiosus*. Three distinct zinc metallopeptidases were characterized and these are an aminoacylase, a lysine aminopeptidase, and an alanine aminopeptidase.

At the beginning of this research project, an aminoacylase had not been isolated from an archaeon or a hyperthermophile. To date, only three have been isolated including the one that was discussed in chapter 2. The recombinant forms of the other two aminoacylases were isolated from *P. horikoshii* and *Thermococcus litoralis*, both of

which are hyperthermophilic archaea. The enzyme isolated from *T. litoralis* is a homotetramer of 43 kDa subunits (Toogood et al. 2002). The enzyme showed the highest specificity using N-acetyl-L-phenylalanine as a substrate. The apoenzyme was 80% active and its activity was stimulated 20% by addition of zinc chloride. In contrast, most mesophilic aminoacylases are not active as the apoenzyme and addition of zinc causes a decrease in activity (Sakanyan et al. 1993; Yang et al. 1994). As expected, the *T. litoralis* enzyme is highly thermostable with a half-life of 25 h at 70 ^{o}C. Crystallization and preliminary X-ray diffraction analyses have been done for this enzyme and the position of a single zinc ion has been determined (Hollingsworth et al. 2002). The aminoacylase isolated from *P. horikoshii* was found to have a dual activity as it functions both as a carboxypeptidase and an aminoacylase. This enzyme contained one zinc ion per subunit and showed a preference for aliphatic and aromatic N-acetylated amino acids. Site-directed mutagenesis studies indicated that the *P. horikoshii* enzyme, like other aminoacylases, contained a highly conserved cysteine residue directly involved in catalysis.

The hyperthermophilic aminoacylases have broad substrate specificities and their physiological roles are not fully understood. The *P. furiosus* enzyme was capable of hydrolyzing a broad range of N-acetylated amino acids and showed highest activity with N-acetyl-L-methionine. As purified, it contains one zinc ion per subunit and is a homotetramer. All aminoacylases that have been isolated and characterized from mesophilic and thermophilic organisms are similar in subunit size (37 to 50 kDa) and most of them are dimers (Yang et al. 1994; Weiss et al. 1995). As shown in Table 5.2, only two are homotetramers, and one is the *P. furiosus* enzyme (Cho 1987). *P. furiosus*

aminoacylase is also capable of hydrolyzing N-formyl-L-methionine as well as other N-acetylated-L-amino acids and is stereospecific for L-isomers. The relatively high K_M values for such substrates indicate that these amino acid derivatives may be present at significant intracellular concentrations within *P. furiosus*, if indeed they are the physiological substrates. There are two possible ways in which such derivatives could be produced, either by cellular protein degradation or degradation of protein/peptide growth substrates. More than likely, the catabolism of protein/peptide growth substrates is the source for several reasons: 1) *P. furiosus* grows fermentatively with peptides as the carbon source (Fiala 1986) and 2) other organisms such as *B. stearothermophilus* (Sakanyan et al. 1993), *A. dentirificans* (Yang et al. 1994), and *P. maltophila* (Wakayama 1998) grow on peptides as well. A metabolic study was done in which *P. furiosus* was grown on different combinations of carbohydrate (maltose), peptides (casein hydrolysate), and S^o (Adams et al., 2001). During this study the activities of 21 enzymes, including aminoacylase, were measured to establish the regulation of fermentative pathways in *P. furiosus*. *P. furiosus* was grown on the following media combinations: maltose and peptides; maltose only, maltose, peptides, and sulfur; maltose plus sulfur; and peptides plus sulfur.

To determine whether or not aminoacylase was involved in one of the major fermentative pathways in *P. furiosus* (saccharolytic or proteolytic), activities were measured under each of these conditions and the results are shown in Table 5.3. It was found that *P. furiosus* aminoacylase did not show any significant changes in activity under these conditions which implies that it is not directly involved in either of the fermentative pathways in *P. furiosus*, or that it is constitutive and not regulated. Based

on genome sequence information, a pathway for the initial steps of proteolytic degradation involving *P. furiosus* aminoacylase was proposed (Fig 2.7, chapter 2) to investigate the role of the enzyme. Preliminary experiments were conducted using a minimal media supplemented with N-acetyl-L-methionine, N-acetyl-L-valine, or N-formyl-L-methionine. No apparent change in aminoacylase activity was detected under these conditions suggesting that enzyme expression is not regulated or that the cell cannot uptake such substrates. However, a more detailed study will have to be conducted to further examine the role of aminoacylase in peptide catabolism possibly by using N-acetylated proteins and other such substrates.

