ROOPALI ROY
Tungsten-Containing Aldehyde Oxidoreductases: A Novel family of Enzymes from Hyperthermophilic Archaea
(Under the Direction of MICHAEL W. W. ADAMS)

The main objective of this research was the molecular, biochemical and kinetic characterization of the members of the AOR family of enzymes and attempt to elucidate the role these enzymes play in the physiology of *Pyrococcus furiosus*. Three different types of tungsten-containing aldehyde oxidizing enzymes aldehyde ferredoxin oxidoreductase (AOR), formaldehyde ferredoxin oxidoreductase (FOR) and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) have previously been isolated from this microorganism. Although *P. furiosus* AOR had been extensively characterized, relatively little was known about the related enzyme FOR. The molecular catalytic and structural properties of this enzyme are presented. Detailed kinetic analyses of FOR indicate that $C_4 - C_6$ dialdehyde or acid-substituted aldehyde may serve as the physiological substrate for this enzyme. Since such semialdehydes are involved in the metabolic pathways of certain amino acids, FOR is proposed to have a role in amino acid metabolism of *P. furiosus*.

The oxygen-inactivated forms of FOR, AOR and GAPOR from *P. furiosus* could be reactivated to 100% of original specific activity by incubation with sodium sulfide under reducing conditions. Loss of sulfide from the W coordination sphere presumably accounts for the loss in specific activity of these enzymes on exposure to oxygen. However, the exact nature of this 'restored' sulfide is not clear at this point. Although, *P. furiosus* cells grown with S° have 3-5 fold higher intracellular concentrations of acid-

labile sulfide and approximately 2-fold higher polysulfide than those grown without S°, these are associated with the high molecular weight fraction of the cell-free extract and not available in the free form. Based on these results, sulfide-activation does not appear to be a physiological reaction.

A survey of the complete genome sequences of numerous microorganisms reveals that tungstoenzymes of the AOR family appear to be widespread among the hyperthermophilic archaea and some bacteria. Two hitherto unknown members have also been identified in the *P. furiosus* genome. Termed *wor4* and *wor5*, these genes are proposed to encode putative tungstoenzymes. One of these tungstoproteins WOR 4 has been isolated from *P. furiosus* cell-free extract and characterized.

INDEX WORDS: Hyperthermophilic Archaea, *Pyrococcus furiosus*, Aldehyde Ferredoxin Oxidoreductase, Formaldehyde Ferredoxin Oxidoreductase, Glyceraldehyde-3-phosphate Ferredoxin Oxidoreductase, Aldehyde Oxidoreductase WOR 4, Tungsten, Tungstopterin, Thermal stability, Oxygen-inactivation, Sulfide-activation, Selenide-activation, Intracellular Sulfide.

TUNGSTEN-CONTAINING ALDEHYDE OXIDOREDUCTASES: A NOVEL FAMILY OF ENZYMES FROM HYPERTHERMOPHILIC ARCHAEA

by

ROOPALI ROY

B.S., Banaras Hindu University, India, 1988

M.S., Bombay University, India, 1991

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ROOPALI ROY

Approved:

Major Professor: Michael W.W. Adams

Committee: Harry Dailey
Michael Johnson
Lars Ljungdahl
Robert Scott

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
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List of Abbreviations

ADH – aldehyde dehydrogenase

AMP – adenosine 5’-monophosphate

AOR – aldehyde ferredoxin oxidoreductase

CAR – carboxylic acid reductase

DMSO – dimethylsulfoxide

EPPS – (*N*-[2-hydroxyethyl]piperazine-*N*’-[3-propanesulfonic acid])

FAD – flavin adenine dinucleotide

Fd – ferredoxin

FDH – formate dehydrogenase

FMDH – formyl methanofuran dehydrogenase

FOR – formaldehyde ferredoxin oxidoreductase

GAP – glyceraldehyde-3-phosphate

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GAPOR – glyceraldehyde-3-phosphate ferredoxin oxidoreductase

GMP – guanosine 5’-monophosphate

IMP – inosine 5’-monophosphate

MFR – methanofuran

NAD – nicotinamide guanine dinucleotid

NADP – nicotinamide adenine dinucleotide phosphate

NADPH – nicotinamide adenine dinucleotide phosphate (reduced)

ORF – open reading frame

PGK – phosphoglycerate kinase

CHAPTER 1

INTRODUCTION

Microorganisms that thrive near the boiling point of water were first isolated in the early 1980s through the pioneering efforts of Stetter and coworkers [Stetter, 1996]. Known as 'hyperthermophiles', these organisms have optimum growth temperatures of at least 80 °C with maximum growth temperatures of 90 °C and above. At present more than 20 different genera of hyperthermophiles are known, and these are listed on Table 1.1. The hyperthermophiles have been isolated from geothermally-heated ecosystems [Adams, 1993]. The majority are from marine habitats, in fact, of the 22 genera listed in Table 1.1, only four of them (*Thermoproteus*, *Thermofilum*, *Sulfophobococcus* and *Methanothermus*) have been found so far exclusively in fresh water environments. Terrestrial habitats include continental hot springs and solfataric fields whereas marine environments include shallow coastal waters and deep-sea hydrothermal vents. The deep-sea vents are formed during sub seafloor volcanic activity by the convective circulation of seawater through newly formed oceanic crust. The vent waters can reach temperatures approaching 400 °C and form so-called 'black smokers' that discharge jets of water blackened by metal sulfide precipitates [Jannasch and Mottl, 1985]. Microorganisms have been isolated both from the spreading plumes of hot water and from inside smoker 'chimneys' at temperatures well in excess of 100 °C. All of the marine hyperthermophiles require quite high salt concentrations to grow but some species of *Desulfurococcus* and *Pyrobaculum* have been isolated from both freshwater and marine ecosystems. Similarly, none of these organisms seem to be dramatically affected

by high hydrostatic pressures, even though such conditions prevail in deep-sea environments. In fact, representatives of marine genera such as *Pyrococcus* and *Pyrobaculum* have been found in both shallow and deep sea-vents [Stetter, 1996].

Two of the hyperthermophilic genera, *Aquifex* and *Thermotoga*, are bacteria; the rest are classified as Archaea (Table 1.1). Archaea constitute the third domain of life and were first recognized in the early 1980s based on 16S rRNA analyses. The pioneering work of C. R. Woese, led to a universal phylogenetic tree for the living world based on 16S rRNA homologous sequences [Woese et al, 1978, 1990]. From this tree three domains are evident: the Bacteria, the Archaea and the Eukarya (Fig.1.1). Based on 16S rRNA analyses, the hyperthermophilic species represent the most slowly evolving organisms within both the Archaea and Bacteria domains [Olsen et al., 1994, Stetter, 1996]. Therefore, it has been proposed that the original organisms on this planet may have first evolved under conditions of high temperature. Present day hyperthermophiles might be the key to understanding the biochemical evolution of the primary metabolic pathways found in the mesophilic organisms, and particularly those in higher life forms or Eucarya, with which the hyperthermophilic archaea have a common ancestor [Woese, 1990].

Phylogenetically the Archaea fall into two major kingdoms: the Euryarchaeota and the Crenarchaeaota. The former group is relatively heterogenous, comprising methanogens, extreme halophiles, sulfate-reducing species and two types of thermophiles (genus *Thermoplasma* and the *Thermococcus-Pyrococcus* group), whereas the Crenarchaeota are a physiologically homogenous group, comprising the relatively tight

clustering of extremely thermophililic archaea, including the *Sulfolobus-Thermoproteus* branch (Fig. 1.1) [Blochl et al., 1995, Woese et al., 1990].

1. Metabolism of Hyperthermophiles

The majority of hyperthermophilic archaea are obligate anaerobes. This property presumably reflects the low availability of oxygen in their natural habitats. In fact, aerobic species are found in only five genera, and three of them (*Pyrobaculum, Pyrolobus* and *Aquifex*) are microaerophiles (Table 1.1). These three genera are also capable of nitrate reduction, obtaining energy by respiratory-type metabolisms. In the absence of oxygen the major electron acceptors are elemental sulfur (S°), sulfate and protons, producing H_2S and H_2. Most of the hyperthermophiles fall into the 'S°-dependent category' as they metabolize S°, reducing it to H_2S. Many species are able to obtain energy for growth only by S° respiration [Stetter, 1996]. However, some species of *Pyrococcus* and *Thermococcus* can grow in the absence of S° by fermentative-type mechanisms (Table 1.1). Further, three recent isolates have been found to be 'S°-independent' as they are unable to utilize S°. These include *Sulfophobococcus* (Hensel et al., 1996) which grows by fermentation, *Aeropyrum* (Sako et al., 1996) which is an obligate aerobe and *Pyrolobus*, which obtains energy for growth by respiratory type metabolism using H_2 as electron donor and nitrate, thiosulfate or low concentration of oxygen as electron acceptors.

Table 1.1. The hyperthermophilic genera: organisms that grow at 90 °C*

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

Iron-oxidizing archaea

Ferroglobus (m)	95°	auto	Fe^{2+}, H_2, S_2^-	NO_3^-, $S_2O_3^{2-}$

Methanogenic archaea

Methanococcus (d/c)	91°	auto	H_2	CO_2
Methanothermus (c)	97°	auto	H_2	CO_2
Methanopyrus (d/m)	110°	auto	H_2	CO_2

Bacteria

Thermotoga (d/m)	90°	hetero	Pep, CBH	S°, H^+
Aquifex (m)	95°	auto	S° (H_2)	mO_2, NO_3^-

*Table adapted from Adams, 1999. Data taken from Kelly and Adams, 1994, Blochl et al., 1997, Sako et al, 1996, Jochinsen et al., 1997 and Hensel et al., 1997.

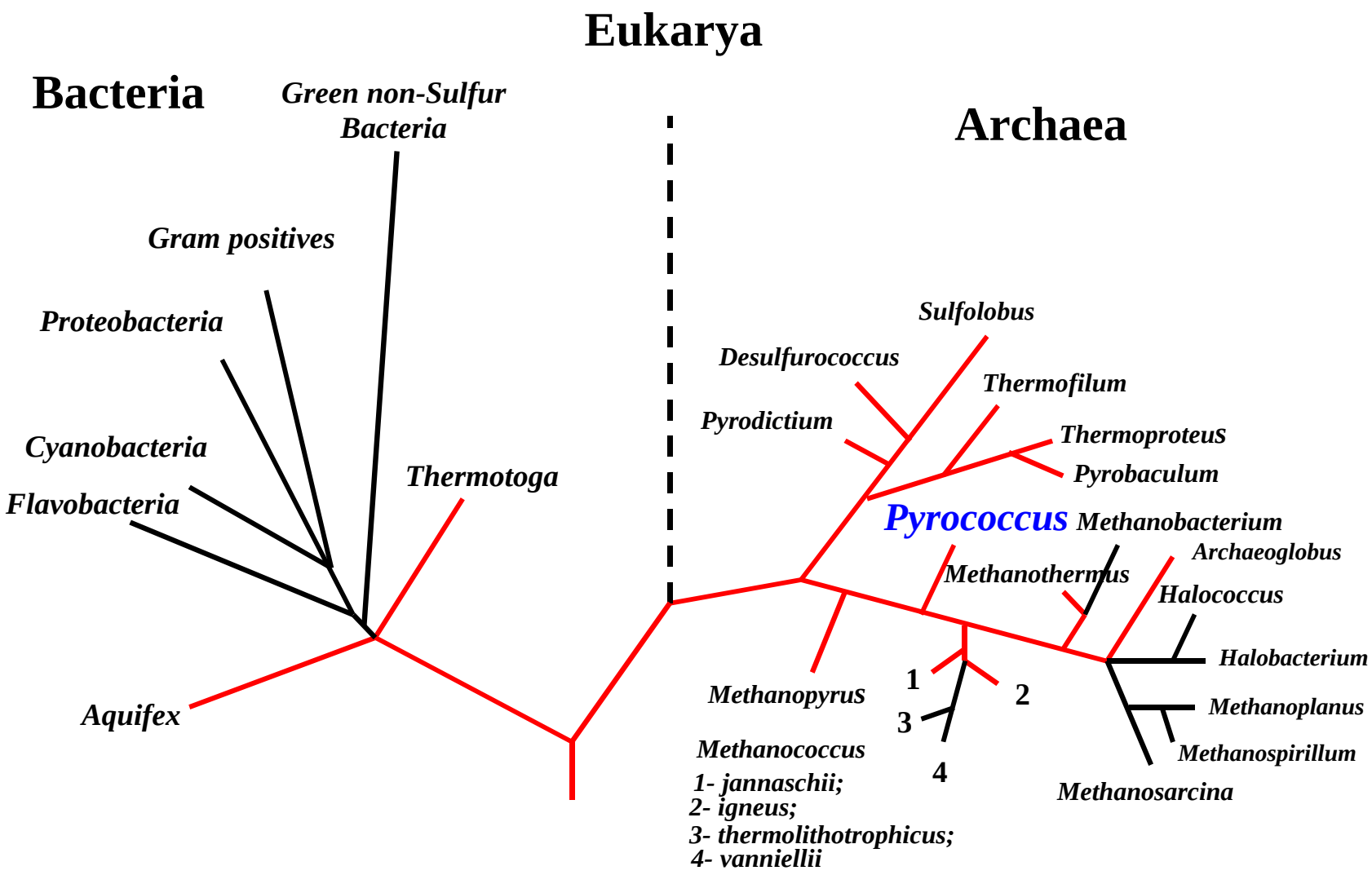
†Maximum growth temperature. ‡Indicates heterotrophic (hetero) or autotrophic (auto) growth mode.

§Growth substrates include peptides (Pep), carbohydrates (CBH), hydrogen (H_2), and elemental sulfur (S°) as electron donors.

¶Species have been found in continental hot springs (c), shallow marine (m) and/or deep sea (d) 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 position of the root was determined by comparing sequences of paralogous genes that diverged from each other before the three primary lineages emerged from their common ancestral condition. Red lines indicate 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
Aquifex
Sulfolobus
Desulfurococcus
Thermofilum
Pyrodictium
Thermoproteus
Pyrobaculum
Pyrococcus
Methanobacterium
Archaeoglobus
Methanothermus
Halococcus
Halobacterium
Methanopyrus
Methanoplanus
Methanospirillum
Methanosarcina
1
2
3
4
Methanococcus
1- jannaschii;
2- igneus;
3- thermolithotrophicus;
4- vanniellii

Remarkably *Pyrolobus fumarii* has an upper growth temperature of 113 °C, the highest yet reported and a significant fraction of exponentially growing cultures of this microorganism can even survive autoclaving (121 °C for 1 hour) [Blochl et al., 1997]. Methanogens comprise three genera of hyperthermophilic archaea (Table 1.1) which like their mesophilic counterparts, produce methane from H_2 and CO_2. Other genera that display alternative, S°-independent modes of metabolism are the sulfate-reducing *Archeoglobus* and the iron-oxidizing *Ferroglobus*. The bacterial species included in the list of hyperthermophiles differ considerably in their metabolic properties: *Thermotoga* species are all fermentative and reduce S° to H_2S but also grow well in its absence. The *Aquifex* species, on the other hand, are microaerophilic denitrifiers.

With the exception of a few autotrophic species, the hyperthermophiles known so far are obligate heterotrophs (Table 1.1). The predominant mode of growth for these organisms is via proteolysis of protein based substrates like yeast, bacterial or meat extracts, peptone and tryptone, all of which sustain good growth. However, their true growth substrates are not known. Although several species (*Pyrococcus, Thermococcus*) have been reported to grow on defined mixtures of amino acids [Watrin et al., 1995, Hoaki et al., 1994, Raven and Sharp, 1997], high cell yields are only obtained on complex media, suggesting that certain factors needed for growth are not provided for in mixtures of amino acids and vitamins [Rinker and Kelly, 1996]. Only a few of the heterotrophic species are saccharolytic. They are able to utilize complex carbohydrates such as starch and glycogen as well as simple disaccharides such as maltose and cellobiose. None of the hyperthermophilic archaea utilize monosaccharides, with the

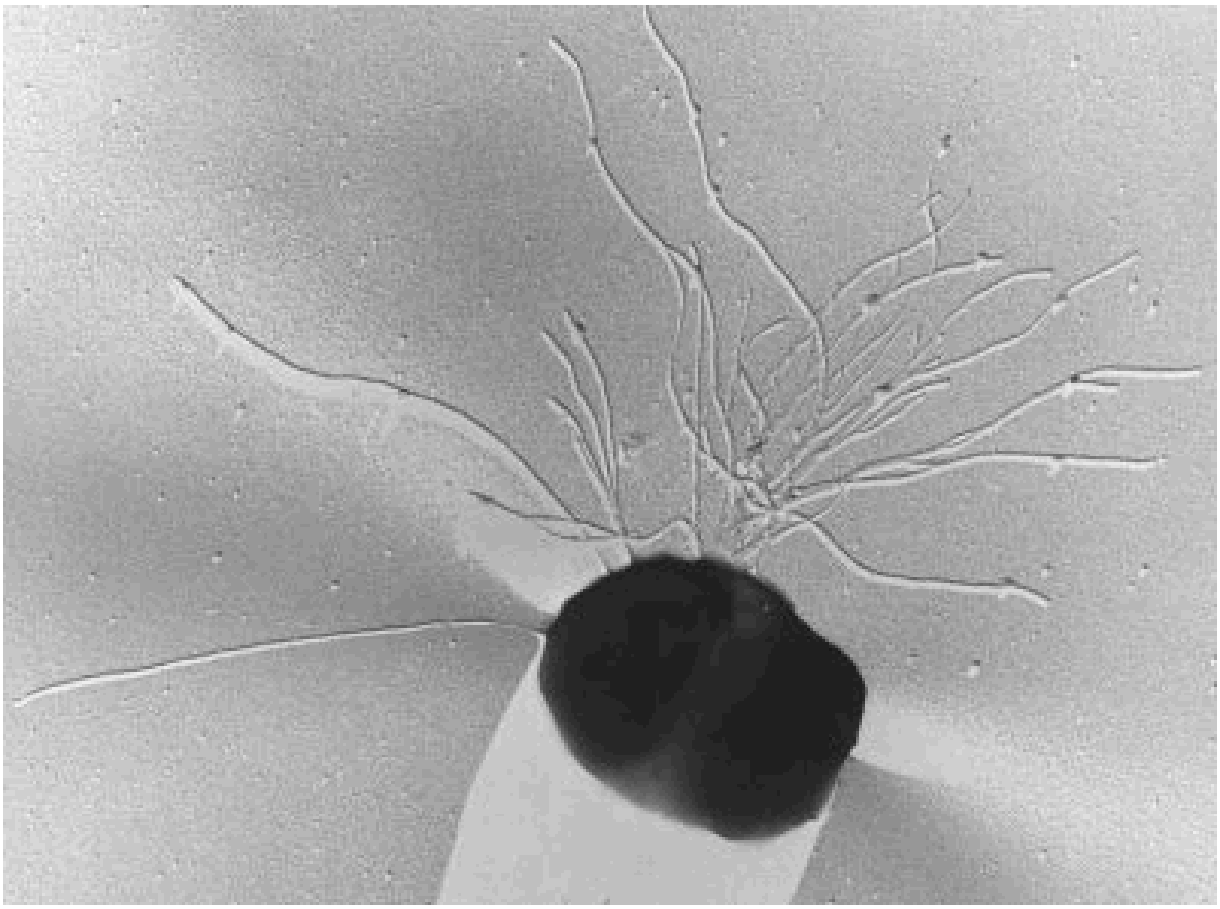
exception of *Thermoproteus*, which can grow using glucose as the sole carbon source. *Thermotoga*, the hyperthermophilic bacterial species grows well on monosaccharides.

In summary, most of the hyperthermophiles isolated so far are anaerobic heterotrophs that are either obligately or facultatively dependent upon S° for optimal growth. For the last decade or so, *Pyrococcus furiosus* has served as the model for the study of physiology and metabolism of hyperthermophilic archaea. *P. furiosus* has been the focus of studies for this research project and the following section provides a brief description of this organism and its mode of metabolism.

P. furiosus was isolated from geothermally-heated marine sediments off the coast of Vulcano, Italy by Stetter and coworkers in 1986 [Fiala and Stetter, 1986]. The organism is spherical shaped, 0.8 to 2.5 μm in width and has monopolar polytrichous flagellation (Fig. 1.2). Its optimal growth temperature is 100 °C at which it has a doubling time of 37 min. *P. furiosus* is a strictly anaerobic heterotroph. It grows fermentatively on complex media containing one or more of a variety of simple and complex saccharides as carbon source, including maltose, starch and cellobiose, and also pyruvate. Optimal growth also requires a source of peptides or proteins (added as yeast extract, tryptone or casein). The main fermentation products are acetate, CO_2, and H_2, and alanine is also produced at high partial pressures of H_2 [Kengen and Stams, 1994]. The organisms can grow with and without S° on sugars but growth on peptides requires S° [Adams et al., 2001]. H_2S is produced if S° is present and there is a comparable decrease in the amount of H_2 evolved. A number of studies have attempted to determine a defined growth medium for this organism [Raven and Sharp, 1997, Krahe et al., 1996, Hoaki et al., 1994].

Fig. 1.2.

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



However, to achieve high density cultures, complex media such as yeast extract or tryptone is essential [Adams et al., 2001]. In addition to the usual trace minerals such as Fe, Mg, Cu, Co, Mn and Zn, *P. furiosus* also requires the element tungsten for optimal growth. In 1989, it was discovered that tungsten, an element rarely used in biological systems, stimulates its growth [Bryant and Adams, 1989].