In fact, comparisons of complete microbial genome sequences support the idea that aminoacylases represented by the *P. furiosus* enzyme are involved in metabolizing protein growth substrates rather than the cell's own proteins (Story, 2001). Similarly, organisms from which aminoacylases have been purified, such as *B. stearothermophilus* (Sakanyan 1993), *A. denitrificans* (Yang 1994), and *Pseudomonas maltophila* (Wakayama 1998) are all capable of peptidolytic growth. For instance, the complete genome sequences of 21 microbial organisms have homologs of the *P. furiosus* aminoacylase in which nineteen of them are capable of growing on peptides. These include *P. horikoshii*, *P. abysii*, *B. stearothermophilus*, *B. subtilus*, *Clostridium acetobutylicum*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *D. radiodurans*, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Treponema denticola*. The other two organisms are *Chlorobium tepidum* and *Synechocystis* sp. (photosynthetic bacteria). To further explore the proposed pathway for the initial steps of protein degradation in *P. furiosus*, a search for genes encoding putative aminopeptidases was carried out using the

Table 5.1. Peptidase families in *P. furiosus*.

Peptidase Family ORF Name	PF Number	Pf enzyme Characterized
C15		
Pyroglutamyl Peptidase I/Pyrrolidone Carboxyl Peptidase	PF1299	Yes
M1		
Putative Aminopeptidase	PF2059	No
Putative Aminopeptidase	PF2063	No
Putative Aminopeptidase/[Tricorn protease interacting factor F2]	PF2065	No
M3,32		
Carboxypeptidase I/Cobalt-activated Carboxypeptidase	PF0456	Yes
M18		
Similar to endo-1,4 beta glucanase/Lysine aminopeptidase	PF1861	Yes
Endoglucanase/Deblocking Aminopeptidase	PF1547	Yes
Probable Endoglucanase/Alanine Aminopeptidase	PF0369	Yes
M19		
Membrane Dipeptidase	PF0874	No

M20		
IAA-amino acid hydrolase/ Aminoacylase	PF0597	Yes
Acetylornithine deacetylase/Carboxypeptidase 2	PF1185	No
Succinyldiaminopimelate desuccinylase	PF2048	No
Acetylornithine deacetylase	PF1686	No
M22		
O-sialoglycoprotein Endopeptidase	PF0473	No
O-sialoglycoprotein Endopeptidase	PF0172	No
M24		
Methionine Aminopeptidase/Methionine Aminopeptidase	PF0541	Yes
XAA-Pro Dipeptidase (proline dipeptidase)/Prolidase	PF1343	Yes
X-Pro Dipeptidase/[Prolidase 2]	PF0702	No
Putative Proline Dipeptidase/[Prolidase 3]	PF0747	No
S9C		
Similar to acylaminoacyl-peptidase/ [Acylamino acid releasing enzyme]	PF0318	No

complete sequence of the *P. furiosus* genome. At the time this work was begun, only three hyperthermophilic aminopeptidases had been isolated and these were from two archaea, one from *Pyrococcus horikoshii* and two from *P. furiosus*.

The two aminopeptidases from *P. furiosus* are a deblocking aminopeptidase (DAP) and a methionine aminopeptidase (Barrett 1995; Tsunasawa et al. 1997; Tsunasawa 1998). *P. horikoshii* is a hyperthermophilic archaeon which grows optimally at 98 ^{o}C (Gonzalez et al. 1998). Unlike *P. furiosus*, this organism can only use peptides as a carbon source. The gene in *P. horikoshii* encodes a protein that is homologous with both an endoglucanase from *Clostridium thermocellum* (based on sequence only) and a deblocking enzyme from *P. furiosus* was cloned and expressed in *E. coli* (Tsunasawa 1998; 2000). The *P. horikoshii* enzyme was found to only have aminopeptidase activity and cleaved N-blocked and non-blocked peptides such as N-acetyl-Ala-Ala-Ala and Ala-Ala-Ala, respectively (Ando et al. 1999). As purified, *P. horikoshii* aminopeptidase contained one calcium ion per subunit. Metal chelators, such as EDTA or o-phenanthroline, did not affect the activity of the enzyme indicating that the calcium ion is not involved in catalysis. Surprisingly, cobalt ions were needed for maximal catalytic activity but no Co^{2+} was found in the purified enzyme. It appears that the enzyme may contain a cobalt-binding site which participates in catalysis. One interesting property of the *P. horikoshii* enzyme is its ability to remove N-acetylated amino acid residues from peptides, although release of the product is slow based on kinetic studies (Ando et al. 1999). The biochemical and kinetic properties of the deblocking aminopeptidase have yet to be reported. The gene for the methionine aminopeptidase was cloned and

Table 5.2. Properties of aminoacylase from mesophilic, thermophilic, and hyperthermophilic sources.

Source	Sp. Act. in Cell Extract (U/mg)	Subunit MW (kDa)	Subunits per monomeric holoenzyme	Zn^{2+} (g atom/subunit)	References
Pig	9.0	43	2	1	(Chen et al. 1997)
Aspergillus oryzae	0.6	36	2	1	(Gentzen et al. 1980)
Alcaligenes denitrificans	0.1	40	2	2	(Yang et al. 1994)
Bacillus thermoglucosidius	0.2	43	4	1	(Cho 1987)
Bacillus stearothermophilus	1.9	45	4	1	(Sakanyan et al. 1993)
Pseudomonas maltophila	0.3	50	2	?	(Wakayama 1998)
P. furiosus	0.3	44	4	1	(Story et al. 2001)

overexpressed in *E. coli*. The enzyme contained two cobalt ions per subunit and it specifically removed methionine residues from the N-terminus of polypeptides.

A search of the *P. furiosus* genomic database revealed three homologs of the *P.horikoshii* aminopeptidase. These included the aforementioned deblocking aminopeptidase (Tsunaswa 1999); a probable endoglucanase (PF0369); and an ORF (PF1861) annotated as similar to endo-1,4, beta glucanase (Table 5.1). The recombinant forms of the latter two were obtained and assayed for activity to determine whether or not one or both had the acyl-aminopeptidase activity proposed in the pathway shown in Fig 2.7 (chapter 2). The genes were cloned separately and expressed in *Esherichia coli* and the recombinant proteins were purified using His-Tag affinity chromatography. These two enzymes belong to the same family of metallopeptidases (M18 family) based on sequence homology, but neither contained endoglucanase activity. Instead, both were found to remove N-terminal amino acid residues from peptide substrates. These two enzymes were distinct in their substrate specificities as one preferred substrates containing a lysine residue at the N-terminus and it was termed lysine aminopeptidase (KAP). The other enzyme was specific for alanine residues (termed AAP). Although it was highly similar (85% sequence identity) to the *P. horikoshii* enzyme, it did not remove N-acetyl groups from acetylated substrates (Table 4.2), like the enzyme from *P. horikoshii*.

The lysine aminopeptidase displayed the highest affinity for substrates containing lysine residues at the N-terminus. Kinetic analysis showed that the enzyme had a higher catalytic efficiency for the synthetic substrate, Lys-pNA as compared to the tripeptide Lys-Gly-Gly (Table 3.4). On the other hand, *P. furiosus* lysine aminopeptidase had the

highest affinity for Lys-Gly-Gly with a K_M value of 1.8 mM. Similar results were found for the recombinant form. These relatively high K_M values suggest that such substrates, if they are physiologically relevant, must be found at high intracellular concentrations. Activities for *P. furiosus* lysine aminopeptidase were also measured in extracts of cells grown under different growth conditions. No significant differences (Table 5.4) were seen indicating that KAP is not directly involved in either of the two metabolic pathways found in *P. furiosus*. The recombinant AAP was specific for substrates containing alanine at the N-terminus. It had a higher specificity for substrates that contained 3 or residues in length with the highest affinity for tri- and tetra-alanine. The catalytic efficiency was relatively low for this enzyme as compared to that of the *P. furiosus* KAP for its substrate. A comparison between the native and recombinant forms will have to be conducted in order to draw further conclusions on the kinetic and biochemical properties of this enzyme.