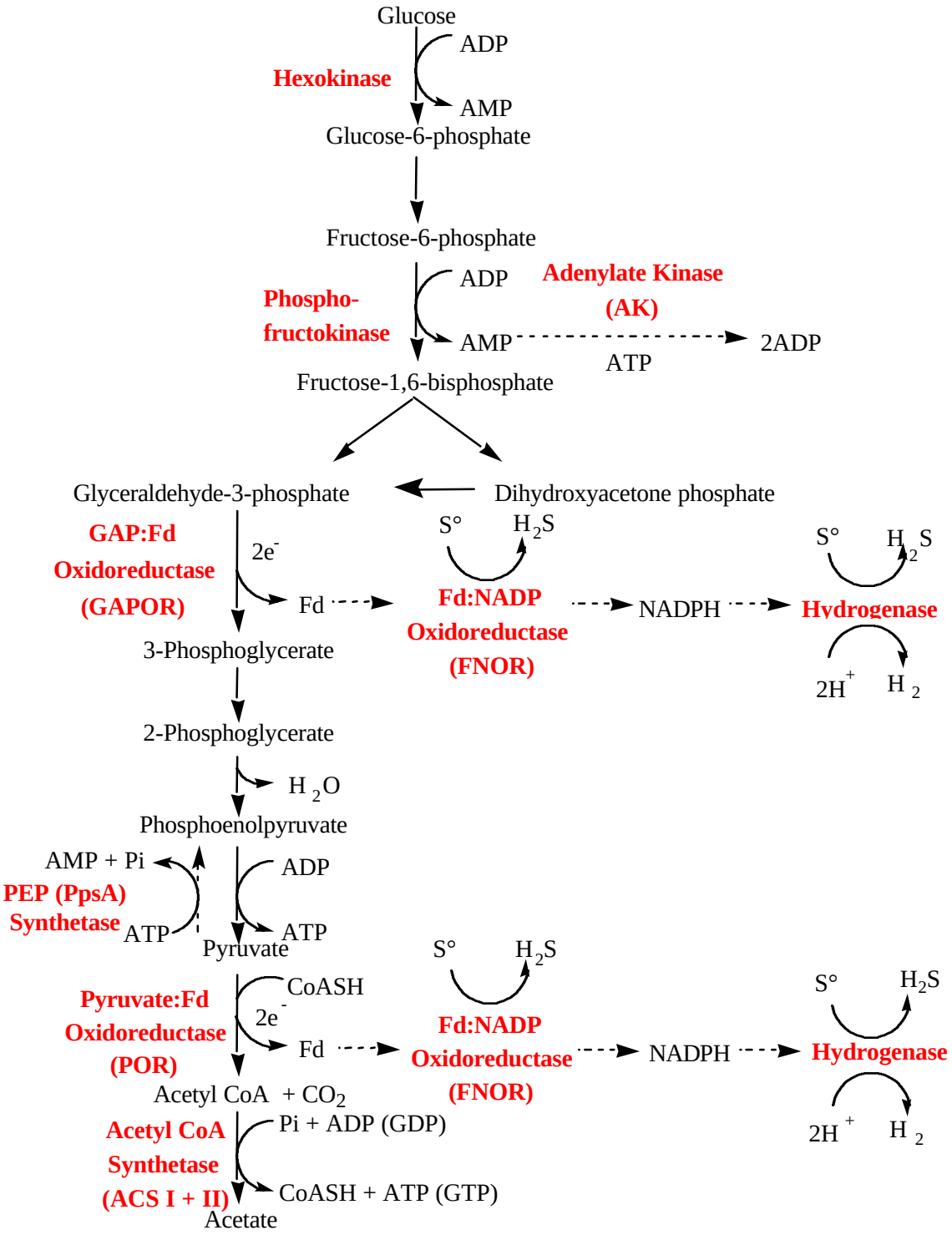
2. Major Metabolic Pathways of *Pyrococcus furiosus*

2.A. Carbohydrate Metabolism

P. furiosus grows on carbohydrates such as maltose, starch and glycogen, but not on monosaccharides. Several of the enzymes involved in their degradation to glucose have been characterized from this organism. It produces several inducible amylolytic enzymes both intracellularly and extracellularly [Sunna et al., 1997], and an intracellular glucosidase to further hydrolyze the products of the amylase reaction, to glucose. The pathway of sugar catabolism in *P. furiosus* has been investigated using ^{13}C NMR spectroscopy [Kengen et al., 1994]. The results demonstrated that the predominant route for sugar metabolism in this organism is an unusual Embden-Meyerhof rather than the modified Entner-Doudoroff type pathway proposed earlier [Schafer and Schonheit, 1992] (Fig. 1.3). However, cell-free extracts of *P. furiosus* have extremely low activity of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In 1995, Mukund and Adams reported that *P. furiosus* contained a new enzyme, glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) which could replace GAPDH in the Embden-Mayerhof pathway (Fig. 1.3).

Fig. 1.3.

Proposed glycolytic pathway in *Pyrococcus furiosus*. Metabolism of glucose to acetate, H_2 and H_2S is shown. Unusual enzymes are highlighted. Fd represents ferredoxin. The mechanism by which H_2S and H_2 are produced are discussed in the text. Modified from Mukund and Adams, 1995.


Glucose
ADP
Hexokinase
AMP
Glucose-6-phosphate
Fructose-6-phosphate
ADP
Adenylate Kinase
(AK)
Phospho-
fructokinase
AMP
2ADP
ATP
Fructose-1,6-bisphosphate
Glyceraldehyde-3-phosphate
Dihydroxyacetone phosphate
GAP:Fd
Oxidoreductase
(GAPOR)
2e-
Fd
S°
H2S
Fd:NADP
Oxidoreductase
(FNOR)
NADPH
S°
H2S
Hydrogenase
2H+
H2
3-Phosphoglycerate
2-Phosphoglycerate
H2O
Phosphoenolpyruvate
AMP + Pi
PEP (PpsA)
Synthetase
ATP
ADP
ATP
Pyruvate
Pyruvate:Fd
Oxidoreductase
(POR)
CoASH
2e-
Fd
S°
H2S
Fd:NADP
Oxidoreductase
(FNOR)
NADPH
S°
H2S
Hydrogenase
2H+
H2
Acetyl CoA + CO2
Acetyl CoA
Synthetase
(ACS I + II)
Pi + ADP (GDP)
CoASH + ATP (GTP)
Acetate

In contrast to GAPDH, GAPOR uses ferredoxin rather than NAD as the electron carrier, and it is proposed to produce 3-phosphoglycerate instead of 1,3-bisphosphoglycerate (the usual product of GAPDH reaction). Therefore, the modified pathway in *P. furiosus* lacks one of the energy conservation steps found in the conventional glycolytic pathway. This is consistent with the reported growth yield data from *P. furiosus* [Kengen et al., 1994].

Three other unusual enzymes are involved in the modified glycolytic pathway; these are (a) hexokinase (b) 2-phosphofructokinase, which are ADP- rather than ATP-dependent enzymes [Kengen et al., 1994], and (c) acetate-producing acetyl CoA synthetase, ACS I and ACS II [Mai and Adams, 1996]. Pyruvate ferredoxin oxidoreductase (POR) catalyzes the second redox step in the pathway (Fig. 1.3) and it produces acetyl CoA from pyruvate. Like GAPOR, it uses ferredoxin as its electron acceptor. Therefore, the excess reductant generated in these two redox steps is in the form of reduced ferredoxin rather than NAD(P)H. The oxidation of reduced ferredoxin is ultimately coupled to the production of H_2, or, if S° is present, to the production of H_2S. However, the pathways by which this occurs are not clear at present. The enzyme ferredoxin NADP oxidoreductase (FNOR) [Ma and Adams, 1994] catalyzes the reduction of NADP using ferredoxin as the electron donor and, *P. furiosus* has two cytoplasmic H_2-evolving hydrogenases, which use NADPH rather than ferredoxin as the electron donor. It was thought that these two enzymes channel electrons from NADPH to either protons or S° to produce either H_2 or H_2S [Ma et al., 1993, 2000]. However, the activities of the hydrogenases are dramatically decreased if S° is present in the growth medium and the nature of the enzyme that reduces S° is not known [Adams et al., 2001]. In addition, *P. furiosus* contains a H_2-evolving, membrane-bound hydrogenase complex, the function of

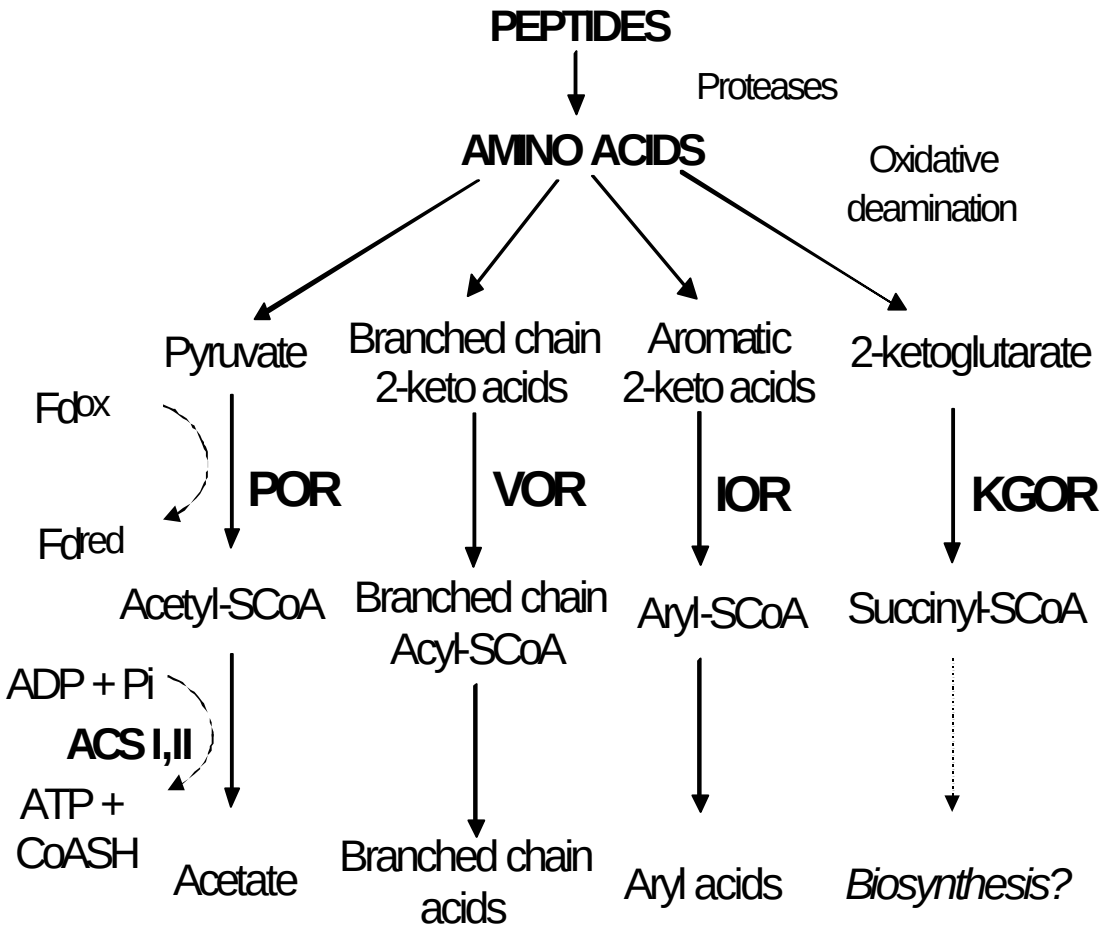
which is not clear at this point, although *in vitro* this enzyme does not reduce S° to H_2S [Sapra et al., 2000].

2.B. Peptide Metabolism

Most of the heterotrophic hyperthermophilic archaea use only peptide substrates as their source of carbon. The first step in the proteolytic pathway is the breakdown of peptides. *P. furiosus* has high intracellular as well as extracellular protease activities [Bauer et al., 1996]. Specific aliphatic and aromatic aminotransferases convert the amino acids generated by the action of proteases to 2-keto acids (Fig.1.4). *P. furiosus* contains four distinct 2-keto acid oxidoreductases to convert transaminated amino acids to their corresponding acyl CoA derivative. These are IOR (indolepyruvate oxidoreductase) which utilizes aromatic 2-keto acids, VOR (2-ketoisovalerate oxidoreductase) which oxidizes branched chain 2-keto acids, KGOR (2-ketoglutarate oxidoreductase) and POR, which oxidizes pyruvate. All the oxidoreductase reactions are coupled to ferredoxin reduction. VOR shows highest affinity for 2-ketoacids derived from valine, leucine and isoleucine, IOR oxidizes similar derivatives from phenylalanine, tyrosine and tryptophan, while KGOR and POR are very specific for 2-keto glutarate and pyruvate, respectively [Adams, 1999]. The acyl CoA intermediates generated by the 2-ketoacid oxidoreductases are further converted to acids with the concomitant production of ATP (Fig. 1.4). ACS I utilizes acetyl CoA and isobutyryl CoA as substrates, like products of the POR and VOR reactions, but it cannot use (aromatic) aryl CoAs. ACS II, on the other hand, can utilize acetyl CoA, branched chain acyl CoAs as well as aryl CoAs.

Fig. 1.4.

Proposed pathway for peptide fermentation from *Pyrococcus furiosus*. IOR, POR, KGOR and VOR represent ferredoxin-dependent oxidoreductases that utilize indolepyruvate, pyruvate, 2-ketoglutarate and 2-ketoisovalerate, respectively. ACS is acyl CoA synthetase and Fd_{ox} and Fd_{red} indicate the oxidized and reduced forms of ferredoxin respectively. Adapted from Mai and Adams, 1996.


PEPTIDES
Proteases
AMINO ACIDS
Oxidative deamination
Pyruvate
Branched chain 2-keto acids
Aromatic 2-keto acids
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
Branched chain acids
Aryl acids
Biosynthesis?

However, neither of these enzymes oxidize succinyl CoA, which is probably directed to biosynthetic pathways [Heider et al., 1996, Mai and Adams, 1996]. Thus, *P. furiosus* generates organic acids during growth on peptides. In fact, when this organism is grown on various combinations of sugars and peptides, the extent of peptide fermentation can be assessed by phenylacetate production in the culture medium and peptides appear to be preferred to sugars such as maltose [Adams et al., 2001].

In a recent detailed study of best growth substrates for *P. furiosus*, it has been reported that this organism can efficiently use peptides for growth only if S° is present in the medium [Adams et al., 2001]. Also growth rates were shown to be much higher with peptides and S°than on maltose (with or without S°). The metabolism of maltose by *P. furiosus* did not show a dependence on S°. Even though it is known that S° (or its metabolites) plays a significant regulatory role in peptide fermentation pathway of this organism, the exact mechanism for S° dependence is not understood at this point.

3. Role of the Transition Element Tungsten

Over the years enzymes containing tungsten (W) have been found to play key roles in the primary metabolic pathways of some hyperthermophilic archaea. Tungsten (W, atomic number 74) and the chemically analogous element molybdenum (Mo, atomic number 42) are both relatively scarce on this planet (Heydeman 1969, Pope 1978). Molybdoenzymes are ubiquitous in nature and virtually all life forms from microorganisms to eukaryotes contain Mo-containing enzymes. They play intimate roles in the global cycles of nitrogen, carbon and sulfur and have been intensively studied in the past 60 years or so (Hille, 1996). Conversely, with its high atomic number, W is an unlikely choice for a

metal with a biological function. Indeed, organisms prefer to use elements with atomic numbers below 35, with the exception of Mo (42) and iodine (53) [da Silva and Williams, 1991]. W belongs to the 5d series in the periodic table and has the electronic configuration $[Xe]4f^{14}5d^{4}6s^{2}$. Its chemical properties are very similar to that of Mo. The atomic and ionic radii of W and Mo, as well as their electron affinity, are virtually identical. In aqueous solution both elements are stabilized at higher oxidation states (IV – VI). The reduction potential for their (V/VI) and (IV/V) redox couples differ only slightly, being more negative for W. The most stable oxidation state for W is W(VI), although certain ligands can stabilize the lower states IV and V. Both W and Mo are known to form double, triple and quadruple metal-metal bonds, although this is probably not relevant in biological systems. Both W and Mo also occur naturally as a mixture of various isotopes. Radioactive isotopes suitable for biological research are available for both elements (^{185}W and ^{99}Mo) in addition to stable nuclear spin isotopes (^{183}W and ^{95}Mo) that are used for spectroscopic studies.

Traditionally W has been regarded as an antagonist of the biological function of Mo. Because the chemistry of W and Mo is so similar, microorganisms grown in the presence of W typically try to incorporate W into their molybdoenzymes. This results in either molybdoenzymes lacking any metal (and therefore any catalytic activity), or in inactive W-substituted molybdoenzymes. Thus, while a requirement for Mo in virtually all biological systems has been known since the 1930's, only in the last two decades has it been realized that W has a positive role in several different biological systems.

In the early 1970s it was reported that W stimulated the growth of certain acetate- and methane-producing microorganisms (Andreesen et al., 1974, Ljungdahl, 1976). A

decade later the first naturally occurring W-containing protein was purified from an acetogen (Yamamoto et al., 1983). Since then this field has grown and at present a dozen or so tungsten-containing enzymes are known. Tungstoenzymes have now been purified from a wide variety of microorganisms. These include hyperthermophilic archaea, which grow at temperatures near 100ºC, methanogens, gram-positive and negative bacteria, and acetylene-utilizing and sulfate-reducing bacteria. Of these, only the hyperthermophilic archaea appear to be obligately dependent on W for growth. All other organisms either have active Mo-isoforms of their 'tungstoenzymes', or express active Mo-substituted counterparts of their tungstoenzymes. So far, a biological role for W has not been recognized in any eukaryote.

The following section of the dissertation gives a brief description of the various classes of tungstoenzymes. This is followed by an overview of what was known about the AOR family of W-containing enzymes at the beginning of this research project.

To date, more than a dozen tungstoenzymes have been isolated and characterized (Table 1.2). The known tungstoenzymes can be classified into three functionally- and phylogenetically-distinct groups termed the AOR, F(M)DH and AH families. The AOR family is represented by aldehyde ferredoxin oxidoreductase (AOR) [Mukund and Adams, 1990, 1991], formaldehyde ferredoxin oxidoreductase (FOR) [Mukund and Adams, 1993] and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) [Mukund and Adams, 1995] from hyperthermophilic archaea, carboxylic acid reductase (CAR) from certain clostridia [White et al., 1989, 1991] and aldehyde dehydrogenase (ADH) from sulfate reducing-bacterium *Desulfovibrio gigas* [Hensgens et al., 1995]. The F(M)DH family includes formate dehydrogenase (FDH) from *Clostridium* species

[Yamamoto et al., 1983, Andreesen and Ljungdahl, 1973], *Methanococcus vannielii* [Jones and Stadtman, 1977] and *Desulfovibrio gigas* [Almendra et al., 1999] and formyl methanofuran dehydrogenase (FMDH) from the thermophilic methanogens, *Methanobacterium thermoautotrophicum* [Bertram et al., 1994] and *Methanobacterium wolfei* [Schmitz et al., 1992]. So far the AH family includes only acetylene hydratase from the acetylene-utilizing anaerobic bacterium *Pelobacter acetylenicus* [Rosner and Schink, 1995].

All of the known tungstoenzymes are listed in Table 1.2. These have been isolated and characterized from anaerobic organisms and most of them are extremely sensitive to inactivation by oxygen. These enzymes are located in the cytoplasm of cells in which they are found – none of them have been shown to be membrane-associated.

3. A. Tungsten enzymes and pterin

All tungsten-containing enzymes isolated so far have a mononuclear tungsten atom at the active site and contain one or more FeS clusters. The cofactor that coordinates the W atom in the tungstoenzymes is the same as that which coordinates the Mo atom in molybdoenzymes. This is an organic moiety known as pterin. The pterin cofactor was first identified by Johnson and Rajagopalan [1982], who isolated and characterized its bis(carboxyamidomethyl) derivative from molybdoenzymes by fluorescence and mass spectroscopy. At that time it was proposed that the pterin ring was a bicyclic structure with a side chain extending from C6 that contained dithiolene, hydroxyl and phosphate groups. The metal (Mo or W) was proposed to be coordinated to the cofactor through dithiolene sulfur atoms.

Table 1.2. Tungsten-containing enzymes.

Organism/enzyme[a]	Holoenzyme M_r (kDa)	Subunits/M_r(kDa)	W content[b]	FeS or cluster content[c]	Pterin cofactor[d]
AOR-family					
Pf AOR	136	α_2 (67)	2	2[Fe_4S_4] + 1Fe	Nonnuc
Pf FOR	280	α_4 (69)	4	4[Fe_4S_4]	Nonnuc
Pf GAPOR	73	α (73)	1	1[Fe_4S_4]	Nonnuc
Ct CAR (form I)	86	$\alpha\beta$ (64,14)	1	~29 Fe, ~25 S	Nonnuc
Ct CAR (form II)	300	$\alpha_3\beta_3\gamma$ (64,14,43)	3	~82 Fe, ~54 S	Nonnuc
Cf CAR	134	α_2 (67)	2	~11 Fe, ~16 S	Nonnuc
Dg ADH	132	α_2 (62)	2	2[Fe_4S_4]	NR
F(M)DH-family					
Ct FDH	340	$\alpha_2\beta_2$ (96,76)	2	20-40 Fe, 2 Se	NR
Mw FMDH	130	$\alpha\beta\gamma$ (64,51,35)	1	2-5 Fe	Nuc[e]

AH-family

Pa AH	73	α (73)	1	1[Fe_4S_4]	Nuc[e]

---

[a]Table modified from Adams and Kletzin, 1996. The abbreviations are : Pf (*Pyrococcus furiosus),* Ct (*C. thermoaceticum*), Cf (*C. formicoaceticum*), Dg (*Desulfovibrio gigas*), Mw (*Methanobacterium wolfei*), Pa (*Pelobacter acetylenicus*). Please see text for details.

[b]Expressed as integer value per mole of holoenzyme.

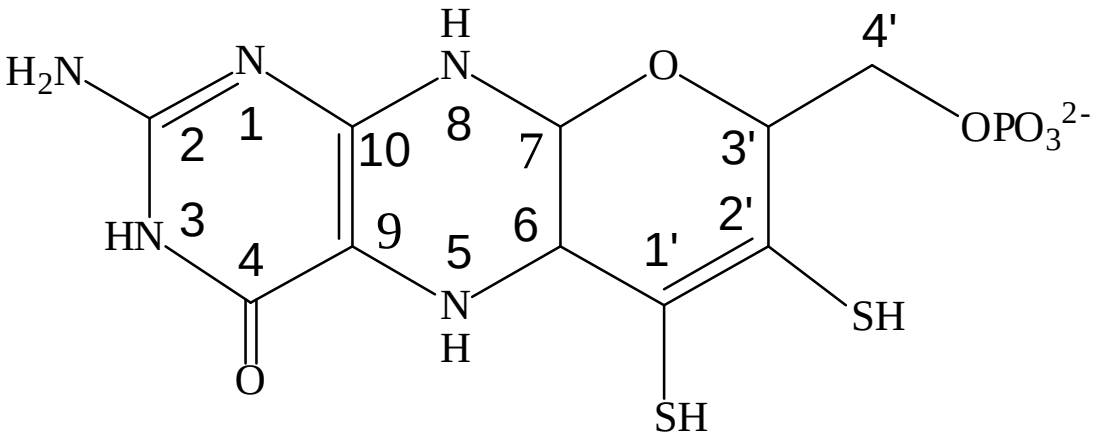
[c]Cluster content expressed per mole of holoenzyme.

[d]Indicates whether the pterin is with (Nuc) or without (Nonnuc) an appended nucleotide. NR, not reported.

[e]Appended nucleotide is GMP.

Fig. 1.5.

Structure of the pterin cofactor. Tautomeric form of the pterin cofactor that coordinates the W (Mo) atom in *P. furiosus* AOR as well as other tungsten- and molybdenum-containing enzymes. The atom numbering scheme of the pterin is indicated.