All three of the aminopeptidases that were characterized from *P. furiosus* were found to be zinc-containing enzymes. The aminoacylase, lysine aminopeptidase, and alanine aminopeptidase contained 1.0, 2.0, and 2.0 g-atoms zinc per subunit, respectively. There are several different motifs found in zinc enzymes and their sequences differ as it relates to catalytic or structural roles of the metal (Christianson 1991). The most highly conserved motif corresponds to the sequence HEXXH, in which X stands for any amino acid. Both of the histidine residues coordinate the same zinc atom, but the glutamate residue does not appear to be directly involved in catalysis. The three enzymes studied in this project do not contain this signature zinc-binding motif. They, therefore represent metallopeptidases which may define a new metal-binding

Table 5.3. Specific activities of aminoacylase in extracts of cells of *P. furiosus* grown under various conditions.

Growth Substrates	Specific Activity (mmoles/min/mg)
Maltose + Peptides	**1.53 ± 0.71**
Maltose	**1.81 ± 0.16**
Maltose + Peptides + S^o	**0.80 ± 0.16**
Maltose + S^o	**1.03 ± 0.78**
Peptides + S^o	**1.03 ± 0.55**

Adapted from Adams et al. 2001.

motif. However, structural interaction from crystallographic analyses must be obtained in order to support this theory.

P. furiosus aminoacylase belongs to the M20 family (ArgE/DAP/E/CPG2/YscS) of metallopeptidases. Enzymes from this family are known to contain either one or two zinc ions that are involved in catalysis (Table 5.1). The ligands involved in metal binding are unknown, although members of this family contain several conserved histidine residues which are possibly involved in metal binding. An alignment of *P. furiosus* aminoacylase with aminoacylases representing archaea, bacteria, and eukaryotes shows three amino acid motifs containing conserved residues (Fig. 5.1). The first motif, **x-x-R-A-D-x-D-A-L-x** (residues 115-125), contains two conserved aspartate residues which might be involved in the binding of metal ions, whereas the second one, **M-H-A-C-G-H-D** (residues 145-152), contains two conserved histidine residues, a conserved cysteine residue, and a conserved aspartate residue. The third motif contains two conserved glutamate residues, **F-Q-P-A-E-E** (residues 182-187). These residues are present in the archaeal and bacterial aminoacylases as well as in the homologs in two plants and in a cyanobacterium (*Synechocystis* sp.), but they are not in the mammalian aminoacylases (human and pig). Although structural analyses will be needed in order to further characterize the metal binding ligands in this family of enzymes, it is proposed that the archaeal, bacterial, and some eukaryal aminoacylases may belong to a metalloexopeptidase family that is quite distinct from those of higher mammals (human and pig aminoacylases).

The amino acid sequence analysis of human and pig aminoacylases were compared with those of several other enzymes including *Saccharomyces cerevisiae*

carboxypeptidase S precursor, succinyl-diaminopimelate desuccinylase from *Escherichia coli*, *Haemophilus influenzae*, and *Corynebacterium glutamicum*, the acetylornithine deacetylase from *Escherichia coli* and *Dictyostelium discoideum*, and *Pseudomonas* sp. carboxypeptidase G_2. All of these enzymes have similar functional and biochemical features (Biagini 2001), although the only one for which a crystal structure has been determined is the *Pseudomonas* carboxypeptidase G_2, which contains two zinc ions. Based on structural data, three metal binding motifs were identified and were largely conserved in the other eight homologs. The three amino acid motifs were: [S, G, A]-H-x-D-x-V; G-x-x-D; and x-E-E. These sequences are distinctly different from that commonly reported for enzymes belonging to the ArgE/DAP/E/CPG2/YscS family (Sakanyan 1993). Furthermore, the conserved sequence in the pig and human enzymes are not present in the *P. furiosus* aminoacylase and its homologs. Their conserved residues are quite different. These findings suggest that the aminoacylase from *P. furiosus* and related enzymes are only distantly related to the homologs of higher mammals, even though they have similar catalytic activities.

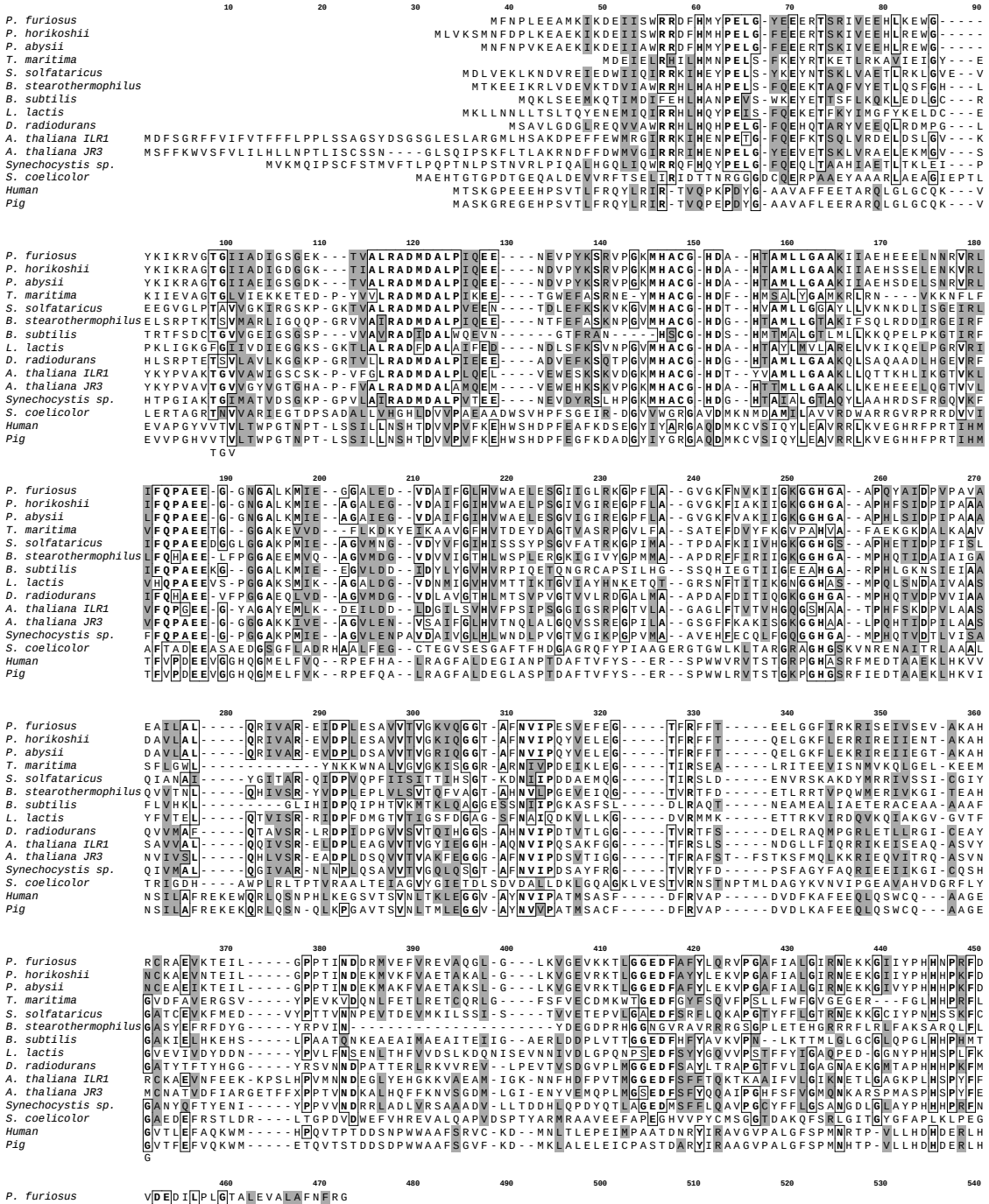
P. furiosus lysine and alanine aminopeptidases belong to the M18 family of metallopeptidases. Only two other members of this family have been isolated, the aminopeptidase I from *Saccharomyces cerevisiae* and an aspartyl aminopeptidase from rabbit (Wilk 1998). The yeast enzyme is a glycoprotein and has a broad substrate range (Metz 1976). It is strongly activated by zinc ions and is post-translationally modified during transport from the cytosol to the vacuole. The aspartyl aminopeptidase is very specific for peptides or proteins with an aspartate or glutamate residue at the N-terminus.