H2N
N
1
2
HN
3
4
O
10
9
H
N
8
N
H
5
7
6
O
4'
3'
2'
1'
OPO3 2-
SH
SH

The structure of the pterin cofactor was first revealed in 1995 by the crystal structure of *P. furiosus* AOR solved at 2.3 Å (Chan et al., 1995). This confirmed the proposed structure with the exception that the cofactor contained a third ring formed by the closure of the side chain hydroxyl with the pterin ring C7 (Fig. 1.5). This forms a non-planar ring structure that binds W via the dithiolene sulfur side chains. Indeed, one of the most surprising features of the AOR structure was that the W atom is coordinated by two dithiolene side chains from two pterins, giving rise to the term bispterin cofactor (see below). Subsequent crystal structures of molybdoenzymes have shown that the pterin cofactor in the two types of enzymes are the same (Romao et al., 1995, Schindelin et al., 1996). The pterin is not covalently attached to any side chain of the protein. Analyses of the cofactor after extraction from the protein has shown that in some tungstoenzymes from bacteria the pterin is usually modified with a mononucleotide (AMP, GMP, CMP or IMP) attached to the terminal phosphate.

The biosynthesis of the pterin cofactor in several organisms has been investigated, both from the standpoint of establishing genes involved in the biosynthetic pathway and the chemical nature of precursors [Rajagopalan and Johnson, 1992, Hille, 1996,].

The role of the pterin cofactor in the reaction mechanisms of mononuclear molybdenum (as well as tungsten) enzymes is not clear at this point. Considering the type of redox reactions catalyzed by these enzymes, it is probable that the pterin cofactor plays an active role in catalysis. However, the stoichiometry of reducing equivalents taken up by these enzymes in the course of catalysis are quantitatively accounted for by their redox-active cofactors, therefore, it is unlikely that the pterin cofactor is itself reversibly reduced in the course of catalysis. On the basis of the crystal structure of *P.*

furiosus AOR (and molybdoenzymes such as *D. gigas* aldehyde oxidoreductase and *R. sphaeroi*des DMSO reductase), two specific roles become clear for the cofactor: mediation of electron transfer to the other redox-active centers and modulation of the W/Mo reduction potential. Remarkably, in *P. furiosus* AOR the amino group of the pterin cofactor is hydrogen bonded to one of the cysteine ligands of a [4Fe-4S] center that is also present in the enzyme. It is thus extremely likely that pterin mediates electron transfer out of the W center of the enzyme in the course of turnover, although not in such a way that it is itself irreversibly reduced.

4. Classification of Tungstoenzymes

4.A. *The AOR family*

The majority of the known tungstoenzymes belong to this family (Table 1.2). Named after the most extensively-studied example of a tungstoenzyme, aldehyde ferredoxin oxidoreductase (AOR) from the hyperthermophilic archaeon *Pyrococcus furiosus*, this family has additional members from both hyperthermophilic (FOR, GAPOR) and mesophilic microorganisms (CAR and ADH). The enzymes in this family are related phylogenetically, and display high sequence similarity at the amino acid level. AOR, FOR and GAPOR are most closely related. For example, AOR and FOR have 40% sequence identity with each other whereas GAPOR is more distantly related with 23% identity with AOR or FOR. However, none of the enzymes of the AOR family show any sequence similarity to any known molybdoenzyme showing that they are phylogenetically distinct.

As the name implies, the enzymes of the AOR family catalyze the oxidation of various types of aldehydes to the corresponding acids according to Eq. 1, where RCHO represents the substrate with the aldehyde functional group and Fd(ox) and Fd(red) represent oxidized and reduced forms of the electron acceptor ferredoxin (Fd), respectively (also see Table 1.3).

$$RCHO + H_2O + 2Fd(ox) \rightarrow RCOOH + 2H^+ + 2Fd(red) \qquad \text{(Eq. 1)}$$

Aldehyde oxidation is a two electron reaction and *in vivo* the redox protein Fd serves as the electron acceptor for AOR, FOR and GAPOR. The physiological electron mediators for ADH and CAR are not known. Although the enzymes in this family catalyze the same type of reaction, they differ in their substrate specificities. In fact, CAR was first isolated based on its ability to catalyze the reductive activation of carboxylic acids (the reverse of the reaction shown in Eq. 1) although it can also carry out aldehyde oxidation. The acid/aldehyde couple has a very low reduction potential, one of the lowest of any biological system. For instance, the E_0' value for the acetaldehyde/acetate couple is –580mV (SHE). Consequently, under biological conditions aldehyde oxidation is much more thermodynamically favorable than acid reduction [Adams and Kletzin, 1996].

With the exception of CAR, the enzymes of the AOR family are comprised of a single type of subunit that contains a mononuclear W site and one FeS cluster. The only other known cofactor is a monomeric Fe site (found in the AOR of *P. furiosus*). On the other hand, CAR is a more complex enzyme with two or more types of subunit, a higher Fe content and it contains flavin as an additional cofactor (Table 1.2).

Table 1.3. Catalytic properties of tungstoenzymes.

Enzyme/source[a]	Reaction catalyzed	K_m (mM)	V_{max}[b]
AOR(*P. furiosus*)	$RCHO^{c} + H_2O + Fd^{d}(ox) \rightarrow RCOOH + 2H^+ + Fd(red)$	00.04[e]	67(80°C)
FOR(*P. furiosus*)	$HCHO^{f} + H_2O + Fd(ox) \rightarrow HCOOH + 2H^+ + Fd(red)$	25.00[g]	62(80°C)
GAPOR(*P. furiosus*)	$R'CHO^{h} + H_2O + Fd(ox) \rightarrow R'COOH + 2H^+ + Fd(red)$	00.03	350(70°C)
CAR (*C. formicoaceticum*)	$RCHO + MV^{++i} + OH^- \leftrightarrow RCOO^- + 2H^+ + MV^+$	00.14[j]	500(40°C)
ADH(*D. gigas*)	$RCHO + BV^{++k} + H_2O \rightarrow RCOOH + 2H^+ + BV^+$	00.01[l]	38(30°C)
FDH (*C. thermoaceticum*)	$CO_2 + NADPH + H^+ \rightarrow HCOOH + NADP^+$	00.11	880(55°C)
FMDH(*M. wolfei*)	$CO_2 + MFR^{m} + 2H^+ \rightarrow CHO\text{-}MFR + H_2O$	00.01	11(65°C)
AH(*P. acetylenicus*)	$C_2H_2 + H_2O \rightarrow CH_3CHO$	00.01	69(30°C)

[a]For source of data see Table 1.2. [b]μmole of substrate utilized min^{-1} mg^{-1} of protein at the indicated temperature. [c]R = H, C1-C4, Ar. [d]ferredoxin. [e]For crotonaldehyde.

[f]R= H, C_1-C_3. [g]For formaldehyde.

[h]R' = $CHOHCH_2OPO_3^{2-}$. [i]Methyl viologen.

[j]For butyraldehyde. [k] Benzyl viologen.

[l]For acetaldehyde.

[m]N-formylmethanofuran.

The AOR-type enzymes do differ in their quaternary structures, for example, GAPOR is a monomer, AOR and ADH are dimers, and FOR is a tetramer. Amongst the tungstoenzymes, AOR, FOR and CAR have the non-nucleotide form of pterin whereas the F(M)DH and AH enzyme families have the modified dinucleotide form.

4.B. *The F(M)DH family*

This family consists of two types of enzymes, both of which use CO_2 as substrate. FDH catalyzes the reversible reduction of CO_2 to formate with the concomitant transfer of two electrons from the appropriate electron carrier, usually NADPH, according to Eq.2.

$$CO_2 + NADPH + H^+ \rightarrow HCOOH + NADP^+ \qquad \text{(Eq. 2)}$$

Typically, FDH obtained from anaerobic organisms is a molybdoenzyme, but in recent years growth studies and biochemical analyses indicate that several types of bacteria and methanogens may have W-containing FDHs [Burke et al., 1980, Girio et al., 1998]. Indeed, the first tungstoenzyme to be isolated and characterized was the W-containing (W-)FDH from the thermophilic acetogenic bacterium, *Clostridium thermoaceticum* [Yamamoto et al., 1983]. However, this organism also contains a Mo-containing FDH. W-FDH from *C. thermoaceticum* is a heterotetramer with two distinct types of subunits (Table 1.2). In addition to the W and FeS centers, this enzyme contains the unusual amino acid selenocysteine (analogous to cysteine but with selenium replacing the sulfur). Both Mo- and W-containing FDHs are reported to be extremely sensitive to oxygen-

inactivation, making it difficult to isolate and characterize them. However, recently the isolation of an air-stable FDH has been reported from the sulfate reducing bacterium *Desulfovibrio gigas* [Almendra et al., 1999]. Like the enzyme from *C. thermoaceticum*, this FDH has multiple types of subunits, it is heterodimeric and has mononuclear W and two [4Fe-4S] clusters per protein molecule. The enzyme was isolated in the presence of oxygen, although activity could be measured only under strictly anoxic conditions. Also, unlike the clostridial FDH, Se was not detected in the *D. gigas* enzyme. The W atom at the active site of the enzyme is coordinated by two pterin cofactors, which are of the dinucleotide form (MGD). It catalyzes the reversible reduction of CO_2 to formate (Table 1.3), which also involves the transfer of two electrons from the appropriate electron carrier. This reaction has a very negative reduction potential (-420 mV, SHE).

Like FDH, the other member of this class of tungstoenzyme, FMDH, is typically a molybdoenzyme. FMDH is found only in the methanogens where it catalyzes the reduction and addition of CO_2 to the organic, methanogenic cofactor, methanofuran (Table 1.3). Methanogens grow on H_2 and CO_2 but, unlike acetogens where the product is acetate, they generate methane. As outlined in Eq.3, FMDH catalyzes the first step in the conversion of CO_2 to methane, where the cofactor methanofuran (MFR^+) is the other substrate. The physiological electron donor for this reaction is not known.

$$CO_2 + MFR^+ + H^+ + 2e^- \Longleftrightarrow CHO\text{-}MFR + H_2O \qquad \text{(Eq.3)}$$

FMDH is usually a molybdoenzyme but in several thermophilic and hyperthermophilic methanogens the enzyme contains W. In fact, *Methanobacterium*

thermoautotrophicum and *M. wolfei* each contain two isoforms of FMDH, one Mo- and one W-containing [Hochheimer et al., 1998]. These are complex multi-subunit enzymes (Table 1.2) that are highly sensitive to oxygen and intrinsically-labile during purification, making it difficult to correctly assess their cofactor contents.

Organisms that exhibit W-dependence for growth do not usually contain W-substituted versions of their molybdoenzymes. Rather, the W-containing FDHs and FMDHs are encoded by specific genes, distinct from the genes that encode the Mo-containing enzymes. However, these tungstoenzymes do show amino acid sequence similarity to their Mo-containing counterparts and it is clear that they are closely related in evolutionary terms. In contrast, the AOR of family of tungstoenzymes discussed above, while showing a high degree of molecular and sequence similarity to each other, show no sequence similarity to the F(M)DH family nor to any class of molybdoenzyme. Therefore, the two main groups of tungstoenzyme diverged very early on the evolutionary timescale, and only one of them shows any relationship to molybdoenzymes.

4.C. *The AH family*

This family of tungstoenzyme is represented by a single member. Isolated from the acetylene-utilizing anaerobic bacterium *Pelobacter acetylenicus*, AH consists of a single type of subunit that contains one W atom and a single FeS cluster (Table 1.2) [Rosner and Schink, 1995, Meckenstock et al., 1999]. The enzyme catalyzes the hydration of acetylene to acetaldehyde, according to Eq. 4.

$$C_2H_2 + H_2O \rightarrow CH_3CHO \qquad \text{(Eq. 4)}$$

Therefore, unlike the other tungstoenzymes, AH does not seem to carry out an overall oxidation or reduction reaction but it is active only in the presence of a strong reducing agent. It therefore seems likely that the tungsten atom and FeS center carry out consecutive reduction, hydration and oxidation reactions. Furthermore, a Mo-containing form of AH has been isolated from *P. acetylenicus* grown in the presence of Mo. The Mo-containing enzyme is fully-active indicating that both W and Mo can catalyze the hydration reaction. An active AH has also been found in an aerobic acetylene-degrading bacterium [Rosner et al., 1997]. However, the expression of the enzyme is molybdenum-dependent, and activity does not require addition of a strong reductant. Therefore, this type of AH appears to be biochemically distinct from the W-containing AH isolated from anaerobic bacteria.

5. Enzymes of the AOR family

5.A. ALDEHYDE FERREDOXIN OXIDOREDUCTASE (AOR)

Molecular properties: AOR has so far been found only in hyperthermophilic archaea and AOR-type enzymes have not yet been reported from mesophiles. AOR was the first W-containing enzyme to be purified from the hyperthermophilic archaeon *P. furiosus*, and it serves as the most extensively studied example amongst all the tungstoenzymes [Mukund and Adams, 1991, Chan et al., 1995]. The enzyme has also been purified and characterized from two other hyperthermophilic archaea *Pyrococcus* strain ES4 [Ma et al., 1996] and *Thermococcus* strain ES1 [Heider et al., 1995]. As discussed in the

preceeding section, *P. furiosus* is an anaerobic heterotroph that grows on both simple and complex carbohydrates as well as peptides (yeast extract, peptone, casein) at temperatures near 100°C. In contrast**,** *Thermococcus* strain ES1 and *Pyrococcus* strain ES4 only use peptides as a carbon source and they require S° for growth, which is reduced to H_2S. AOR is extremely oxygen-sensitive and occurs in the cytoplasmic fraction of the cell. It is an abundant protein, for example, in T*hermococcus* strain ES1 it constitutes approximately 1% of the total protein content in the cell [Heider et al., 1995] and the intracellular concentration of this protein in *P. furiosus* is about 110 μM. Based on sequence comparisons, homologs of AOR are present in the genomes of several other hyperthermophilic archaea including *Pyrococcus horikoshii* [Kawarabayasi et al., 1998], *Archaeoglobus fulgidus* [Klenk et al., 1997] and *Pyrobaculum aerophilum* [Fitz-Gibbon et al., 1997]. However, this is based solely on similarity at the amino acid sequence level and whether the enzymes encoded by the predicted ORFs in these organisms really are AOR-type enzymes must await their isolation and characterization.

Metal content and cofactors: Elemental analyses of pure preparations of AOR from *P. furiosus* [Mukund and Adams, 1991] and *Thermococcus* ES1 [Heider et al., 1995], by inductively coupled plasma emission spectroscopy and colorimetric assays reveal the presence (in g-atom per subunit) of approximately 4 Fe, 1 W, 4 S, 1 Mg and 2 P. The only other metals detected in significant amounts (> 0.1 g-atom/subunit) are Ca and Zn, present at approximately 0.7 and 0.2 g-atom/subunit, respectively. The presence of an organic, pterin-type cofactor in AOR was demonstrated by extracting the enzyme in the presence of iodine and measuring the fluorescence spectra of the resulting bis-

(carboxyamidomethyl) pterin derivative. Similar spectra have been reported for all molybdenum-containing enzymes that have been examined (with the exception of nitrogenase) [Johnson and Rajagopalan, 1982]. The structure of the pterin cofactor found in AOR is shown in Figure 1.5. As discussed below, the tungsten atom of AOR is coordinated to the cofactor through the dithiolene sulfurs. The other cofactor in AOR is a [4Fe-4S] cluster present in each subunit of the enzyme, together with a single metal atom, presumed to be iron, that bridges the two subunits of the homodimer.

Catalytic properties and physiological role: AOR oxidizes a broad range of both aliphatic and aromatic aldehydes to their corresponding acids. This is a two-electron oxidation, and under physiological conditions Fd serves as the electron acceptor. The Fd of *P. furiosus* contains a single [4Fe-4S] cluster and this undergoes a one-electron redox reaction. Consequently, assuming each W-containing subunit of AOR and also FOR and GAPOR, functions independently, one catalytic turnover per subunit requires the reduction of two molecules of Fd. It is proposed that during the reaction each Fd molecule is reduced sequentially rather than at the same time.

Although AOR has the ability to oxidize a wide range of aldehyde substrates, the enzyme shows the highest catalytic efficiency (K_{cat}/K_m) with acetaldehyde, isovaleraldehyde, indoleacetaldehyde and phenylacetaldehyde. The K_m values are less than 100µM (Table 1.4) [Mukund and Adams, 1991, Heider et al., 1995], although higher concentrations (in the mM range) inhibit the enzyme. These aldehyde substrates are derivatives of the common amino acids such as alanine, valine, tryptophan and phenylalanine. These are produced when *P. furiosus* grows on peptides and are by-

products of keto acid oxidoreductases (POR, IOR, VOR and KGOR) which oxidize pyruvate, branched-chain 2-keto acids and aromatic 2-keto acids to their coenzyme A derivative. A fraction of the keto acid substrates are converted by non-oxidative reaction to the corresponding aldehyde [Ma et al., 1997]. Inside the cell, it is thought that AOR functions to oxidize the aldehydes derived from pyruvate (when *P. furiosus* grows on carbohydrates) or aldehydes generated during amino acid oxidation in peptide metabolism (in proteolytic organisms) (see Fig. 1.6). However, metabolic labelling studies are needed to confirm the proposed role of AOR in the peptide fermentation pathway of *P. furiosus*.

Structure: The crystal structure of *P. furiosus* AOR, resolved at 2.3 Å resolution, was the first for a tungsten-containing protein and for a pterin-containing protein (and the first for an enzyme from an organism that can grow at 100°C [Chan et al., 1995]). AOR is a homodimer where each subunit (M_r ~ 66 kDa) contains one mononuclear W atom and a [4Fe-4S] cluster in close proximity. The X-ray structure of *P. furiosus* AOR revealed a mononuclear metal site, most likely Fe, positioned at the dimer interface (Fig 1.7). However, this site is situated more than 20 Å from the W and probably has a purely structural function.

Each subunit of AOR comprises three domains, with binding sites for the tungstopterin cofactor and [4Fe-4S] cluster located at the interface of these domains. The tungsten ion in AOR is symmetrically coordinated by the four dithiolene sulfurs from the two pterins bound to each subunit (Fig. 1.8).

Table 1.4. Substrate specificity of AOR.

Substrate[a]	Apparent K_m (mM)	$k_{cat}(s^{-1})$	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Formaldehyde	1.42	950	0.7
Acetaldehyde	0.02	343	22.0
Propionaldehyde	0.15	1,100	7.5
Crotonaldehyde	0.14	269	2.0
Benzaldehyde	0.06	720	13.0
Isovaleraldehyde	0.03	272	10.0
Phenylacetaldehyde	0.08	960	13.0
Indoleacetaldehyde	0.05	55	1.1
Salicylaldehyde	0.06	13	0.2
Glyceraldehyde[b]	0.20	3	0.01

[a]Reactions were carried out using *Thermococcus* strain ES1 AOR at 80°C in 100mM EPPS buffer (pH 8.4) with benzyl viologen (1.6 mM) as the electron acceptor.

[b]Assayed at 45°C.

Fig. 1.6.

Proposed physiological role of AOR in the catabolism of glucose and amino acids.

Taken from Heider et al., 1995 and Ma et al., 1997.

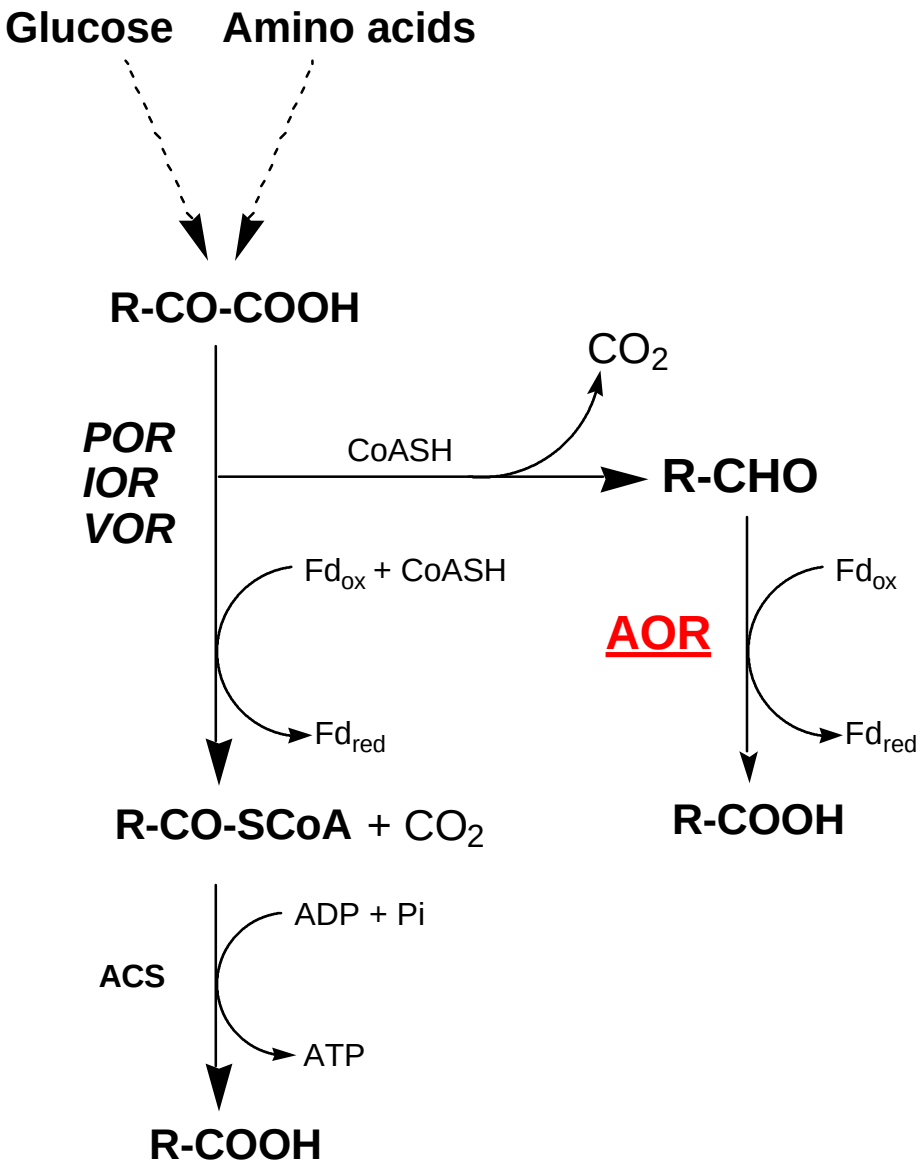

Glucose
Amino acids
R-CO-COOH
POR
IOR
VOR
CO2
CoASH
R-CHO
Fdox + CoASH
Fdred
AOR
Fdox
Fdred
R-CO-SCoA + CO2
R-COOH
ADP + Pi
ACS
ATP
R-COOH

Fig. 1.7.

Schematic representation of the AOR dimer. The two subunits are shown with the associated metallocenters. The secondary structures of each subunit are color coded with α–helices, β-sheet and 'coil' structures represented with cyan, red and green, respectively. Adapted from Chan et al., 1995.