Table 5.4. Specific activities of lysine aminopeptidase in extracts of cells of *P. furiosus* grown under various conditions.

Growth Substrates	Specific Activity (mmoles/min/mg)
Maltose + Peptides	24.4
Maltose	21.5
Maltose + Peptides + S^o	15.7
Maltose + S^o	18.9
Peptides + S^o	22.7

Fig. 5.1.

Amino acid sequence alignment. Comparison of the amino acid sequence of *P. furiosus* aminoacylase with those of *P. horikoshii*, *P. abysii*, *T. maritima*, *S. solfataricus*, *B. stearothermophilus*, *B. subtilis*, *L. lactis*, *D. radiodurans*, *A. thaliana* ILR1, *A thaliana* JR3, *Syncechocystis* sp., *S. coelicolor*, human and pig. Boxed residues represent residues that are identical whereas the shaded regions represent similar residues.


P. furiosus
P. horikoshii
P. abysii
T. maritima
S. solfataricus
B. stearothermophilus
B. subtilis
L. lactis
D. radiodurans
A. thaliana ILR1
A. thaliana JR3
Synechocystis sp.
S. coelicolor
Human
Pig

To date, there are no structural data for the yeast or rabbit aminopeptidases. The *P. furiosus* lysine and alanine aminopeptidases have 22% similarity to both of these enzymes.

Based on sequence analyses, the lysine and alanine aminopeptidases of *P. furiosus* are also homologous to endoglucanases with more than 40% identity. Endoglucanases are enzymes which hydrolyze β-1, 4-glycosidic bonds within polysaccharide chains. A search of the genomes of *P. horikoshii*, and *P. abysii* reveals three homologs in each organism of the lysine and alanine aminopeptidases and of the deblocking aminopeptidase in each organism (Tsunasawa 1998). However, of these six homologs, only the deblocking aminopeptidase of *P. horikoshii* has been characterized (Ando 1999). Its genome also contains two genes annotated as Frv proteins. These are types of cellulases, but the information presented in Table 5.5 suggests that the products of these genes do not have such activity. The three corresponding genes in *P. abysii* have been annotated as aminopeptidases. Each enzyme appears to have a homolog in all three species (Table 5.5). For instance, the *P. furiosus* lysine aminopeptidase has 94% and 96% similarity to the *P. horikoshii* Frv protein, and that encoded by *P. abysii* gene 2096, respectively. On the other hand, the *P. furiosus* alanine aminopeptidase is highly similar to the *P.horikoshii* deblocking aminopeptidase and the *P. abysii* gene 2375. It is possible that this family of enzymes may have a bifunctional role serving as both endoglucanases and aminopeptidases, although this would seem unlikely. More biochemical and kinetic are needed in order to reveal their true physiological roles within these organisms.

Metal analyses of the both the native and recombinant forms of the lysine aminopeptidase of *P. furiosus* indicate that it contains two zinc ions per subunit. The

same results were obtained for the recombinant alanine aminopeptidase, although the native enzyme should be characterized before any conclusions can be drawn as there are reports in which recombinant proteins contain metals that differ from those found in the native enzyme (D'souza 2002). For example, the recombinant methionine aminopeptidases that have been crystallographically characterized from *E. coli* (Roderick and Matthews 1993; Lowther et al. 1999), *Homo sapiens* (Liu et al. 1998), and *P. furiosus* (Tahirov et al. 1998) show a dinuclear cobalt site. However, based on the results of studies of whole cell metal analyses for the *E. coli* methionine aminopeptidase, it was suggested that the in vivo metal for these enzymes was either Fe^{2+} or Mn^{2+} (D'Souza V and Holz 1999). Extensive metal analyses have shown that these enzymes are active in the presence of iron or manganese and are maximally stimulated upon addition of two equivalents of metal ion. However, the *in vivo* metal has yet to be determined. In order to establish whether or not the metal found in recombinant proteins is indeed the correct one, both native and recombinant forms of the enzyme must be isolated and their metal dependence must be determined. Such is the case for the native and recombinant forms of *P. furiosus* lysine aminopeptidase in which both forms contain two zinc ions per subunit. These findings provide evidence that zinc is most likely the naturally occurring metal in this enzyme. However, structural analyses will be required to determine the nature of the zinc sites in this enzyme.