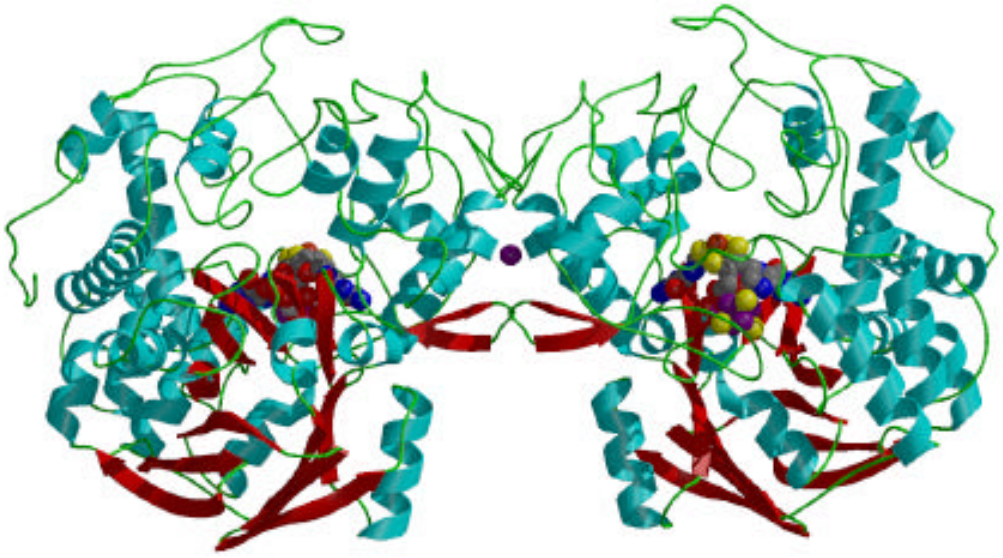
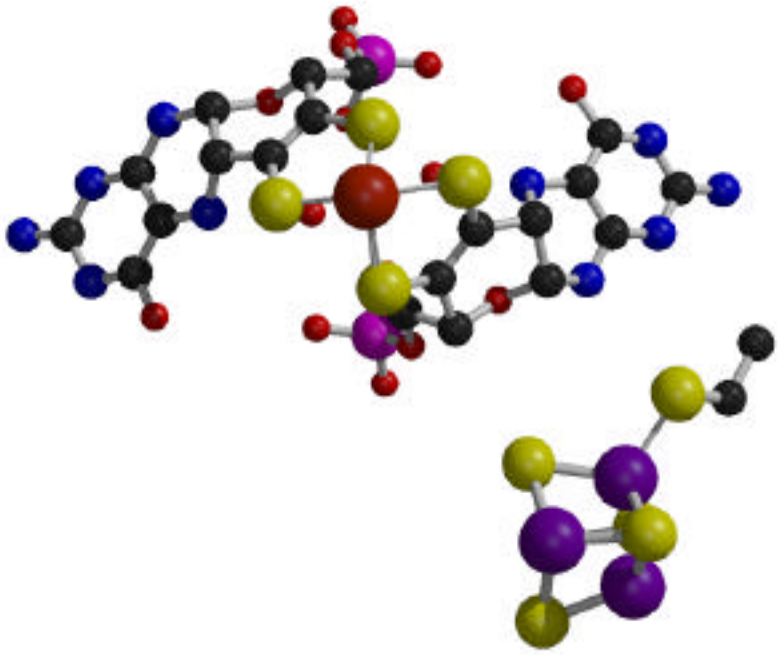


Fig. 1.8.

Ball-and-stick representation of the pterin cofactor and the [4Fe-4S] cluster of AOR. The sidechain of Cys494 that coordinates the cluster and accepts a hydrogen bond from the N8 of the pterin cofacotr is indicated. Taken from Chan et al., 1995.



The presence and location of oxo groups coordinated to the tungsten have not been definitely established, perhaps due to either heterogeneity of the AOR active site or crystallographic problems associated with locating light atoms in the vicinity of heavy metal at moderate resolution [Schindelin et al., 1997]. No protein ligands are coordinated to the tungsten. The arrangement of the tungsten and the two pairs of dithiolene sulfurs may be described as a distorted square pyramid, with the base of the pyramid defined by pairs of sulfurs separated by ~3.0 Å on an edge, for sulfurs both in the same and in different dithiolene groups. The tungsten is positioned ~1 Å above the least squares plane defined by the four sulfurs.

As the first crystal structure for a pterin-containing enzyme, one surprising result was the revelation that the W atom is coordinated by two dithiolene side chains from not one but two pterin cofactors, giving rise to the term bispterin cofactor. The two pterins are linked to each other through their terminal phosphate groups that coordinate the same Mg ion. The tungstopterin site is buried deep within the protein, and located approximately 10 Å away from the [4Fe-4S] cluster (Fig. 1.8). The cluster is closer to the protein surface, serving as an intermediary in the electron transfer between the W active site and the physiological electron acceptor, Fd. The [4Fe-4S] cluster is coordinated by the protein via four cysteine ligands. One of these Cys forms a hydrogen bond with a pterin ring nitrogen, indicating that the pterin might have an active role in the redox chemistry of the enzyme. The coordination sphere around the active site W in AOR includes four dithiolene sulfur atoms and an oxo ligand. However, it is difficult to establish the exact nature of the ligands near the W atom due to either heterogeneity of the AOR active site and/or crystallographic problems associated with locating light atoms

in the vicinity of heavy metals at moderate resolution [Schindelin et al., 1997]. X-ray absorption spectroscopy (XAS) studies on reduced active form of AOR reveals a single W=O, 4 or 5 W-S and possibly an additional W-O/N coordination, the oxidized, catalytically inactive form of AOR, in contrast, has 2 W=O, 3W-S and possible a W-O/N [Mukund and Adams, 1990, George et al., 1992]. This suggests that in the oxidized form the enzyme exists in the W(VI) oxidation state. No protein ligands are coordinated to the tungsten. A hydrophobic channel of ~15 Å in length leads from the surface of the protein to active site tungsten, which is at the bottom of the cavity. The channel is large enough to accommodate both small and large substrates, consistent with the ability of AOR to oxidize both aliphatic and aromatic aldehydes.

5.B. FORMALDEHYDE FERREDOXIN OXIDOREDUCTASE (FOR):

Molecular properties: The second member of the AOR family to be identified, FOR has been found only in hyperthermophilic archaea. To date, the enzyme has been isolated and characterized from two species, from *Thermococcus litoralis* [Mukund and Adams, 1993] and *P. furiosus* [Mukund, 1995]. *T. litoralis*, like *P. furiosus* is an obligate heterotroph and can grow on complex protein sources, as well as simple and complex carbohydrates both with and without S°. Like the other members of the AOR family, FOR is extremely oxygen sensitive and is a cytoplasmic enzyme.

Metal content and cofactors: Elemental analysis of *P. furiosus* FOR by both plasma emission spectroscopy and colorimetric assays reveals that the enzyme contains (in g-atom per subunit of ~ 68 KDa) approximately 4 Fe, 1 W, 4 S^{2-}, 1 Mg, 2 P [Mukund,

1995]. No other metals are present in significant amounts. *P. furiosus* FOR contains a pterin cofactor, like other tungstoenzymes in its class. The presence of this cofactor has been confirmed by extracting the pterin in presence of iodine and measuring the fluorescence spectra of the resultant derivative, based on the method of Johnson and Rajagopalan. Each subunit of FOR has a mononuclear W atom coordinated by pterin, in addition to a [4Fe-4S] cluster.

Catalytic properties: Originally isolated based on its ability to oxidize formaldehyde, FOR can also use other short chain (C_1-C_4) aldehydes as substrates. However, the K_m values for such substrates are very high (> 10 mM), especially for formaldehyde (25 mM), indicating that these are not unlikely to be physiological substrates (Table 1.3). This enzyme oxidizes a variety of short chain aldehydes such as acetaldehyde, propionaldehyde and glyceraldehyde although again high apparent K_m values preclude these as good substrates for FOR. With increasing chain length (e.g. crotonaldehyde, butyraldehyde) the activity decreases suggesting that the active site on FOR is probably only accessible to short chain aldehydes [Mukund and Adams, 1995].

During purification of FOR from *P. furiosus* (and also from *T. litoralis*), a significant loss of activity was observed, even when strictly anaerobic conditions are maintained. Only 5-7% of the original enzyme activity is recovered. This loss can be reversed, by treating the enzyme with sulfide under highly reducing conditions, a process termed ‘sulfide-activation’ [Mukund, 1995]. Incubation of FOR with excess sodium sulfide (20mM) and sodium dithionite (20mM) at room temperature (pH 8.0) results in a

four- to fivefold increase in specific activity over a period of 6 hours. This sulfide-activation effect is not observed if either reagent is omitted.

5.C. GLYCERALDEHYDE-3-PHOSPHATE FERREDOXIN OXIDOREDUCTASE (GAPOR):

Molecular properties: GAPOR is the third tungstoenzyme to be isolated and characterized from *P. furiosus* [Mukund and Adams, 1995]. It is a monomeric protein of 73 kDa and is extremely oxygen sensitive. It is the least characterized of the three tungstoenzymes that have been purified from *P. furiosus,* in part because its crystal structure has not yet been determined. GAPOR is a monomeric enzyme by biochemical analysis. Metal analyses of the pure enzyme shows the presence of one W and approximately six Fe atoms. It also contains two Zn atoms per subunit, the function of which is not known.

Catalytic properties and physiological role: GAPOR is absolutely specific for its substrate glyceraldehyde-3-phosphate (GAP), which it oxidizes to 3-phosphoglycerate (Table 1.3). So far this is the only substrate known that can be oxidized by the enzyme. Like AOR and FOR, GAPOR uses ferredoxin as its physiological electron carrier and does not use NAD or NADP [Mukund and Adams, 1995]. GAPOR is proposed to have a key role in the glycolytic pathway during carbohydrate (maltose) metabolism in *P. furiosus* wherein it replaces the more conventional enzymes GAP dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), and converts GAP directly to 3-phosphoglycerate, by-passing the intermediate 1,3-bisphosphoglycerate. Consistent with

this proposal, the activities of GAPDH and PGK are very low in maltose-grown *P. furiosus* [Mukund and Adams, 1995].

6. Proposed Research

The previous part of the Introduction provides a summary of what was known about aldehyde oxidoreductases from *Pyrococcus furiosus* at the time that the following research project was initiated. *P. furiosus* AOR was the most extensively-characterized example with detailed molecular, structural and spectroscopic properties. However, several key areas remained unexplored and several key questions remained open. Although FOR had been obtained from *P. furiosus* [Mukund, 1995], a routine purification procedure had not been established, and the molecular properties of the enzyme had not been reported in detail. In addition, although formaldehyde was routinely used as a substrate for this enzyme, the high K_m values for this aldehyde suggested that it was not the true substrate for FOR. In Chapter 2 – 4, procedures to purify FOR, AOR and GAPOR from the same batch of *P. furiosus* cells is presented, together with in depth analysis of the molecular properties and substrate specificity of FOR, including results from crystallographic analyses of the enzyme in the presence of a putative substrate. The possible physiological role of this enzyme is also discussed. Related to this issue is the ability of these aldehyde oxidoreductases to undergo activation upon incubation with sulfide under reducing conditions. Chapter 5 describes the kinetics of activation by sulfide, the properties of activated enzymes and address, the issue of whether this phenomenon has any relevance inside the cell.

With the advent of the so-called ‘genome era’, the complete genome sequences of a multitude of organisms have become available in the last few years, and this includes that of *P. furiosus*, as well as several other hyperthermophilic archaea. This poses the question of whether *P. furiosus* contains other members of the AOR family, and to what extent this family is present in other organisms. Suffice to say here that when the *P. furiosus* sequence database was searched for AOR-type sequences, two hitherto unknown genes (*wor4* and *wor5*) were discovered, and the details of this are described Chapter 6. Chapter 7 describes the isolation and purification of WOR 4, the fourth-member of the AOR family.

CHAPTER 2

THREE ALDEHYDE OXIDOREDUCTASES FROM *Pyrococcus furiosus*[1]

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[1]Roy, R., A. L. Menon, and M. W. W. Adams. 2001. *Methods In Enzymology*. 331:132-144. Reprinted here with permission of publisher.

Introduction

An early study with *Pyrococcus furiosus* showed that growth of this hyperthermophilic archaeon is stimulated by the addition of tungsten to the medium (1). Subsequently, three distinct tungsten-containing enzymes were purified from this and related organisms. They are aldehyde ferredoxin oxidoreductase (AOR), which has been purified from *P. furiosus* (2), *Pyrococcus* strain ES-4 (3) and *Thermococcus* strain ES-1 (4), formaldehyde ferredoxin oxidoreductase (FOR), which has been purified from *Thermococcus litoralis* (5) and *P. furiosus* (6), and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR), which has been obtained so far only from *P. furiosus* (7). All three enzymes catalyze the oxidation of various types of aldehyde using ferredoxin (Fd) as the physiological electron acceptor (Eq. 1, where Fd_{ox} and Fd_{red} are the oxidized and reduced forms).

$$R.CHO + H_2O + 2Fd_{ox} \rightleftharpoons R.COOH + 2H^+ + 2Fd_{red} \qquad \text{(Eq. 1)}$$

The three aldehyde-oxidizing enzymes differ in their substrate specificities. AOR oxidizes a wide range of both aliphatic and aromatic aldehydes to the corresponding acids and it has been proposed that such aldehydes are derived from amino acids during peptide fermentation (4). FOR has a more limited substrate range and oxidizes C_5-C_6 di- and semialdehydes and C_1-C_3 aldehydes (5,6). Various C_4-C_6 semialdehydes are involved in the metabolism of some amino acids, such as Arg, Lys and Pro (8,9), suggesting that FOR may function in the catabolism of one or more of these amino acids. GAPOR, on

the other hand, specifically oxidizes glyceraldehyde-3-phosphate yielding 3-phosphoglycerate. This is a key enzyme in the sugar fermentation pathway (7,10).

AOR, FOR and GAPOR are members of one of three distinct groups of tungsten-containing enzymes know as the "AOR family". They all consist of a single type of subunit of approximately 67 kDa in size and their amino acid sequences show high sequence similarity (11). The crystal structures of both AOR and FOR from *P. furiosus* have been determined and these show that their tertiary structures are very similar (12,13). Each subunit contains a mononuclear tungsten coordinated by four dithiolene sulfur atoms from two pterin molecules, together with a single [4Fe-4S] cluster coordinated by four sulfur atoms from four cysteine residues. The overall sequence similarity between structurally uncharacterized GAPOR from *P. furiosus* and AOR/FOR (50% similarity) is lower than that between AOR and FOR (61%). Nevertheless, the amino acid residues involved in binding the pterins and the four cysteines coordinating the iron-sulfur cluster in AOR and FOR are conserved in GAPOR, indicating that GAPOR also contains a tungstobispterin cofactor and a single [4Fe-4S] cluster (6,10). The three enzymes do differ, however, in their quaternary structures. GAPOR is thought to be monomeric, AOR is dimeric and FOR exists as a tetramer.

In addition to these three tungsten-containing enzymes that have been characterized from *P. furiosus*, the genome sequence of this organism contains two additional genes, *wor*4 and *wor*5, that are also thought to encode tungstoenzymes (6). WOR 4 and WOR 5 have predicted molecular weights comparable to those of AOR, FOR and GAPOR and show high sequence similarity to them although as yet there is no indication as to the function of these putative enzymes.

This chapter describes the purification of AOR, FOR and GAPOR from *P. furiosus* and summarizes some of their properties.

Assay Methods

The activities of AOR, FOR and GAPOR are routinely assayed by the conversion of various aldehydes to the corresponding acid. Although ferredoxin is the proposed physiological electron carrier, the artificial dye benzyl viologen (BV) is more conveniently used in the assays. Aldehyde oxidation is monitored by BV reduction which can be easily measured spectrophotometrically at 600 nm by the appearance of blue color. Strictly anaerobic conditions must be maintained during the assay as reduced benzyl viologen is instantly oxidized by oxygen and the enzymes themselves are irreversibly inactivated by oxygen (see below).

Reagents

The following stock solutions are prepared in 100 mM N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid, EPPS) buffer, pH 8.4

Benzyl viologen (BV), 100mM

Sodium dithionite (DT), 100mM

Glyceraldehyde-3-phosphate, 88 mM (for GAPOR)

Formaldehyde, 1.5M (for FOR)

Crotonaldehyde, 1.0 M (for AOR)

EPPS buffer is prepared from a 1.0 M stock solution. The buffer is degassed thoroughly on a vacuum manifold and flushed with Ar. Appropriate amounts of BV, DT are weighed out in powder form and degassed in empty 8 ml serum-stoppered vials before

EPPS is added by syringe under Ar. Glyceraldehyde-3-phosphate, formaldehyde and crotonaldehyde are degassed in the liquid form in the vial prior to adding buffer.

Procedure

A serum-stoppered cuvette containing 2 ml of anaerobic 100 mM EPPS buffer (pH 8.4) under Ar is incubated for three minutes at 70° (for GAPOR) or 80°C (for AOR and FOR) in a Spectronic 500 (Fisher Scientific, Atlanta, GA) spectrophotometer equipped with a thermostated cuvette holder and a thermoinsulated cell compartment. To this is added 50 µl of 2.5 mM BV. At this point it is important to make sure that the assay solution is anaerobic. This is done by adding a few µl of DT (100 mM) by syringe. This should turn the solution a light blue color (reduced BV) indicating no oxygen contamination. The cell-free extract or enzyme and the aldehyde substrate are then added with thorough mixing after each addition. The final substrate concentration for GAPOR, AOR and FOR are 250 µM, 200 µM and 50 mM, respectively. Benzyl viologen reduction is measured by the increase in visible absorption at 600 nm. Enzyme activity for all three enzymes is calculated from the initial rate of BV reduction, which is measured over a period of 30s or less, using a molar absorbance of 7,400 $M^{-1}cm^{-1}$ (6). Assays are performed at 80°C for FOR and AOR, but glyceraldehyde-3-phosphate is unstable at such temperatures so GAPOR assay is carried out at 70°C (7). Activities are expressed in units (U), where 1 U is the amount of enzyme catalyzing the oxidation of 1 µmole of substrate (2 µmole of BV) per minute under standard assay conditions. Protein concentrations are routinely estimated by the Bradford method (14). Under these standard assay conditions the specific activity determined for all three enzymes is dependent on the amount of protein added to the assay, with higher activities obtained

with lower protein concentrations over a range of approximately four-fold. Therefore, to enable comparisons to be made for the same enzyme with different substrates or between the three enzymes, comparable enzyme concentrations should be used. Recommended concentrations for all three enzymes are 35 – 50 μg/ml of the assay mixture.

Purification of AOR, FOR and GAPOR from the same batch of *Pyrococcus furiosus* cells.

The oxidation of glyceraldehyde-3-phosphate is specific for GAPOR and the oxidation of crotonaldehyde is specific for AOR as neither reaction is catalyzed by the other two tungstoenzymes. However, formaldehyde oxidation, the assay for FOR, is catalyzed by AOR, so the formaldehyde oxidation activity measured in cell-free extracts is the sum of the activity of both enzymes. FOR can be distinguished from AOR by using crotonaldehyde as substrate, but the two enzymes are separated from each other, and from GAPOR, by subjecting the cell-free extract to anion-exchange chromatography. The three enzymes are then purified separately, as described in the flow chart shown in Figure 2.1.

P. furiosus (DSM 3638) is obtained from the Deutsche Sammlung von Mikroorganismen, Germany. It is routinely grown at 90°C in a 600-liter fermentor with maltose as the carbon source as described previously (15,16). The three tungstoenzymes are routinely purified from 500 g (wet weight) of cells at 23°C under strictly anaerobic conditions. The procedures to prepare the cell-free extract and to carry out the first chromatography step are described elsewhere in this volume (15). In brief, cells are thawed in (1 g per 3 ml) of 50 mM Tris-HCl pH 8.0 containing 2 mM sodium dithionite, 2 mM dithiothreitol

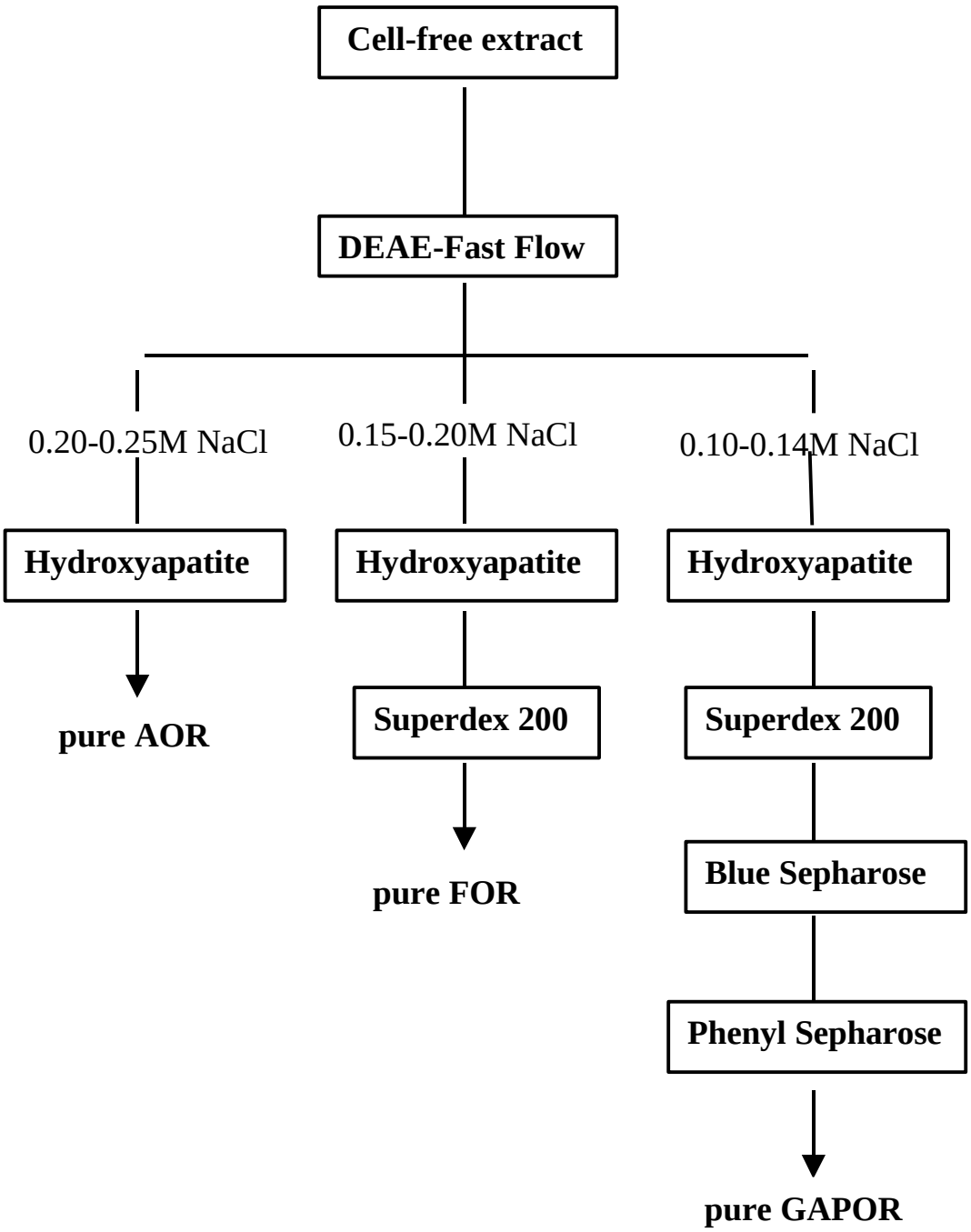
and 0.5 µg/ml DNase I. After incubation at 37°C for 1 hr and centrifugation at 30,000 x *g* for 1 hr, the cell-free extract is loaded onto a column (10 x 20 cm) of DEAE-Sepharose FF (Pharmacia Biotech) equilibrated with 50 mM Tris-HCl containing 2 mM sodium dithionite and 2 mM dithiothreitol. The extract is diluted three-fold with the buffer as it is loaded. The bound proteins are eluted with a linear gradient (15 liters) from 0 to 0.5 M NaCl in the equilibration buffer and 125 ml fractions are collected. GAPOR, FOR and AOR elute as 130-190, 160-200 and 190-260 mM NaCl is applied to the column, respectively. Although there is some overlap, the majority of the activity of each of the three enzymes can be separated from the others. These are then purified as outlined in Figure 2.1.

Aldehyde Ferredoxin Oxidoreductase

Fractions from DEAE-Fast Flow column with AOR activity are loaded at 4 ml/min onto a column (5 x 25 cm) of Hydroxyapatite (American International Chemicals) column previously equilibrated with 50 mM Tris-HCl, pH 8.0, containing 2mM sodium dithionite and 2 mM dithiothreitol (buffer A). The column is washed with two column volumes of buffer A and a linear gradient (1.5 liters) from 0 to 0.2 M potassium phosphate in buffer A is applied at a flow rate of 4 ml/min. Fractions containing AOR activity, elute out when 3 mM potassium phosphate is applied. The purity of the AOR samples is judged by SDS-PAGE using 10% (w/v) acrylamide. The pure protein gives rise to a single band of ~ 65 kDa. Fractions judged homogenous are combined and concentrated by ultrafiltration to 20 mg/ml using a PM-30 membrane (Amicon, Bedford, MA) and stored as pellets in liquid N_2.

Figure 2.1.

Purification of AOR, FOR and GAPOR from the same cell-free extract of *P. furiosus.* Range of salt concentration at which each enzyme elutes from DEAE-Fast Flow is indicated. Boxes denote the successive columns used during chromatography.


Cell-free extract
DEAE-Fast Flow
0.20-0.25M NaCl
0.15-0.20M NaCl
0.10-0.14M NaCl
Hydroxyapatite
Hydroxyapatite
Hydroxyapatite
pure AOR
Superdex 200
Superdex 200
pure FOR
Blue Sepharose
Phenyl Sepharose
pure GAPOR

This procedure yields 120 mg of AOR with a specific activity of ~ 80 units/mg. This is a 30% yield, based on the activity in the cell-free extract.

Formaldehyde Ferredoxin Oxidoreductase

Fractions from initial DEAE Fast Flow column that have FOR activity (but not AOR activity) are combined and loaded directly onto a column (5 x 25 cm) of Hydroxyapatite (American International Chemicals) column which has been previously equilibrated with buffer A. After washing the column with at least five column volumes of buffer A, the adsorbed proteins are eluted with a gradient (4.0 liters) from 0 to 0.2 M potassium phosphate in buffer A at a flow rate of 4 ml/min. FOR activity elutes as 0.05 to 0.14 M phosphate is applied and fractions of 100 ml are collected. Active fractions are combined, concentrated to 12 ml by ultrafiltration with a PM-30 membrane. The concentrated sample is applied to a column (6 x 60 cm) of Superdex 200 (Pharmacia LKB) equilibrated with buffer A containing 0.2 M KCl at 2ml/min. Fractions with FOR activity that are judged pure by SDS-PAGE are combined, concentrated by ultrafiltration to 20 mg/ml using a PM-30 membrane (Amicon, Bedford, MA) and stored as pellets in liquid N_2. With SDS-PAGE FOR migrates as a single band of ~ 68 kDa. This procedure yields 50 mg of FOR with a specific activity of ~ 50 units/mg and a recovery of activity of 7% (excluding the formaldehyde oxidation activity in the cell-free extract due to AOR).

Glyceraldehyde-3-phosphate Ferredoxin Oxidoreductase

GAPOR-containing fractions from DEAE-Fast Flow column are loaded onto a column (5 x 25 cm) of Hydroxyapatite (American International Chemicals) previously equilibrated with 50 mM Tris/HCl, pH 8.0, containing 2 mM dithiothreitol (buffer B) at 4 ml/min. Adsorbed proteins are eluted with a linear gradient (1.0 liter) from 0 to 0.4 M potassium phosphate in buffer B at the same flow rate and 100 ml fractions are collected. GAPOR activity begins to elute as 0.1 M phosphate is applied. Active fractions are pooled and concentrated to approximately 10 ml by ultrafiltration using an Amicon-type PM 30 membrane. The concentrated sample is applied to a column (6 x 60 cm) of Superdex 200 (Pharmacia LKB) equilibrated with buffer B containing 0.2 M KCl at 0.3 ml/min. The GAPOR-containing fractions are diluted five-fold with buffer B and are loaded on to a column (5 x 10 cm) of Blue Sepharose (Pharmacia LKB) equilibrated with buffer B. The column is washed with one column volume of buffer B and adsorbed proteins are eluted with a linear gradient (2.0 liters) from 0 to 0.6 M KCl in buffer A. GAPOR begins to elute as 0.3 M KCl is applied. Fractions containing GAPOR activity are pooled, diluted two-fold with buffer B containing 2.0 M ammonium sulfate, and loaded onto a column (3.5 x 10 cm) of Phenyl Sepharose equilibrated with buffer B containing 1.0 M ammonium sulfate. A linear gradient (1.0 liter) from 0.5 to 0 M ammonium sulfate in buffer B is applied at 5ml/min. GAPOR activity elutes as 350 mM ammonium sulfate is applied. The purity of GAPOR-containing fractions is assessed by SDS-PAGE (10%, w/v acrylamide). The pure enzyme gives rise to a single band of ~ 60 kDa. Pure fractions are combined, concentrated by ultrafiltration. This procedure yields

35 mg of GAPOR with a specific activity of ~ 25 units/mg and a recovery of activity of 5% relative to the cell-free extract.

Properties of AOR, FOR and GAPOR

All three tungsten-containing oxidoreductases are oxygen-sensitive (see Table 2.1) and must be purified under strictly anaerobic conditions. All buffers contain sodium dithionite to remove any trace oxygen contamination, and dithiothreitol to protect exposed thiol groups (2). However, GAPOR is inhibited by sodium dithionite and this reagent is not added to buffers once the enzyme is separated from AOR and FOR.

The molecular properties of the three tungstoenzymes are listed in Table 2.1. AOR is the best-characterized example of the "AOR-family" of tungstoenzymes. Its gene sequence has been determined (17) and crystallographic analysis has established that it is a homodimer (12). The AOR gene encodes 605 amino acids which corresponds to a protein of M_r 66,630 Da. Crystallographic analyses show that each subunit contains a mononuclear W atom coordinated via pterin cofactor, together with a [4Fe-4S] cluster located 10 Å away that is coordinated by four cysteine residues (12). The pterin cofactor, shown in Figure 2.2, consists of an organic tricyclic ring structure with dithiolene and phosphate side chains. The W atom is coordinated to two cofactors through their dithiolene sulfur atoms giving rise to a bispterin site. A Mg atom coordinates the phosphate moieties from the two pterins. The two pterin-binding motifs and four cysteinyl residues involved in binding the 4Fe-cluster are conserved in FOR and GAPOR from *P. furiosus* (6). Crystallographic analysis of AOR also revealed that it contains a metal site, probably iron, that bridges the two subunits.

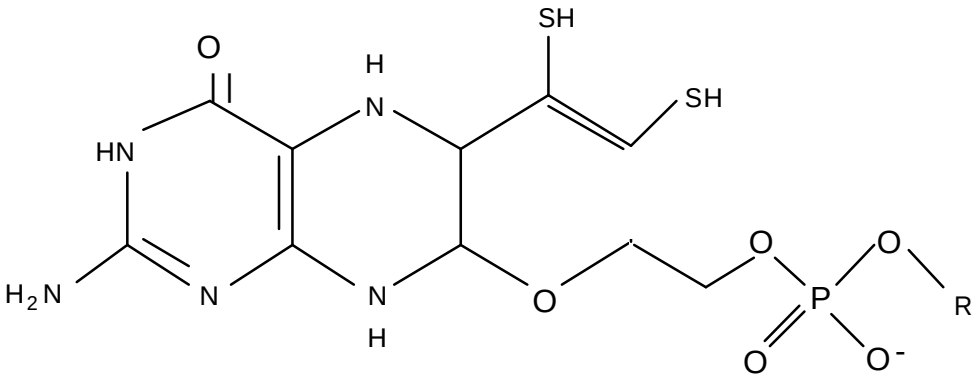
Table 2.1. Molecular properties of tungstoenzymes from *P. furiosus*

Properties	**AOR**	**FOR**	**GAPOR**
Holoenzyme (kDa)	136(α_2)	280(α_4)	73(α)
Subunit (kDa)	67	69	73
Metal content/ subunit[a]			
W	1	1	1
FeS cluster[b]	1 x [Fe_4S_4]	1 x [Fe_4S_4]	1 x [Fe_4S_4]
Other Fe	+ 1Fe		+2Fe?
Other metals	1Mg	1Mg	1Mg
	2P	2P	2P
		1Ca	2Zn
Pterin cofactor	MPT[c]	MPT	MPT
Thermal stability at 80°C ($t_{1/2}$)	15min	6 hr	15min
O_2 sensitivity at 23°C ($t_{1/2}$)	30min	9 hr	6 hr
references	2,12	6,13	7

[a]Metal content expressed as an integer value per mole of monomer.
[b]Cluster content is expressed per mole of monomer.
[c]MPT stands for metal binding pyranopterin dithiolate, also indicates presence of pterin without appended nucleotide.

Figure 2.2.

Structure of pterin cofactor in *P. furiosus* AOR. The W atom is coordinated by the dithiolene side-chains.


SH
O
H
N
SH
HN
O
P
O
R
H_2N
N
N
O
H
O
O^-

The Fe atom is coordinated by two EXXH motifs in each subunit and it is thought to play a structural rather than catalytic role.

FOR from *P. furiosus* is a homotetramer with a molecular weight of 280 kDa (Table 2.1). The crystal structure (13) reveals that the four monomers are arranged around a central cavity measuring 60 Å in diameter. Like AOR, each subunit of FOR has one [4Fe-4S] cluster situated 10 Å from a mononuclear W atom coordinated by dithiolene ligands to a bispterin cofactor. The FOR subunits are not bridged by Fe atoms and they lack the EXXH motifs that coordinate the monomeric Fe site in AOR. FOR has one Ca atom per subunit not present in AOR and this situated near one of the pterin rings. It probably has a structural rather than a catalytic role, like the Fe site in AOR (13).

GAPOR is a monomeric enzyme by biochemical analyses but is the least characterized of the three tungstoenzymes. From its gene sequence it consists of 653 amino acids with a predicted Mr of 73,942 Da (7,10). The pterin- and cluster binding motifs of AOR and FOR are conserved in GAPOR. Although a crystal structure for this enzyme is not available, metal analysis of the pure enzyme shows the presence of one W and approximately six Fe atoms by direct metal analyses. It also contains two Zn atoms per subunit, the function of which is not known

The aldehyde substrates oxidized by AOR and FOR are listed in Table 2.2. AOR oxidizes a broad range of both aliphatic and aromatic aldehydes, with acetaldehyde, isovaleralaldehyde, phenylacetaldehyde and indoleacetaldehyde being the best substrates. These correspond to the aldehyde derivatives of the amino acids alanine, leucine, phenylalanine and tryptophan, respectively. Based on these kinetic analyses AOR is thought to play a key role in peptide fermentation and to oxidize the aldehydes that are

generated by the decarboxylation of 2-keto acids derived from amino acids (4,18). AOR cannot couple aldehyde oxidation to the reduction of either NAD or NADP but shows a high affinity for *P. furiosus* ferredoxin, consistent with this redox protein being the physiological electron carrier (4).

Although formaldehyde is used routinely to assay FOR during purification, the high K_m that this enzyme displays towards this compound (Table 2.2) suggests that it is not its physiological substrate (5). Similarly, FOR has very high apparent K_m values for acetaldehyde and propionaldehyde (Table 2.2) so they are unlikely to be of physiological significance (6). The inability of FOR to oxidize longer/branched chain aldehydes indicates that the catalytic site of the enzyme is only accessible to short-chain aldehydes. This is supported by a lack of detectable activity seen with aromatic substrates like benzaldehyde, salicaldehyde and 2-furfuraldehyde. FOR does oxidize short-chain aromatic aldehydes like phenylacetaldehyde, phenylpropionaldehyde and indole-3-acetaldehyde, although the high K_m values again suggest that such aromatic substrates are not physiologically-relevant. C_4-C_6 aldehydes with associated acid or aldehyde groups serve as the most efficient substrates for FOR as indicated by the low K_m values (Table 2.2). Thus, FOR rapidly oxidizes succinic semialdehyde (C_4) and glutaric dialdehyde (C_5), yet the similarly-sized unsubstituted aldehydes are very poor substrates (6). Various C_4, C_5 and C_6 semialdehydes are involved in the metabolism of basic amino acids, Arg and Lys, and also of Pro (8,9). It is therefore thought that FOR has a role in peptide metabolism, although this has yet to be proven. Like AOR, FOR cannot use either NAD or NADP as electron acceptors for aldehyde oxidation, but can use native *P. furiosus* ferredoxin for which it has a K_m of 100μM (6).

Table 2.2. Substrate specificities of AOR and FOR

Substrate[a]	AOR (ES1)[4]	FOR (Pf)[6]
	Apparent K_m (mM)	
Formaldehyde	1.42	25.00
Acetaldehyde	0.02	60.00
Propionaldehyde	0.15	62.00
Crotonaldehyde	0.14	ND[b]
Benzaldehyde	0.06	ND
Isovaleraldehyde	0.03	ND
Phenylacetaldehyde	0.08	ND
Phenylpropionaldehyde	NA[c]	15.00
Indoleacetaldehyde	0.05	25.00
Succinic semialdehyde	NA	8.00
Glutaric dialdehyde	NA	0.80

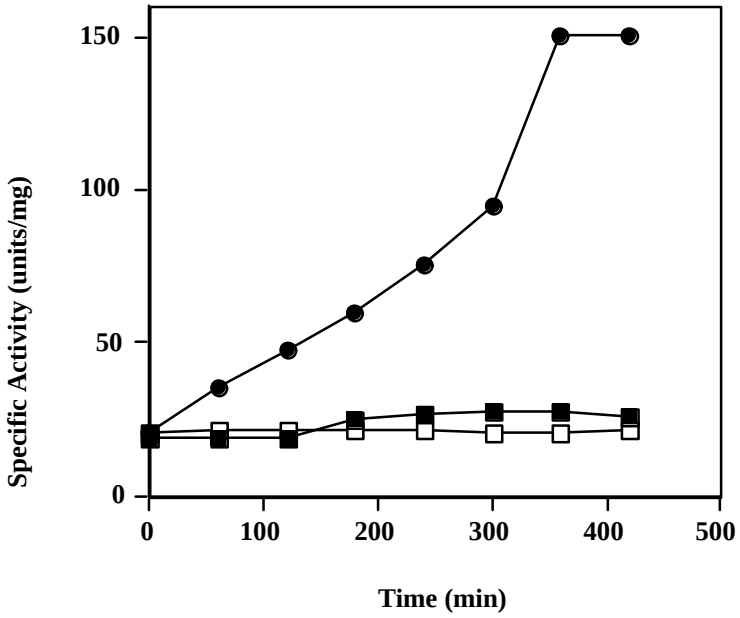
[a]Reactions were carried out at 80°C in 100mM EPPS buffer (pH 8.4) with BV (2.5mM) as the electron acceptor.
[b]Activity not detectable.
[c]Data not available.

During the purification of FOR from *P. furiosus* (and also from *T. litoralis*) a significant loss of activity is observed, even when strictly anaerobic conditions are maintained. This loss can be reversed by treating the enzyme with sulfide under highly reducing conditions (6). Incubation of FOR with excess sodium sulfide (20 mM) and sodium dithionite (20 mM) at room temperature (pH 8.0) results in a 4-5 fold increase in specific activity over a period of 5 hrs (see Figure 2.3). This 'sulfide-activation' effect is not observed if either reagent is omitted. When the enzyme is activated with sulfide for 5 hours, after which the excess sulfide is removed, the specific activity of the enzyme decreases by about 20%, but thereafter it stays the same under anaerobic conditions. The sulfide-activated form of the enzyme is more sensitive to oxygen than the as purified form of the enzyme. The substrate specificity is virtually the same for the as-purified and sulfide-activated forms.

GAPOR oxidizes its substrate, glyceraldehyde-3-phosphate (GAP), with apparent V_{max} and K_m values of 350 units/mg and 30 μM, respectively (at 70°C). So far this is the only substrate known to be oxidized by this enzyme. It shows no activity with formaldehyde, acetaldehyde, glyceraldehyde, benzaldehyde, glucose, glucose-6-phosphate or glyoxalate. Like AOR and FOR, GAPOR uses ferredoxin as its physiological electron carrier and it does not use NAD or NADP as electron acceptors (7). The product of GAP oxidation by the enzyme is thought to be 3-phopshoglycerate rather than 1,3-bisphosphoglycerate. GAPOR is a glycolytic enzyme that functions in place of GAPDH and phosphoglycerate kinase (7). This is substantiated by the fact that both these enzyme activities are very low in maltose grown *P. furiosus*, when assayed in the glycolytic direction (7).

Figure 2.3.

Activation of *P. furiosus* FOR by sulfide. The enzyme (10 mg/ml in 50 mM Tris/HCl, pH 8.0) was incubated at 23°C with either sodium sulfide (20 mM, open squares), sodium dithionite (20 mM, solid squares) or both (solid circles). At the indicated times, samples were removed and the residual activity was determined using formaldehyde as the substrate under standard assay conditions.


150
100
50
0
Specific Activity (units/mg)
0
100
200
300
400
500
Time (min)

In addition, the cellular activity of GAPOR has been shown to be about five-fold higher during growth on cellobiose as compared to pyruvate, and expression of the gene encoding GAPOR is significantly induced after the addition of cellobiose to pyruvate grown cultures of *P. furiosus* (10).

Hence, three members of the "AOR family" of tungstoenzymes have been purified from *P. furiosus* and characterized. Analysis of the genome sequence of *P. furiosus* reveals the two additional ORFs that appear to encode the fourth and fifth members of this family. These genes are termed *wor4* and *wor5* and are predicted to encode 622 and 582 amino acids, which correspond to proteins of 69 and 65 kDa, respectively. The similarities (identities shown in parentheses) of the sequence of WOR 4 to those of FOR, AOR and GAPOR are 57% (36%), 58% (37%) and 49% (25%), respectively, and those of the WOR 5 protein are 56% (33%), 58% (36%) and 49% (25%), respectively. Hence both WOR 4 and WOR 5 are more closely related to AOR and FOR than they are to GAPOR. The sequences of the two putative tungstoenzymes contain the conserved motifs that bind the bispterin cofactor and the [4Fe-4S] cluster in AOR and FOR (and presumably in GAPOR). Homologs of the three known and two putative tungstoenzymes in *P. furiosus* are present in the genomes of other hyperthermophilic archaea, including *P. horikoshii* (19), *P. aerophilum* (20), *P. abyssi* (21), *M. jannaschi* (22) and *A. fulgidus* (23). Tungstoenzymes of the “AOR family” appear to be widespread amongst the hyperthermophilic archaea although precisely what all of them do is still somewhat of an unanswered question.

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CHAPTER 3

PURIFICATION AND MOLECULAR CHARACTERIZATION OF THE TUNGSTEN-CONTAINING FORMALDEHYDE FERREDOXIN OXIDOREDUCTASE FROM THE HYPERTHERMOPHILIC ARCHAEON *Pyrococcus furiosus*[1]

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ABSTRACT

P. furiosus is a hyperthermophilic archaeon which grows optimally near 100°C by fermenting peptides and sugars to produce organic acids, CO_2, and H_2. Its growth requires tungsten, an element rarely used in biology, and two different types of tungsten-containing enzymes, aldehyde ferredoxin oxidoreductase (AOR) and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR), have been previously purified from this organism. These two enzymes are thought to function in the metabolism of peptides and carbohydrates, respectively. A third type of tungsten-containing enzyme, formaldehyde ferredoxin oxidoreductase (FOR), has now been characterized. FOR is a homotetramer of mass 280 kDa and contains approximately 1 W, 4 Fe and 1 Ca atom per subunit, together with a pterin cofactor. The low recovery of FOR activity during purification was attributed to loss of sulfide, since the purified enzyme was activated up to five-fold by treatment with sulfide (HS^-) under reducing conditions. FOR uses *P. furiosus* ferredoxin as an electron acceptor (K_m = 100 μM) and oxidizes a range of aldehydes. Formaldehyde (K_m = 15 mM for the sulfide-activated enzyme) was used in routine assays, but the physiological substrate is thought to be an aliphatic C_5 semi- or dialdehyde, e.g., glutaric dialdehyde, K_m = 800 μM. Based on its amino terminal sequence, the gene encoding FOR (*for*) was identified in the genomic database, together with those of AOR and GAPOR. The amino acid sequence of FOR corresponded to a mass of 68.7 kDa and is highly similar to those of the subunits of AOR (61%; 40% identity) and GAPOR (50%; 23% identity). The three genes are not linked on the *P. furiosus* chromosome.

INTRODUCTION

In the last decade, several species of anaerobic sulfur-dependent heterotrophic hyperthermophiles have been isolated from solfataric fields and submarine hydrothermal systems (5,46). These organisms exhibit optimal growth at temperatures of 90°C or above, and most of them belong to the domain Archaea rather than Bacteria (50). With the exception of the methanogenic species, many of the hyperthermophiles are able to utilize peptides as their sole carbon source, although a few saccharolytic species are also known. One of the most extensively studied of these organisms is the archaeon *Pyrococcus furiosus* (T_{opt} 100°C: 14). This anaerobic heterotroph grows on peptides (casein, peptone, yeast extract) and utilizes both simple (maltose, cellobiose) as well as complex sugars (starch, glycogen). It metabolizes carbohydrates by an unusual fermentative-type pathway in which acetate, CO_2, H_2, and alanine are the products (26, 27). Although the growth of many of the hyperthermophilic archaea is dependent upon elemental sulfur (S°), which is reduced to H_2S, *P. furiosus* is one of the few that can grow well in the absence of S° (14, 44).