Another interesting feature of the *P. furiosus* lysine and alanine aminopeptidases is their dependence on Co^{2+} ions for maximal activity whereas these ions cannot be replaced by Zn^{2+} ions or any other divalent cations. As mentioned above, metal analyses indicate that these are zinc-containing enzymes; therefore, at this point the role of cobalt

for activity is not clear. However, stimulation of activity or hyperactivity by cobalt has also been observed in some leucine aminopeptidases, enzymes that remove N-terminal leucine residues from polypeptides and proteins. Leucine aminopeptidase is a hexameric protein found in animals, plants and microorganisms (Kim and Lipscomb 1994). Structural and metal analyses of the bovine lens leucine aminopeptidase indicate that it contains two zinc ions at the active site (Kim and Lipscomb 1993). These two sites differ dramatically in their affinities for various divalent metal cations. In aqueous solution, one site (site 1) easily exchanges its bound zinc ion for such cations as Mn^{2+}, Mg^{2+}, Co^{2+}, whereas the second site (site 2) binds its zinc ion more tightly thereby making the metal less exchangeable. Furthermore, it has been noted that site 2 is more specific for Zn^{2+} than site 1. Interestingly, of the different divalent cations that can replace zinc in site 1, cobalt ions are the only cations that also bind stoichiometrically in site 2 (Thompson and Carpenter 1976). The same exchange of metals has also been observed in the two metal binding sites of the porcine kidney leucine aminopeptidase (Van Wart and Lin 1981). Furthermore, each metal substitution results in a change in the enzyme activity for both enzymes and structural analyses does not indicate which zinc ion is bound more tightly than the other.

Based on these facts, it seems likely that *P. furiosus* lysine and alanine aminopeptidases may bind Co^{2+} in a manner similar to that of leucine aminopeptidases. In other words, these enzymes contain a binuclear zinc site in vivo and this site remains intact upon purification. However, one of the zinc atoms is easily replaced by a Co^{2+} ion, leading to increased activity. Thus, these are really zinc metalloenzymes and it appears that the cobalt-effect may be an *in vitro* artifact. Of course, structural analyses of both

the native and Co-treated enzymes will have to be carried out in order to reveal the role of Co^{2+} ions in these enzymes and how Co^{2+} stimulates enzyme activities.

Table 5.5. % Identity of *P. furiosus*[a] aminopeptidases and homologs found in *P. horikoshii*[b] and *P. abysii*[b].

Organism/ Enzyme Name	Pfu DAP	Pfu AAP	Pfu KAP	Pho operon FrvX Protein	Pho operon FrvX Protein II	Pho AP	Pab AP 2096	Pab AP 0437	Pab AP 2375	Calc. Mol. Wt. (kDa)
Deblocking Aminopeptidase (DAP)	100									38.5
Alanine Aminopeptidase (AAP)	52	100								36.7
Lysine Aminopeptidase (KAP)	69	50	100							38.2
[operon FrvX Protein]	68	49	94	100						39.0
[operon FrvX Protein II]	95	52	67	66	100					39.0
Aminopeptidase	50	85	49	47	49	100				36.9
[*P. abysii* 2096]	69	50	96	94	68	48	100			38.1
[*P. abysii* 0437]	96	52	69	67	96	49	68	100		38.6
[*P. abysii* 2375]	50	87	49	49	68	91	49	49	100	36.6

[a] **http://comb5-156.umbi.umd.edu/genemate**
[b] **http://www.tigr.org/**

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