The growth of *P. furiosus* is dependent upon tungsten (8), an element rarely used in biological systems (24, 30). Two tungsten-containing, aldehyde-oxidizing enzymes, aldehyde ferredoxin oxidoreductase (AOR: 36) and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR: 38) have been previously purified from this organism. AOR has a broad substrate specificity but is most active with aldehydes derived from amino acids (via transamination and decarboxylation: 21). AOR is thought to play a key role in peptide fermentation by oxidizing aldehydes generated by the four types of 2-keto acid oxidoreductase present in this organism (1, 22, 35). In contrast,

GAPOR uses only glyceraldehyde-3-phosphate as a substrate and it functions in the unusual glycolytic pathway that is present in *P. furiosus,* replacing the expected glyceraldehyde-3-phosphate dehydrogenase (38).

The hyperthermophilic archaeon *Thermococcus litoralis*, has been reported to contain another type of tungsten-containing, aldehyde-oxidizing enzyme, termed formaldehyde ferredoxin oxidoreductase (FOR: 37). This organism, like *P. furiosus,* ferments peptides and certain sugars and requires S° for optimal growth (7, 39), although *T. litoralis* shows essentially no similarity (3%) with *P. furiosus* at the DNA level (39). *T. litoralis* FOR is distinguished from AOR and GAPOR of *P. furiosus* by its substrate specificity. FOR was reported to oxidize only small (C_1-C_3) aliphatic aldehydes (37). However, dramatic losses of FOR activity occurred during purification from *T. litoralis* (> 90% after two chromatography steps) leading to a virtually inactive enzyme (37). Herein we show that *P. furiosus* also contains FOR and report on its biochemical and molecular properties, including the gene-derived amino acid sequence. This enzyme also undergoes significant inactivation during purification, but the results presented herein indicate that this is due to the loss of sulfide. In addition, the substrate specificity of FOR has been evaluated, and although its function *in vivo* is still unclear, the enzyme shows a high catalytic efficiency with aliphatic dialdehydes.

MATERIALS AND METHODS

Growth of the Organism and Enzyme Purification. P. furiosus (DSM 3638) was grown in the absence of S° at 90°C in a stainless steel fermentor using maltose as the carbon source as previously described (8). FOR was routinely purified from frozen cells (500 g,

wet weight) under strictly anaerobic conditions at 23°C. The buffer used throughout the purification was 50 mM Tris-HCl, pH 8.0, containing 2 mM dithiothreitol. Where indicated (see Results section), 10% glycerol [v/v] and/or 2 mM sodium dithionite were also present. The techniques and procedures were the same as those used to purify AOR from *P. furiosus*, up to the first chromatography step (36). In a typical purification, the cell-free extract prepared from up to 500 g (wet weight) of frozen cells was loaded on to a column (10 by 14 cm) of DEAE Fast Flow (Pharmacia-LKB) equilibrated with buffer. FOR eluted from the column at 150-205 mM NaCl using a gradient (10 liters) from 0 to 0.5 M NaCl in buffer. The flow rate was 20 ml/min and 125 ml fractions were collected. Fractions from this column with FOR activity were combined (1.2 liters) and loaded directly onto a column (5 x 25 cm) of Hydroxyapatite (American International Chemical). The adsorbed proteins were eluted with a gradient (4.0 liters) from 0 to 0.2 M potassium phosphate in buffer at a flow rate of 4.0 ml/min. Fractions of 100 ml were collected and FOR activity eluted as 55-140 mM phosphate was applied. Fractions containing FOR activity were combined and concentrated to approximately 12 ml by ultrafiltration with an Amicon type PM-30 membrane. The concentrated sample of FOR was applied to a column (6 x 60 cm) of Superdex 200 (Pharmacia LKB) equilibrated with buffer containing KCl (200 mM). Fractions containing pure FOR as judged by SDS-electrophoresis were combined (150 ml), concentrated by ultrafiltration to approximately 5 ml, and stored as pellets under liquid N_2.

Enzyme Assays. FOR activity was routinely determined at 80°C using formaldehyde (50 mM) as the substrate with benzyl viologen (3 mM) as the electron acceptor (37). Where indicated, methyl viologen (3 mM) replaced benzyl viologen. The reduction of *P.*

furiosus ferredoxin by FOR was measured as described previously (8). Crotonaldehyde (250 µM) and glyceraldehyde-3-phosphate (300 µM) were used as substrates in the assays for AOR and GAPOR, respectively. Results are expressed as units/mg of protein where 1 unit equals the oxidation of 1 µmol of substrate/min/mg protein. Hydroxycarboxylate-viologen oxidoreductase assays were carried out at 80°C in 100 mM EPPS, pH 8.4, using either 2-hydroxyacids as substrates with oxidized benzyl viologen (3 mM) as the electron acceptor, or with 2-ketoacids as substrates and reduced benzyl viologen (1 mM) as the electron donor (45). For the latter assay, benzyl viologen was reduced using sodium dithionite. Amino acid oxidoreductase assays were carried out at both 50 and 80°C in 100 mM EPPS, pH 8.4, using various amino acids as substrates (0.1 - 5 mM) and benzyl viologen (3 mM) as the electron acceptor. Pyruvate formate lyase activity was determined by a two step assay modified from that previously described (31). The assay mixture contained 100 mM EPPS, pH 8.4, sodium pyruvate (2 or 50 mM), CoA (55 or 100 µM) and FOR (see below). This mixture was incubated anaerobically at 80°C for 10 min and then rapidly cooled to 4°C. Formate was then measured at 37°C using an NAD-dependent formate dehydrogenase from yeast (F8649, Sigma Chemical Co., St. Louis, MO). NAD (1 mM) and formate dehydrogenase (50 µg) were added to the cooled reaction mixture and NADH production was measured by the increase in absorbance at 340 nm. A standard curve was prepared using 50 - 300 µM formate. For the hydroxycarboxylate-viologen oxidoreductase, amino acid oxidoreductase and pyruvate formate lyase assays, the final reaction volumes were 2 ml and all assays contained either pure FOR (50 µg) or a cell-free extract of *P. furiosus* (50 µl of ~ 15 mg/ml).

Other methods. Iron (33), acid-labile sulfide (11), cysteine (2, 43), pterin (51) and protein (6, 34) were measured as described previously. Molecular weight estimations (32, 37), plasma emission spectroscopy (37) and the N-terminal sequence analysis (13) were all carried out as described in the references. Ferredoxin (2), AOR (36) and GAPOR (38) were purified from *P. furiosus* as described previously. The DNA sequences of the genes encoding FOR is available from GenBank under accession number AF102769. The DNA sequence for AOR and GAPOR (40) can be found in the EMBL data bank under the accession numbers X79777 and U74298 respectively. Amino acid sequence was analyzed using the computer software program GCG and MacVector (International Biotechnologies, INC., New Haven, CT).

RESULTS

Purification of FOR. The ability of FOR to catalyze the formaldehyde-dependent reduction of benzyl viologen was used to detect the enzyme during its purification from *P. furiosus* cells. Cell-free extracts contained 4.3 ± 1.8 units/mg of formaldehyde-oxidizing activity, which is much higher than that found in cell-free extracts of *T. litoralis* (0.6 ± 0.2 units/mg). However, AOR (but not GAPOR) of *P. furiosus* also uses formaldehyde as a substrate (36), so the apparent FOR activity observed in the extracts of *P. furiosus* is the sum of the formaldehyde-oxidizing activities of both FOR and AOR. The AOR and FOR activities were easily separated by the first chromatography step using DEAE Sepharose. FOR activity eluted at 150 mM NaCl whereas the AOR activity eluted much later at 210 mM NaCl. FOR judged homogeneous by gel electrophoresis was obtained by two further chromatography steps (hydroxyapatite and gel filtration). It

should be noted that substantial losses in activity were observed throughout the purification procedure. The highest recovery of activity (~ 6%) was obtained when sodium dithionite (2 mM), dithiothreitol (2 mM) and glycerol (10%, v/v) were included in all buffers (see Table 3.1). This decreased to 2% if dithionite was omitted, hence, *P. furiosus* FOR undergoes substantial inactivation during purification, even under strictly anaerobic conditions. Possible reasons for this are discussed below.

Approximately 50 mg of pure FOR were obtained from 500 g (wet wt) of *P. furiosus* cells (Table 3.1). This compares with yields of 120 and 35 mg of AOR and GAPOR, respectively, from the same cell mass. Using benzyl viologen as the electron acceptor, the specific activity of pure FOR ranged from 35 - 45 units/mg. These values are in the same range as those reported for AOR (80 units/mg with crotonaldehyde as the substrate) and GAPOR (25 units/mg using glyceraldehyde-3-phosphate as the substrate) when assayed under standard conditions at 80°C. With methyl rather than benzyl viologen as the acceptor, the activities of both FOR (24 units/mg) and AOR (8 units/mg) decreased, while that of GAPOR (28 units/mg) was unaffected. Based on the total amount of activity in a cell-free extract and assuming an intracellular volume of 5 µl/mg (42), the intracellular concentrations of FOR, AOR and GAPOR were estimated to be 46, 112 and 112 µM, respectively. These calculations are based on the holoenzyme forms (tetramer, dimer and monomer, respectively, see below), and takes into account the formaldehyde oxidation activity of AOR in determining the FOR concentration. Hence, in *P. furiosus*, the amounts of these three aldehyde-oxidizing enzymes appear to be comparable, at least in cells grown using maltose as the primary carbon source.

Table 3.1. Purification of FOR from *P. furiosus*

Step	Protein (mg)	Activity (units)	Sp. activity (u/mg)	Recovery (%)	Purification (fold)
Cell-free extract	14,000	35,600	2.5	100	1
DEAE-Sepharose	5,300	26,700	5.0	75	2
Hydroxyapatite	1,100	10,186	9.2	28	4
Superdex-200	51	2,142	42.0	6	17

Molecular properties. The apparent molecular weight of *P. furiosus* FOR as determined by gel filtration was 275,000 ± 20,000 (data not shown). After treating the enzyme with SDS (1%, w/v) at 100°C for 10 min, the sample after SDS-gel electrophoresis gave rise to two bands (Fig. 3.1) which corresponded to molecular weights of 265,000 ± 20,000 and 68,000 ± 4000. A single protein band corresponding to molecular weight of 68,000 ± 4000 was observed if the sample was heated with SDS at 100°C for 30 min (Fig. 3.1). These results suggest that FOR is a homotetrameric protein (M_r ~ 272,000) with subunits of M_r ~ 68,000. The latter value is similar to the subunit molecular weights reported for AOR (66,630 from the gene sequence; 29) and GAPOR (63,000 by gel filtration and electrophoresis: 38), although in contrast to FOR, AOR is a dimer (10) while GAPOR appears to be a monomer (38). The high purity of *P. furiosus* FOR and the presence of a single subunit were confirmed by N-terminal amino acid sequencing. This gave rise to a single sequence that showed similarity to the N-terminal sequences of AOR and GAPOR from *P. furiosus* (Fig. 3.2). The N-terminal sequence (29 residues) of FOR was used to search the genomic sequence database of *P. furiosus*, which is nearing completion using multiplex sequencing methods (12). The N-terminus matched exactly the translated 5' end of one ORF, now termed *for*, which contained 619 codons corresponding to a protein with a molecular weight of 68,724 (see below), which is in good agreement with that (68,000) estimated by biochemical analyses.

Colorimetric analyses indicated that *P. furiosus* FOR contained 3.8 ± 0.3 mol of Fe, 2.9 ± 0.5 g mol of acid-labile sulfide, and 6 ± 1 cysteine residues per subunit. The latter value agrees with that (6 Cys/subunit) determined from the amino acid sequence (see below).

Figure 3.1.

SDS-gel analysis of purified *P. furiosus* FOR. Samples of FOR (8.5 mg/ml) were incubated with an equal volume of SDS (1%, w/v) at 100°C for 10 min (lane 2) or 30 min (lane 3) prior to electrophoresis on a 10% (w/v) acrylamide gel. Lane 1 contained marker proteins with the indicated molecular weights (in kDa, from top to bottom) myosin (202), β-galactosidase (116), phosphorylase b (97), bovine serum albumin (67), ovalbumin (45), and carbonic anhydrase (29).


1
2
3

An elemental analysis of FOR by inductively coupled plasma emission spectroscopy revealed the presence (in g-atoms per subunit) of 3.8 ± 0.3 mol Fe, 0.9 ± 0.1 mol W, 1.5 ± 0.1 mol Mg, 1.4 ± 0.1 mol P, and 0.4 ± 0.1 mol Ca. No other metals were present in significant amounts (> 0.1 g-atom/subunit).

The presence of a pterin cofactor in FOR was confirmed by extracting it in the presence of iodine and measuring the fluorescence of the resulting derivative. The spectrum (not shown) was virtually identical to that previously reported for the cofactor extracted from *P. furiosus* AOR (36), as well as to those obtained from various molybdenum-containing enzymes (except nitrogenase) when they are treated in a similar fashion. Such spectra originate from the so-called Form A derivative of the pterin cofactor (41).

Catalytic properties. FOR was purified by its ability to oxidize formaldehyde and reduce the artificial electron carrier benzyl viologen. Under standard assay conditions (80°C, pH 8.4), the specific activity of pure FOR was dependent, for reasons as yet unknown, upon the enzyme concentration in the assay mixture, with a four-fold increase in going from 0.01 to 0.15 mg/ml. Hence, to enable meaningful comparisons, and unless otherwise stated, FOR was used at a protein concentration of 0.025 mg/ml in all assay mixtures.

The activity of the pure enzyme increased more or less linearly with pH over the range 5.5 (1.5 units/mg) to 10.0 (55 units/mg) at 80°C (data not shown), and over the temperature range from 60°C (13 units/mg) to 90°C (58 units/mg) at pH 8.4 (data not shown). FOR activity, albeit low, was measurable at 25°C (~ 1 unit/mg, pH 8.4). The enzyme was quite thermostable, with a time required for a 50% loss of activity ($t_{50\%}$) at 80°C of about 8 hr (using 5 mg/ml in 100mM EPPS, pH 8.4, containing 2 mM sodium dithionite and 2 mM DTT).

Figure 3.2.

N-terminal amino acid sequence analyses of *P. furiosus* FOR and related enzymes. The abbreviations used are: Pf FOR, *P. furiosus* formaldehyde ferredoxin oxidoreductase (this work); ES AOR, ES-1 aldehyde ferredoxin oxidoreductase (21); Pf AOR, *P. furiosus* aldehyde ferredoxin oxidoreductase (36); Tl FOR, *T. litoralis* formaldehyde ferredoxin oxidoreductase (37); Cf CAR, *C. formicoaceticum* carboxylic acid reductase (49); Pf GAP, *P. furiosus* glyceraldehyde-3-phosphate ferredoxin oxidoreductase (38); Ct CAR, *C. thermoaceticum* carboxylic acid reductase (α-subunit; 47); HVOR, *P. vulgaris* hydroxycarboxylate viologen oxidoreductase (48).


Pf FOR
ES AOR
Pf AOR
Tl FOR
Cf CAR
Pf GAP
Ct CAR
HVOR

For comparison, the values for AOR and GAPOR when treated under the same conditions were 15 min and 1 hr, respectively. Note that activity values at 80°C (and above) were determined for all three enzymes from initial rates of benzyl viologen reduction, which were measured over a period of 30s or less.

For FOR under standard assay conditions, a linear Lineweaver Burk plot was obtained when the formaldehyde concentration was varied from 0.5 to 100 mM. The apparent K_m and V_m values were 25 mM and 62 units/mg, respectively. The former value suggests that formaldehyde is unlikely to be the physiological substrate. As shown in Table 3.2, FOR also oxidized various C_1-C_3 aldehydes when used at either low (0.3 mM) or high (50 mM) concentrations. The exceptions were glyoxal, which inhibited the enzyme at concentrations above 1 mM (data not shown), and methyl glyoxal, which was not oxidized at a detectable rate. However, the apparent K_m values, calculated from linear double reciprocal plots, for the most active of the C_1-C_3 aldehydes, acetaldehyde and propionaldehyde (Table 3.2), were above 60 mM (Table 3.3), suggesting that these reactions also are not of physiological relevance. As shown in Table 3.2, FOR exhibited low activity using butyraldehyde (C_4) or crotonaldehyde (C_4) as a substrate, but was not active with isovaleraldehyde (C_5), indicating that the catalytic site of the enzyme is accessible only to short chain (C_1 - C_3) aliphatic aldehydes. This notion was supported by a lack of detectable activity with the aromatic substrates, benzaldehyde, salicaldehyde and 2-furaldehyde. On the other hand, FOR did oxidize short-chain aldehydes with associated aromatic groups, such as phenylacetaldehyde, phenylpropionaldehyde and indole-3-acetaldehyde (Table 3.2). In fact, the kinetic constants determined for indoleacetaldehyde and phenylacetaldehyde were comparable to those obtained with

acetaldehyde (Table 3.3), although the high apparent K_m values again suggest that the oxidation of aromatic acetaldehyde derivatives is not the *in vivo* function of FOR.

A clue to other potential substrates for *P. furiosus* FOR came from the fact that the N-terminal sequences of the AOR-family of tungstoenzymes, which includes FOR (Fig. 3.2), were previously reported (30) to have similarity with that of the molybdoenzyme, hydroxycarboxylate-viologen oxidoreductase (HVOR). HVOR, which has been purified from *Proteus vulgaris* (48) and *Clostridium tyrobutyricum* (3), has a broad substrate specificity and catalyzes the reversible oxidation of 2-hydroxycarboxylic acids using benzyl viologen as the electron carrier. However, *P. furious* FOR did not oxidize lactate, 2-hydroxybutyrate, 2-hydroxyvalerate, 2-hydroxycaproate or 1-ethyl-2-hydroxycaproate, nor did it reduce pyruvate, 2-ketobutyrate, 2-ketovalerate, 1-ethyl-3-ketovalerate, 1-ethyl-4-ketovalerate, or 2-ketocaproate (using these substrates at 5 or 50 mM final concentration). In addition, FOR was unable to oxidize amino acids (glycine, alanine, serine, threonine, aspartate, glutamate and sarcosine) or formate (to CO_2) using benzyl viologen or NAD as the electron acceptor, nor did it exhibit pyruvate formate lyase activity. Furthermore, none of these activities were detected in a cell-free extract of *P. furiosus.*

Thus, of all the compounds listed above, *P. furiosus* FOR oxidizes only short-chain (C_4) unsubstituted aldehydes, but even these are poor substrates, as shown by the high apparent K_m values. Our attempts to uncover related substrates for which the enzyme had a much greater affinity were limited by the availability of substituted compounds of this type

Table 3.2. Substrate specificity of *P. furiosus* FOR

Substrate	Specific activity (U/mg)[a]			
	Purified FOR at substrate concentration (mM):		Sulfide-activated FOR at substrate concentration (mM):	
	0.3	50	0.3	50
Formaldehyde (C_1)	0.49 ± 0.24	35.50 ± 2.46	2.30 ± 0.09	85.00 ± 7.5
Formamide (C_1)	1.06 ± 0.12	0.64 ± 0.24	0.44 ± 0.13	0.20
Acetaldehyde[b] (C_2)	0.30 ± 0.06	8.03 ± 3.87	2.45 ± 0.08	23.22 ± 6.0
Glyoxal (C_2)	2.20 ± 0.05	nd[c]	2.45 ± 0.08	nd
Methyl glyoxal (C_3)	0.04	nd	0.23	nd
Pyruvaldehyde (C_3)	0.47 ± 0.03	2.44 ± 0.44	1.92 ± 0.36	7.48 ± 0.57
Glyceraldehyde (C_3)	0.20 ± 0.03	1.02 ± 0.05	0.55 ± 0.02	3.49 ± 0.15
Propionaldehyde (C_3)	0.61 ± 0.20	5.16 ± 2.10	0.32 ± 0.15	14.40 ± 0.88

Crotonaldehyde (C_4)	0.18 ± 0.06	0.59 ± 0.08	0.09 ± 0.04	0.36 ± 0.13
Butyraldehyde (C_4)	nd	0.29 ± 0.13	nd	0.35 ± 0.08
Isovaleraldehyde (C_5)	nd	nd	nd	0.33 ± 0.14
Phenylacetaldehyde	nd	0.72 ± 0.01	nd	nd
Phenylpropionaldehyde	0.31 ± 0.13	19.81 ± 4.87	0.62 ± 0.19	30.81 ± 7.33

[a]Reactions were carried out at 80°C in 100 mM EPPS buffer (pH 8.4) using benzyl viologen (3 mM) as the electron carrier with the substrate concentration of 0.3 or 50 mM.

[b]Measured at 65°C.

[c]nd, no activity was detected.

Table 3.3. Kinetic constants for the purified and sulfide-activated forms of *P. furiosus* FOR.

Substrate[a] (mM)	As-isolated FOR			Sulfide-activated FOR		
	Apparent K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	Apparent K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Formaldehyde (0.5 - 80)[b]	25	7.1×10^4	3.00	15	1.3×10^5	8.46
Acetaldehyde[c] (1 - 250)	60	4.4×10^4	0.73	8	2.8×10^4	3.50
Propionaldehyde (1 - 200)	62	1.1×10^4	0.17	25	2.3×10^4	0.92
Phenylpropionaldehyde (0.9 -80)	15	2.5×10^4	1.70	10	2.2×10^4	2.22
Indole-3-acetaldehyde (0.5 - 80)	25	2.3×10^3	0.09	12	1.1×10^3	0.10
Succinic semialdehyde (0.5 - 30)	8	5.7×10^3	0.71	6	1.1×10^4	1.90
Glutaric dialdehyde (0.01 - 30)	0.8	4.2×10^4	60.00	1	5.7×10^4	57.00

[a]Reactions were carried out at 80°C in 100 mM EPPS buffer (pH 8.4) using benzyl viologen (3 mM) as the electron acceptor.[b]Values in parentheses indicate the concentration range in mM used to obtain the kinetic constants.[c]Measured at 65°C.

Nevertheless, FOR did catalyze the oxidation of succinate semialdehyde (C_4) at a reasonable rate and the apparent K_m value was almost an order of magnitude lower than that of propionaldehyde (Table 3.3). Moreover, while butyraldehyde (C_4) was oxidized by FOR only at extremely high concentrations (Table 3.2), the presence of a terminal aldehyde group, as in glutaric dialdehyde (C_5), resulted in a substrate that was oxidized at a rate comparable to that obtained with acetaldehyde, which is the highest with the unsubstituted aldehydes, and with an apparent K_m value almost of two orders of magnitude lower (60 mM *vs.* 0.8 mM, Table 3.3). Increasing the chain length to C_6 (adipic acid semialdehyde, methyl ester) resulted in an activity similar to that obtained with succinic semialdehyde, but with a much higher K_m value (Table 3.3). From these analyses we conclude that the true substrate for FOR is therefore most likely a C_5 di- or semi-aldehyde, although its precise nature is still unclear.

The activity of FOR was largely unaffected when it was assayed in the presence of various mono- and di-acids (formate, acetate, succinate, citrate) when they were used at concentrations of 10 mM. The exception was glutarate, which resulted in ~ 15% inhibition under standard assay conditions. At a higher concentration of the acids (100 mM), the enzyme was inhibited by 35, 32, 36, 75 and 70% in the presence of formate, acetate, succinate, glutarate and citrate, respectively. These data again suggest that C_5 or C_6-type aldehydes might be physiologically relevant. With either formaldehyde (50 mM) or glutaric dialdehyde (30 mM) as the substrate for FOR, linear double reciprocal plots were obtained with *P. furiosus* ferredoxin in place of benzyl viologen as the electron acceptor (concentration range, 10 - 200 µM). The apparent K_m value for the ferredoxin was approximately 100 µM using both substrates. Thus, while the aldehyde substrate in vivo for FOR remains unknown, in contrast to AOR and GAPOR, like them FOR utilizes ferredoxin as its physiological electron carrier.

FOR was routinely purified in the presence of glycerol (10%, v/v) as this appeared to stabilize the enzyme. That is, when purified in the absence of this reagent, the final recovery of FOR activity decreased by about two-fold, as did the specific activity of the pure enzyme. However, although glycerol is a potential substrate analog it had little effect on FOR activity. For example, when "glycerol-free" FOR was incubated (at 23°C in 50 mM Tris/HCl buffer, pH 8.0) for up to 6 hr with up to 40% (v/v) glycerol, there was no significant loss of activity (determined in the absence of glycerol). The enzyme was inhibited when 60% (v/v) glycerol was used, as about 60% of the activity was lost after 6 hr. Under the same conditions, *P. furiosus* AOR was much more sensitive to inhibition by glycerol, with a loss of 25 and 80% of the initial activity after a 4 hr incubation with 20 and 40% (v/v) glycerol, respectively. *P. furiosus* GAPOR was even more sensitive to inhibition by glycerol, with a 50% loss of activity after 30 and 10 min in the presence of 40 and 60%, respectively. Hence, although all three enzymes are routinely purified in the presence of glycerol (10%, v/v), significantly higher concentrations are required for inhibition.

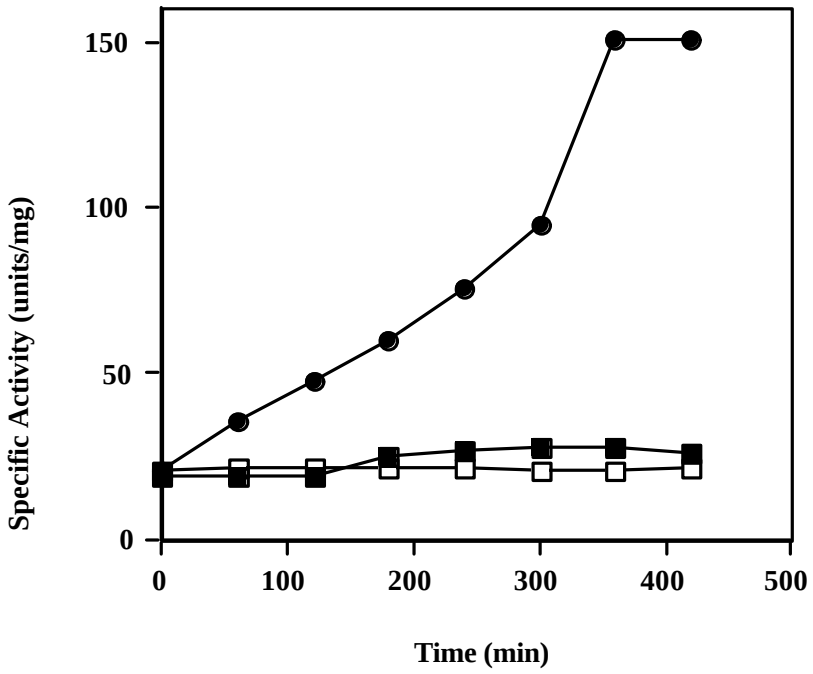
P. furiosus FOR as purified under standard conditions was oxygen-sensitive, with a $t_{50\%}$ value of about 12 hr when exposed to air (no activity was lost under anaerobic conditions). For comparison, a similar value was obtained with *P. furiosus* GAPOR, whereas AOR from *P. furiosus* was much more sensitive to oxygen with a $t_{50\%}$ value of about 30 min in air. *P. furiosus* FOR was not inhibited when it was incubated for up to 24 hr with iodoacetate, arsenite or cyanide (each at a concentration of 5 mM) prior to assaying under standard conditions in the absence of these reagents. In contrast, both AOR and GAPOR of *P. furiosus* were inhibited by all of these reagents under the conditions used for FOR. Thus, when AOR was incubated with either potassium cyanide, sodium arsenite or sodium iodoacetate (each 5 mM), the time required

for a 50% loss of activity was 13 hr, 10 min and 5 min, respectively. With GAPOR under the same conditions, the times were 30 min, 12 hr and 20 min, respectively. Thus, while GAPOR is very sensitive to inhibition by cyanide and AOR is readily inhibited by arsenite and iodoacetate, FOR was not affected by these reagents.

Properties of sulfide-activated FOR. A troubling aspect concerning the characterization of *P. furiosus* FOR was the significant loss of activity during purification. This occurred even when the procedure was carried out under the most rigorous of anaerobic conditions with reducing agents (DTT and sodium dithionite) in all buffers. A clue to the mechanism involved was the finding that the enzyme can be activated by sulfide. As shown in Fig. 3.3, incubation of FOR with excess sodium sulfide (20 mM) and sodium dithionite (20 mM) at 23°C (at pH 8.0) resulted after a 6 hr period in a more than 5-fold increase in specific activity. No activation was observed even after 8 hr if either reagent was omitted. The degree of activation varied from preparation to preparation but all samples analyzed showed at least a 3-fold increase in activity after a 6 hr period and thereafter showed little if any further increase, even after 24 hr. The time course and degree of activation was similar when sodium dithionite was replaced by titanium (III) citrate (20 mM) but DTT (20 mM) was ineffective as the reductant in the activation reaction. When the enzyme was incubated with sulfide for 6 hr and the sulfide was then removed (by gel filtration using 50 mM Tris/HCl, pH 8.0, containing 2 mM sodium dithionite, 2 mM DTT and 10%, v/v, glycerol), the specific activity of the resulting sulfide-free form of the activated enzyme decreased by ~ 20%, but this remained unchanged even after a further 24 hr under anaerobic conditions (in the absence of sulfide).

Figure 3.3.

Activation of *P. furiosus* FOR by sulfide. The enzyme (10 mg/ml in 50 mM Tris/HCL, pH 8.0) was incubated at 23°C with either sodium sulfide (20 mM, open squares), sodium dithionite (20 mM, solid squares) or both (solid circles). At the indicated times, samples were removed and the residual activity was determined using formaldehyde as the substrate under standard assay conditions.


150
100
50
0
Specific Activity (units/mg)
0
100
200
300
400
500
Time (min)

The sulfide-free, activated form of FOR was much more sensitive to inactivation by oxygen than the as-purified form, with a decrease in the $t_{50\%}$ value from 12 to about 4 hr. Similarly, in contrast to as-purified form, sulfide-activated FOR was sensitive to inhibition by cyanide. For example, while untreated FOR was unaffected by a 4 day incubation with cyanide (5 mM), the activated form (10 mg/ml in 50 mM Tris/HCL, pH 8.0) lost approximately 70% of its activity after a 16 hr incubation under the same conditions.

Thus, treating FOR with sulfide results in a more active enzyme that is more sensitive to inactivation by both cyanide and oxygen. In this respect, sulfide-activated FOR more resembles AOR and GAPOR than does the as-purified form of FOR. Moreover, it seems reasonable to suggest that the larger than expected decrease in activity of FOR during purification is due, in substantial part, to loss of sulfide.

While the mechanism of the sulfide-dependent effects are currently under study, of more relevance to the present work was the possibility that the substrate specificity of the sulfide-activated form of FOR was significantly different from that of the as-purified or "native" form of the enzyme. As shown in Table 3.2, however, this was not the case. The only exception is phenylacetaldehyde, which at high concentrations was oxidized, albeit at a low rate, by the native enzyme but not by the sulfide-activated form. In addition, the latter form of the enzyme had a lower apparent K_m value for formaldehyde (15 mM) compared to native FOR (25 mM), although for glutaric dialdehyde the apparent K_m value was similar (1 mM, see Table 3.3).

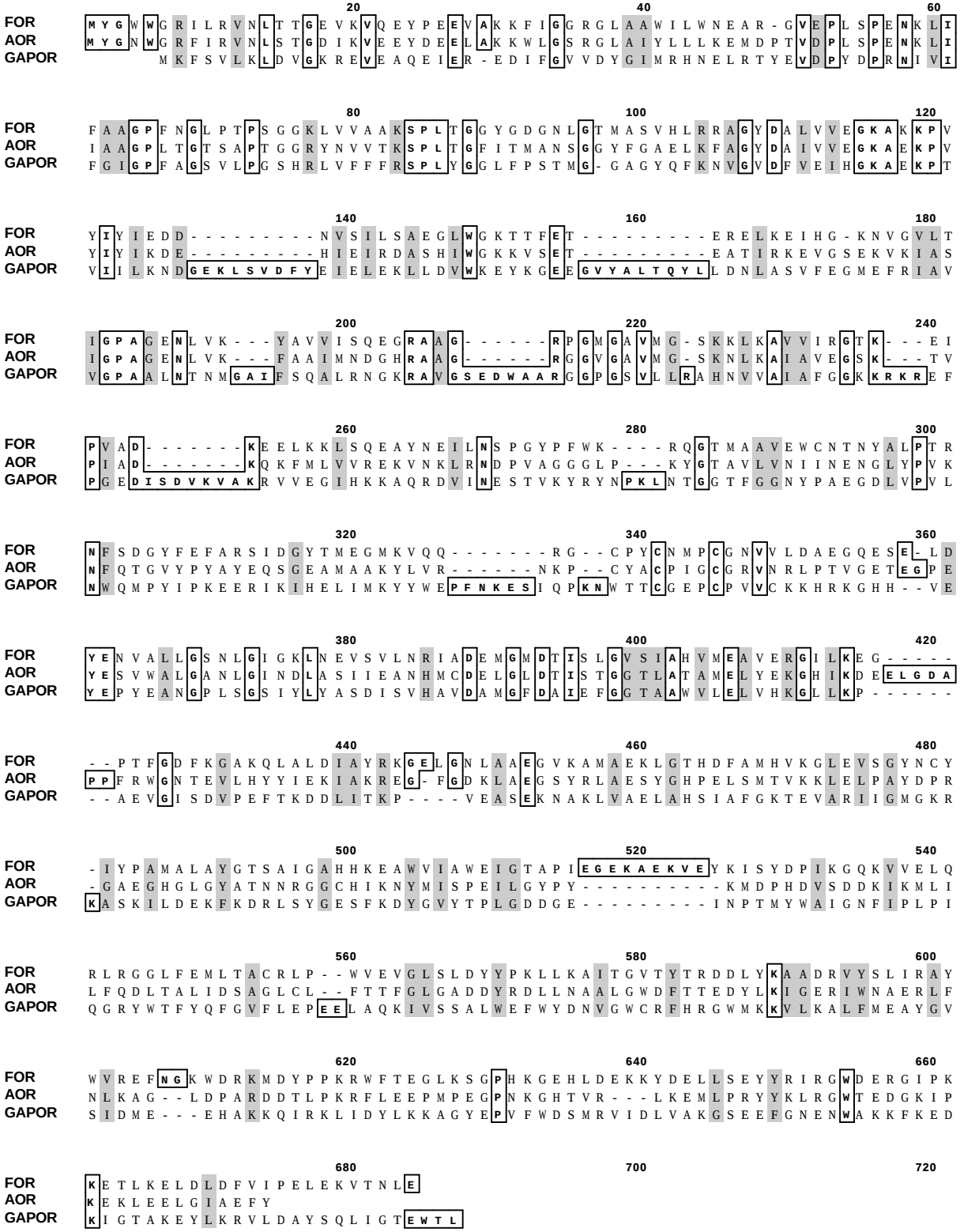
Gene sequences of related enzymes. With the availability of the complete amino acid sequence of FOR, the genomic sequence database of *P. furiosus* (12), while still incomplete, was also searched for related enzymes. The N-terminal sequence (45 residues) of *P. furiosus* GAPOR

(38) matched exactly the translated 5' end of one ORF, now termed *gor*. The gene contained 653 codons corresponding to a protein with a molecular weight of 73,850. This value is in reasonable agreement with that (63,000) estimated by biochemical analyses (38). The gene (*aor*) encoding AOR was located in the database using the previously published sequence (29) and the two nucleotide sequences were identical. The gene encoding FOR (*for*), *aor* and *gor* were spatially separated on the genome and, except for the previously identified cofactor-modifying (*cmo*) gene (29), none of the ORFs immediately adjacent to the *for, aor* and *gor* genes appear to be related to the synthesis or function of these three tungstoenzymes in *P. furiosus*. The complete sequences of the three enzyme are aligned in Fig. 3.4. FOR and AOR are highly similar (61%, 40% identity) with GAPOR somewhat less closely related (50% similarity and 23% identity with FOR, and 45% similarity and 25% identity with AOR).

A search of the genome database with the complete amino acid sequences of FOR, AOR and GAPOR revealed the presence of two additional ORFs and these appear to encode the fourth and fifth members of this enzyme family. Tentatively termed *wor4* and *wor5* (to depict genes encoding putative oxidoreductases within the tungstoenzyme family), these genes are also spatially separated from those encoding the other three tungstoenzymes and adjacent genes appear to be unrelated (although obviously the functions of these putative enzymes are as yet unknown). The *wor4* and *wor5* genes encode 622 and 582 codons, corresponding to proteins with molecular weights of 69,363 and 64,889 respectively. The similarity (identity) of the sequences of the WOR 4 and WOR 5 proteins to those of FOR, AOR and GAPOR are 57 (36), 58 (37) and 49 (25), and 56 (33), 58 (36) and 49 (25), respectively. Hence, both WOR 4 and WOR 5 appear to be more closely related to AOR and FOR, than they are to GAPOR (see Chapter 6 & 7 for details).

Figure 3.4.

Alignment of the sequences of FOR, AOR and GAPOR and putative gene products WOR 4 and WOR 5 from *P. furiosus*. Identical residues are boxed and similar residues are shaded.


FOR
AOR
GAPOR

The complete genomes of several hyperthermophilic archaea are now, or soon will be, available and these were searched for relatives of FOR and other members of the tungstoenzyme family found in *P. furiosus*. These include the genomes of *P. horikoshii* (19,25), *Pyrobaculum aerophilum* (15, 16), *Methanococus jannaschii* (9) and *Archaeoglobus fulgidus* (28), all of which contained genes encoding putative tungstoenzymes.

DISCUSSION

FOR is the third distinct type of tungsten-containing aldehyde-oxidizing enzyme, in addition to AOR and GAPOR, to be characterized from the hyperthermophile, *P. furiosus*. Their cellular concentrations appear to be comparable, at least in cells grown with maltose as the primary carbon source. The Fe, W, P and Mg content of FOR is similar to that of AOR (10, 36) and also GAPOR, but FOR also contains Ca (apparently one atom/subunit) while GAPOR also contains Zn (two atoms/subunit) (38). The function of Ca in FOR (and of Zn in GAPOR) is as yet unknown. From crystallographic analysis, AOR is known to contain one [4Fe-4S] cluster and a mononuclear tungstobispterin cofactor (10). The two pterin molecules are each bound non-covalently by the motif DxxGLC/D in AOR, where the Cys residue is one of the four that coordinate the [4Fe-4S] cluster. These two pterin-binding motifs and all four of the Cys residues in AOR are conserved in FOR, indicating that it too contains one [4Fe-4S] cluster and a mononuclear tungstobispterin cofactor. This agrees with the measured cofactor content, which includes the presence of more than one P atom per subunit of FOR (a bispterin site contains two phosphate groups). AOR also contains a second mononuclear metal site, most likely iron, which bridges the two subunits, and this is coordinated by two ExxH motifs from each subunit. The subunits of FOR do not appear to be bridged by a metal ion since these motifs are absent in the

FOR subunit, and this conclusion is supported by the iron content of FOR. The sequence of the FOR of *T. litoralis* is also available (29) and, in spite of the low similarity in the overall DNA sequences of *P. furiosus* and *T. litoralis,* it is virtually identical to the *P. furiosus* enzyme with 92% similarity (87% identity) in their amino acid sequences. In fact, AOR and FOR of *P. furiosus* are less similar (50% at the amino acid level) than are *P. furiosus* AOR and *T. litoralis* FOR (59% similarity: 29).

The other tungstoenzyme purified from *P. furiosus,* GAPOR, appears from its amino acid sequence to also have the same cofactor content per subunit as FOR and AOR. GAPOR contains two pterin-binding motifs, one of which contains a conserved Cys residue, and the other three Cys residues that coordinate the [4Fe-4S] cluster are also conserved. In fact, the spacing (number of residues) between these motifs is very similar in GAPOR, FOR and AOR, suggesting that their subunits have very similar tertiary structures (see Chapter 6). GAPOR contains one of the two ExxH motifs found in AOR, but since GAPOR is thought to be a monomer (in contrast to dimeric AOR and the putative tetrameric FOR), the function of such a motif, if any, is not clear.

Homologs of the three characterized tungstoenzymes of *P. furiosus,* as well as the two putative tungstoenzymes in this organism, were identified in the genomes available for other hyperthermophilic archaea. Thus, *P. horikoshii* has representative sequences of tungstoenzymes in all three main groups, namely, FOR, AOR and GAPOR, as well as homologs of the putative *P. furiosus* WOR 4 and WOR 5 enzymes. This is consistent with the physiological similarities between the two organisms (8, 19). Both are obligate heterotrophs that can grow fermentatively on a mixture of complex carbohydrates and peptides. Similarly, the presence of homologs of FOR and AOR, both of which have proposed roles in peptide metabolism, in *Pm. aerophilum*

and *A. fulgidus*, is consistent with the reported ability of both organisms to use peptides as a carbon source, even though their modes of energy conservation are quite different (by respiring nitrate or oxygen, and by sulfate reduction, respectively). On the other hand, the finding of a GAPOR-type sequence in *M. jannaschii* is puzzling. This methanogenic organism is thought to grow only on H_2/CO_2, yet GAPOR is a key enzyme in the glycolytic pathway of heterotrophic hyperthermophiles. Either GAPOR has another function in methanogens, or else *M. jannaschii* can perhaps grow on other carbon substrates.

The true role of FOR in *P. furiosus* also remains a mystery, in spite of the extensive analyses of potential physiological substrates reported herein. Compared to formaldehyde, FOR exhibited a higher k_{cat}/K_m value only with glutaric dialdehyde, a C_5-compound, for which the value was 20-fold higher, but this compound does not lie on a known biochemical pathway. However, various C_4-C_6 semialdehydes are involved in the metabolism of certain amino acids such as Arg, Lys and Pro (4, 20, 52), and although such compounds are not commercially available, it should be possible to investigate whether related enzymes are present in cell extracts of *P. furiosus*, and such studies are underway. Indeed, it should be noted that *P. furiosus* appears to utilize AOR and GAPOR for very different purposes, namely, in peptide catabolism and glycolysis, respectively, yet these two enzymes have very similar molecular properties, and presumably are also closely related phylogenetically. Thus, it is hard to rationalize a potential physiological role for FOR merely based on its similarity to AOR and GAPOR. Similarly, while an aldehyde-oxidizing enzyme with a substrate specificity analogous to that of AOR has been found in mesophilic clostridia (47, 49), an enzyme with the substrate range of FOR has not been reported from any organism.

Lastly, we turn to the finding that FOR is dramatically activated by incubation with sulfide under reducing conditions. Although loss of sulfide can account for the major loss of activity observed during the purification of FOR, it raises several issues, such as what is the nature of the sulfide that is lost, and is sulfide activation a physiologically-relevant reaction ? While there is no experimental evidence to address the latter question, the sulfide that is lost is presumably associated with the catalytic site. By analogy with AOR (10) and from the above discussion, FOR is expected to contain a tungstobispterin site where the W atom is coordinated in part by four S atoms from the dithiolene groups of two pterin molecules, with water, hydroxide and/or a terminal oxo group completing the coordination sphere. Interestingly, mononuclear molybdopterin-containing enzymes such as xanthine oxidase lose sulfur as thiocyanate when the oxidized forms are treated with cyanide (see, for example, 23). The resulting “desulfo”-enzymes are activated by treatment with sulfide, which is accomplished by conversion of a terminal Mo=O species to Mo=S, which does not involve the dithiolene sulfurs. So, is FOR as purified equivalent to the desulfo-state, and does sulfide activation involve W=O to W=S conversion? It is noteworthy that upon sulfide-activation, FOR became more sensitive to inactivation by O_2 and by cyanide, suggesting that both may effect a W=S to W=O conversion. However, for AOR, there was no evidence for a W=S bond either from crystallography (10) or X-ray absorption spectroscopy (18). Thus, the precise mechanism by which sulfide activates FOR remains to be determined, and spectroscopic analyses using both X-ray absorption and resonance Raman (see reference 17) of the enzyme are in progress to address these issues.

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CHAPTER 4

EFFECTS ON FOR AND AOR ACTIVITY OF THE GROWTH CONDITIONS OF *Pyrococcus furiosus*[1]

[1]Roy, R. and M. W. W. Adams. To be submitted to *J. Bacteriol*.

Introduction

Hyperthermophiles are microorganisms that grow optimally at 80°C and above (Stetter, 1996, 1996). Most of them are strictly anaerobic heterotrophs. The major carbon source for these heterotrophs is peptides, and most use elemental sulfur (S°) as a terminal electron acceptor, reducing it to H_2S. However, a few species are saccharolytic and are capable of metabolizing poly- and oligosaccharides, in addition to peptides. A few examples of such species are *Pyrococcus furiosus*, *P. woesei* and *P. glycovorans* [Fiala and Stetter, 1986, Blamey et al., 1999, Barbier et al., 1999]. *P. furiosus* serves as the model organism for the study of anaerobic, heterotrophic hyperthermophilic archaea. This microorganism grows on peptides such as casein, peptone or yeast extract and utilizes both simple (maltose, cellobiose) and complex (starch, glycogen) sugars. It metabolizes carbohydrates by an unusual fermentative pathway with acetate, CO_2, H_2 and alanine as end products [Kengen and Stams, 1994, Kengen et al., 1994]. The pathways of peptide and carbohydrate metabolism have been well studied in *P. furiosus* [de Vos et al., 1998, Adams, 1999, 2001]. Glycolysis occurs via a modified Embden-Meyerhof pathway [Kengen et al., 1994, Mukund and Adams, 1995]. This differs from the classical glycolytic pathway in that the hexose kinase and phosphofructokinase steps are ADP- rather than ATP-dependent and a ferredoxin-dependent oxidoreductase, glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) replaces the conventional enzymes glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase.

Growth of *P. furiosus* on peptide substrates is dependent on the presence of S° in the medium whereas growth on sugars does not require sulfur [Adams et al., 2001]. Amino acid catabolism in this microorganism is though to involve four distinct 2-keto

acid oxidoreductases that convert transaminated amino acids into their corresponding coenzyme A (CoA) derivative [Blamey and Adams, 1993, Heider et al., 1996, Mai and Adams, 1996]. These CoA derivatives are then transformed to the corresponding organic acids by two acetyl-CoA synthetases, with concomitant ATP production by substrate-level phosphorylation [Mai and Adams, 1996].

The growth of *P. furiosus* is stimulated by tungsten [Bryant and Adams, 1989]. Three tungsten-containing, aldehyde-oxidizing enzymes, aldehyde ferredoxin oxidoreductase (AOR; Mukund and Adams, 1990, 1991), formaldehyde ferredoxin oxidoreductase (FOR; Mukund, 1995, Roy et al., 1999) and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR; Mukund and Adams, 1995) have been purified from this organism. All three enzymes use aldehydes as substrates, which they reduce to the corresponding acids, although they differ considerably in their substrate specificity. AOR, the most extensively studied tungstoenzyme, has a broad substrate specificity. The enzyme can oxidize a wide range of aliphatic and aromatic aldehyes, however, it is most active with aldehydes derived from amino acids (via transamination and decarboxylation) during peptide metabolism [Heider et al., 1995]. Therefore, AOR is proposed to play a key role in peptide fermentation by oxidizing aldehydes generated by the four types of 2-keto acid reductases present in this organism. In contrast to AOR, GAPOR uses only glyceraldehyde-3-phosphate as a substrate and it functions in the unusual glycolytic pathway that is present in *P. furiosus* [Mukund and Adams, 1995].

FOR has a more limited substrate range and oxidizes C_1-C_3 aldehydes to the corresponding acids, however, the enzyme displays a high K_m for these substrates suggesting that they might not be the true substrates for this enzyme. The enzyme is

unable to oxidize longer or branched chain aldehydes indicating that the catalytic site of the enzyme is only accessible to short-chain aldehyde substrates. C_4-C_6 aldehydes with associated acid or aldehyde groups serve as the most efficient substrates for FOR [Roy et al., 1999]. The enzyme rapidly oxidizes substrates such as succinic semialdehyde (C_4) and glutaric dialdehyde (C_5), however, similarly-sized unsubstituted aldehydes are very poor substrates. Further, FOR activity is inhibited in the presence of acids such as formate, acetate, glutarate and citrate. These data suggest that C_4- or C_6- type aldehydes might be physiologically-relevant substrates for this enzyme.

Structural analyses of FOR also supports such a bifunctional aldehyde substrate. The crystal structure of *P. furiosus* FOR has been resolved at 1.8 Å [Hu et al., 1999]. FOR is tetrameric, with four identical subunits arranged around a channel of ~27Å diameter. As observed in the structure of the related enzyme AOR [Chan et al., 1995], each monomer of FOR comprises a mononuclear W atom coordinated to a bispterin cofactor and a [4Fe-4S] cluster. FOR crystals were soaked with the dicarboxylic acid, glutarate, which is the product of glutaric dialdehyde oxidation [Hu et al., 1999]. Glutaric dialdehyde was chosen since the K_m value is the lowest for any of the substrates characterized so far. In the crystal structure, the glutarate molecule is clearly visible at the active site of FOR. One carboxylate group of the glutarate is located near the W site, stabilized by hydrogen bond interactions with side chain carboxylate groups of residues Glu, Tyr and His. The second carboxylate group of glutarate is bound to the protein through electrostatic interactions with the side chains of two Arg residues. This structure lends credence to the proposal that a C_4-C_6 substituted aldehyde might be the physiological substrate for FOR.

Various C_4, C_5 and C_6 semialdehydes are involved in the metabolism of amino acids such as Arg, Lys and Pro [Bender, 1985, Gottschalk, 1986]. Therefore, we decided to investigate the metabolism of these amino acids in *P. furiosus*, and whether FOR (and AOR) could play a role in the process. Of these amino acids, the metabolism of Arg has been the most extensively studied. This amino acid is used as a source of carbon, nitrogen and energy by various microgorganisms by several different pathways. These involve enzymes such as arginine succinyltransferase (AST), arginine deiminase (ADI), arginine dehydrogenase, arginine decarboxylase, and arginase [Park et al., 1997]. *P. furiosus* genome was searched for the presence of homologs of the key enzymes in these pathways, but none were found. Of course, this does not mean that such enzymes do not exist in this organism. We will focus on arginine utilization via ornithine, as the genome did contain a homolog (ORF #1334722) of ornithine aminotransferase (OAT). A possible pathway for arginine utilization is given in Fig. 4.1. This is based on that proposed in *Klebsiella aerogenes*, which can utilize arginine as a source of carbon, energy and nitrogen, and converts arginine to glutamate via ornithine [Friedrich and Magasanik, 1978]. Note that the product of the OAT is a C_5-semialdehyde, glutamate-5-semialdehyde, a possible substrate for FOR. Similarly, proline dehydrogenase produces pyrroline-5-carboxylate that spontaneously converts to glutamate-5-semialdehyde. In addition, lysine is commonly degraded via the saccharopine pathway and this also contains semialdehydes [Papes et al., 1999]. This pathway produces one molecule of acetyl CoA per molecule of lysine metabolized. *P. furiosus* genome has homologs of the first two enzymes in the lysine catabolic pathway, saccharopine dehydrogenase and amino adipate reductase. Therefore, the general catabolic pathways for all three of these

amino acids have the potential to contain semialdehyde intermediates that could possibly serve as substrates for FOR. Unfortunately, such compounds are not available commercially, and are not readily synthesized, so it is not possible to simply assay FOR with such substrates. We therefore carried out physiological and enzymatic studies to test our hypothesis that FOR is involved in amino acid metabolsim.

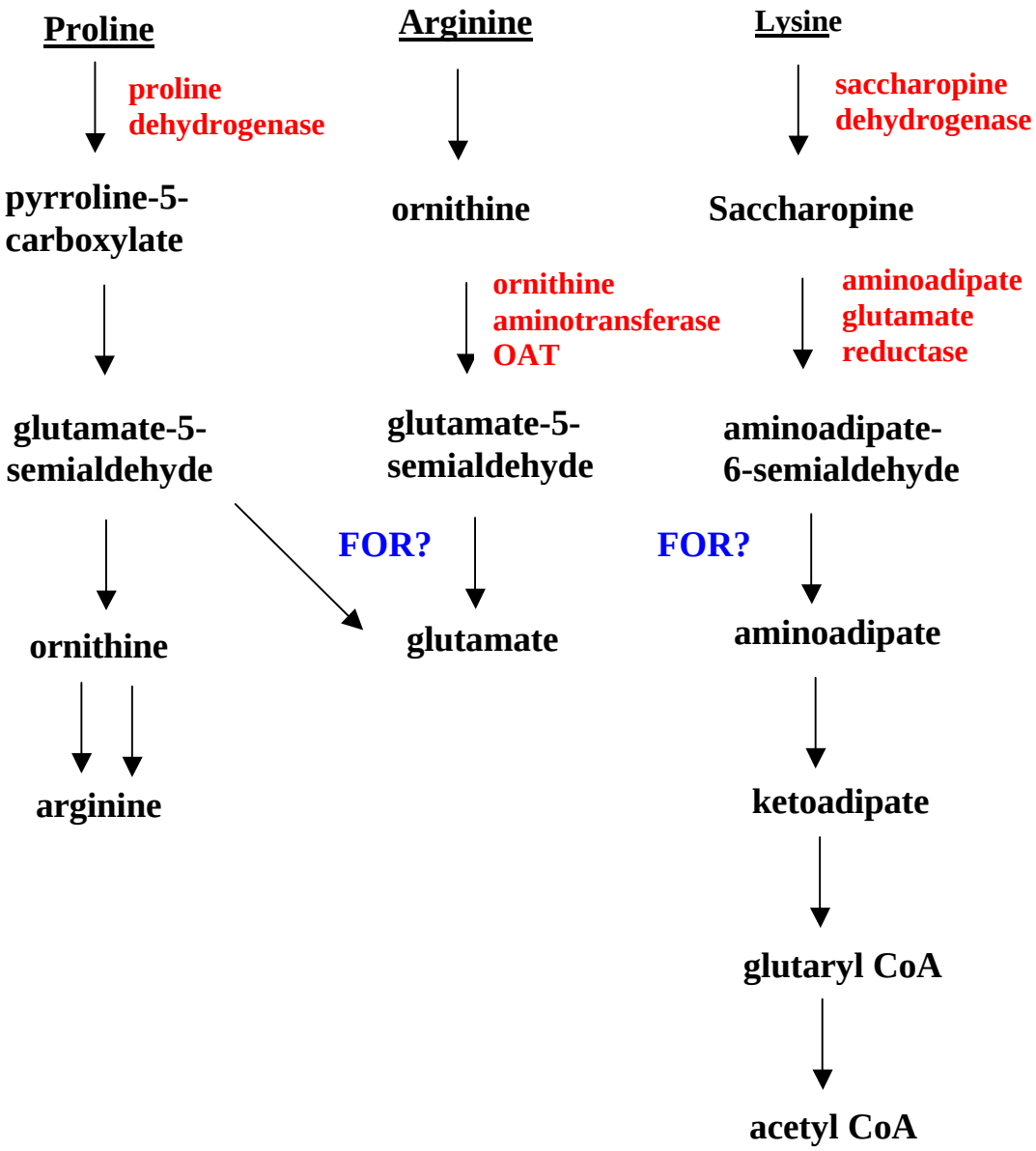
P. furiosus was grown on various combinations of carbohydrate (maltose), peptides (casein hydrolysate), and S° and activities of FOR, AOR and GAPOR were measured in the cell-free extracts (Adams et al., 2001). Further, to test whether FOR had a role in the metabolism of certain amino acids, *P. furiosus* was grown on a minimal maltose medium supplemented with Arg (or its precursor ornithine) Lys or Pro. In addition to measuring their effects on cell growth, the activity of FOR, and its relatives, AOR and GAPOR, were determined.

Materials and Methods

Growth conditions: *P. furiosus* (DSM 3638) was grown in a 20-liter fermentor containing 15 liters of medium, prepared as described previously [Verhagen et al., 2001]. It contained a base salt solution and either maltose or peptides, or a combination of both, as the carbon source [Adams et al., 2001]. The maltose-based medium contained 0.5% (w/v) maltose, and 0.1% (w/v) elemental sulfur was added to give the maltose-plus-S° medium. The peptides-plus-S° medium contained 0.5% (w/v) casein hydrolysate plus 0.1% (w/v) S°. The peptides–plus-maltose medium contained 0.5% (w/v) maltose and 0.5% (w/v) casein hydrolysate, together with 0.5% (w/v) yeast extract.

Fig. 4.1.

Probable catabolic pathways of basic amino acids in *P. furiosus*. Key enzymes for which homologs exist in the *P. furiosus* genomic database are shown in red [Adapted from Park et al., 1997, Bender, 1985 and Gottschalk, 1986].


Proline
proline dehydrogenase
pyrroline-5-carboxylate
glutamate-5-semialdehyde
ornithine
arginine
Arginine
ornithine
ornithine aminotransferase OAT
glutamate-5-semialdehyde
FOR?
glutamate
Lysine
saccharopine dehydrogenase
Saccharopine
aminoadipate glutamate reductase
aminoadipate-6-semialdehyde
FOR?
aminoadipate
ketoadipate
glutaryl CoA
acetyl CoA

Elemental sulfur (0.1%, w/v) was added to give the peptides-plus-maltose-plus-sulfur medium. The headspace of the fermentor was flushed with N_2-CO_2, and L-cysteine-HCl and Na_2S were added as reducing agents to remove residual O_2. The pH of the medium was maintained at 6.8 (± 0.1) by the addition of 5% (w/v) $NaHCO_3$ and the temperature was kept at 95 °C. *P. furiosus* was grown under each of these conditions in triplicate [Adams et al., 2001].

To study the effects of amino acid supplementation in the growth medium of *P. furiosus*, small-scale cultures (50 ml) were grown initially. The media were prepared according to previously established protocols [Verhagen et al., 2001, Adams et al., 2001]. In an effort to establish carbon-limiting conditions for cell growth, various combinations of maltose and yeast extract were used to prepare various media. Maltose concentrations were 0.4, 0.2 or 0.1% (w/v), and yeast extract concentrations were 0.05, 0.025 or 0.01% (w/v) respectively. The small-scale cultures were sealed with N_2-CO_2 in the headspace and a combination of L-cysteine-HCl and Na_2S was used as reductant. The pH was adjusted to 6.8 using NaOH but was not controlled during growth.

P. furiosus cultures that had undergone at least three successive transfers (50 ml cultures) on each experimental medium were used to innoculate 1 liter cultures. During growth, samples were withdrawn from the cultures and used to measure cell count and pH. Cells were counted using a Petroff-Hausser counting chamber and phase contrast microscopy. The growth rate was calculated by measuring the slope of a best-fit line through the exponential portion of the growth curve. Cells were harvested in the late-logarithmic phase (1×10^8 to $2 \times 10^8 \cdot ml^{-1}$). The cultures were cooled, and the cells were harvested by centrifugation at 10,000 x *g* for 15 min (Beckman J2-21 centrifuge, JA-10 rotor) at 4

°C, then resuspended in anoxic 50mM Tris-HCl bufffer (pH 8.0) containing 2mM sodium dithionite (DT) and 2mM dithiothreitol (DTT), and frozen with liquid nitrogen and stored at –80 °C.

Preparation of cell-free extract: All sample transfers and manipulations were carried out in an anaerobic chamber and all buffers were degassed and flushed with argon and contained 2mM sodium dithionite and 2mM dithiothreitol. The cell suspension was thawed, and deoxyribonuclease I in buffer was added to a final concentration of 0.0002% (w/v). The cell suspension was incubated at room temperature, the cells were then disrupted anaerobically by sonication for 30 min (Branson sonifier 450) by placing the sample vial in an ice-water slurry with the sonicator probe. Cell lysis was verified using phase-contrast microscopy. Debris and unbroken cells were removed by centrifugation (10,000 x *g* for 15 min in a Beckman L8-M ultracentrifuge with a 60 Ti rotor), and a portion of the supernatant was used as the whole-cell extract (WCE). The remainder was centrifuged at 100,000 x *g* for 45 min, and the supernatant was used as the cytoplasmic protein fraction.

Enzyme assays: Activities are expressed in units (U) per milligram of protein where 1 U is equivalent to 1 μmol of substrate transformed min^{-1} at 80 °C. Protein concentrations were estimated using Bradford method [Bradford, 1975] using bovine serum albumin as a standard. All the assays were carried out anaerobically in rubber stopper-sealed glass cuvettes that had been degassed and flushed with argon. The buffer used was 50 mM *N*-(2-hydroxyethyl) piperazine-*N*'-3-propanesulfonic acid (EPPS) buffer (pH 8.4). FOR, AOR and GAPOR activities were determined by measuring the reduction of 3 mM benzyl viologen (BV) at 600 nm [ε= 7,400 (M^{-1} cm^{-1})]. FOR activity was determined

with formaldehyde (50 mM) or glutaric dialdehyde (20 mM) as the substrate whereas AOR and GAPOR activities were measured using 0.3 mM crotonaldehyde and 0.4 mM glyceraldehyde-3-phosphate, respectively [Mukund and Adams, 1991 and 1995].

Results

The growth rate of *P. furiosus* in 20 liter cultures at 95 °C using media containing maltose, maltose + S° and maltose plus peptides were not significantly different from each other [Adams et al., 2001]. Doubling times were all in the range 65.2 ±1.5 min. Growth was most rapid when cultures were grown on peptides plus S° (both with and without maltose) with doubling times of ~ 45 min. In fact, growth was extremely poor when cultures were grown in peptide medium without S° after the first transfer, and no significant growth occurred after a second transfer, suggesting that S° is required for growth of *P. furiosus* on peptides but not on maltose.

The specific activities of AOR, FOR and GAPOR were measured in cells grown under five different conditions in the 20 liter cultures (Fig. 4.2). All three aldehyde oxidoreductases are known to be present in the cytoplasmic fraction and were assayed in the cell-free extract and in the supernatant fractions. As shown in Fig. 4.2, AOR specific activity was virtually the same in cells grown on maltose or peptides-plus-S° (with and without maltose) but decreased significantly when *P. furiosus* was grown on maltose plus S°. FOR specific activity was comparable in cells grown on maltose and maltose-plus-peptides (rich medium), but increased almost two-fold when the medium contained peptide + S° or maltose-plus-peptides + S°. The specific activities of both FOR and AOR decreased considerably when S° was added to the maltose only medium. The specific

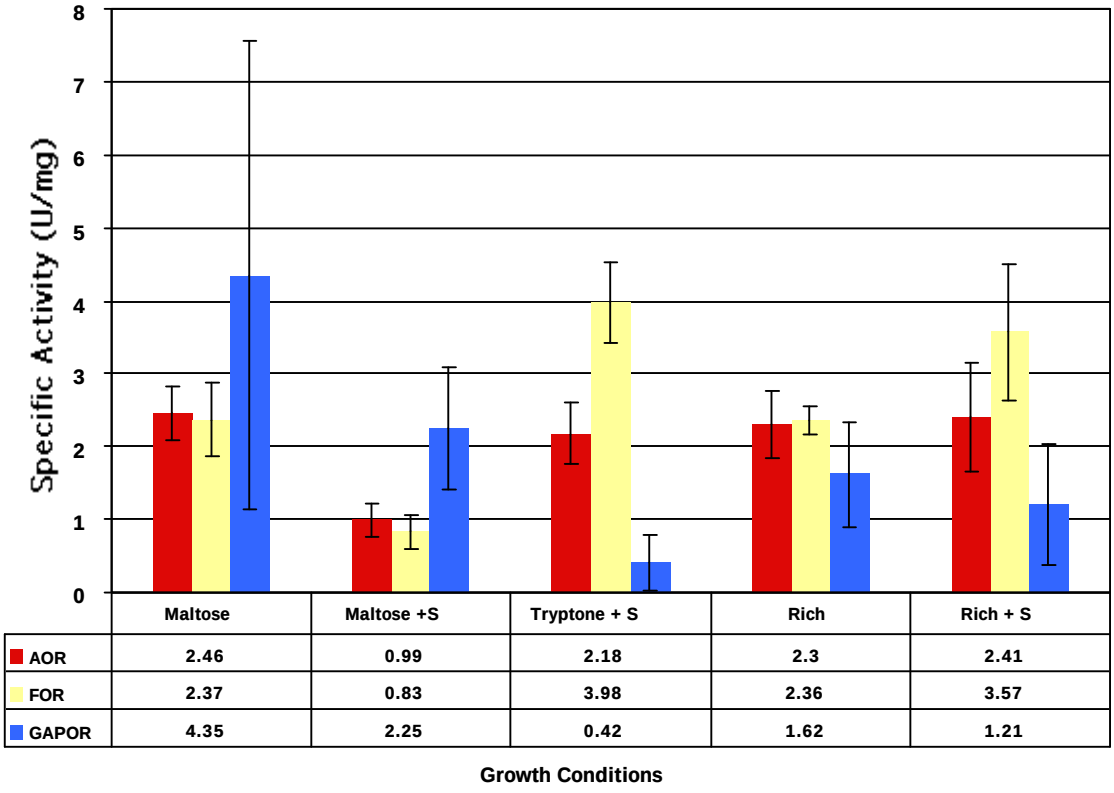
activity of the glycolytic enzyme, GAPOR, was the lowest in cells grown on the peptides-plus-S° medium and highest in maltose-grown cells. However, the values obtained varied considerably with the different growth conditions probably because of the inhibitory effect of sodium dithionite (which was present in all buffers) on the enzyme [Mukund and Adams, 1995].

To measure the effects of adding amino acids Arg, Pro, Lys and ornithine to the *P. furiosus* growth medium on the activity of the aldehyde oxidoreductases, we initially determined the amount of growth substrate that limited growth. Under our usual growth conditions for *P. furiosus*, the major carbon source is 0.5% (w/v) of either maltose or peptides (casein hydrolysate). Since the goal was to determine if *P. furiosus* would use these amino acids as a carbon source, carbon-limited growth conditions had to be defined. Maltose was used as the carbon source and low concentrations (≤ 0.05%) of yeast extract were also added to provide vitamins and factors essential for growth. *P. furiosus* cells were grown in media according to Table 4.1. The cells underwent at least four successive transfers in each medium and the final cell density for each is presented in Table 4.1.

Figure 4.3 outlines cell densities of *P. furiosus* cells in three successive transfers. Cultures 1 and 2 (see Table 4.1) with maltose and yeast extract concentrations of 0.4%/0.05% and 0.4%/0.025% respectively, displayed growth rates and cell densities very similar to that of *P. furiosus* cultures grown under the usual conditions [Adams et al., 2001]. Cultures 3 and 4 with maltose/yeast extract compositions of 0.4%/0.01% and 0.2%/0.05% respectively, initially grew slowly but seemed to become adapted by the third transfer and grew to cell densities comparable to those of cultures 1 and 2.

Fig. 4.2.

Specific activities of AOR, FOR and GAPOR in *P. furiosus* cells grown on various substrates. All enzyme activities were measured in the cytoplasmic fraction. Rich medium indicates a combination of maltose, casein hydrolysate and yeast extract each at 0.5% (w/v) concentration. The values for the specific activity of each of the three enzymes under the different growth conditions are presented. The results are an average of three independent determination and standard deviation values are indicated. Data taken from Adams et al., 2001.



	Maltose	Maltose +S	Tryptone + S	Rich	Rich + S
AOR	2.46	0.99	2.18	2.3	2.41
FOR	2.37	0.83	3.98	2.36	3.57
GAPOR	4.35	2.25	0.42	1.62	1.21

Table 4.1. Various combinations of substrates used for *P. furiosus* cultures

Medium composition[a]	**Maltose % (wt/vol)**	**Yeast extract % (wt/vol)**	**Cell count**[b] **($x10^8$ ml^{-1})**
1.	0.4	0.05	7.06
2.	0.4	0.025	5.46
3.	0.4	0.01	3.90
4.	0.2	0.05	4.73
5.	0.2	0.025	1.99
6.	0.2	0.01	1.08
7.	0.1	0.05	1.31

[a]The only variables in the experimental media were the maltose and yeast extract concentrations. All other constituents remained the same.

[b]Denotes maximum cell density of culture transferred three times in the indicated medium.

Cultures 5, 6 and 7 displayed slow growth rates with lower cell densities and then remained unchanged with subsequent transfers.

Cultures 4, 5, 6 and 7 were chosen to study the effects of amino acid addition studies, as these appeared to be carbon-limited for growth. To each of these cultures a combination of amino acids (Arg, Lys, Pro and ornithine) was added to a final concentration of either 1 mM or 5 mM. Otherwise, the media remained the same. The cells were grown and transferred twice in this medium. They were harvested in the late-log phase. Cultures without supplemented amino acids served as control. Cell-free extract prepared from these cells was assayed for activity of FOR, AOR and GAPOR. Since growth rates for the media with 1 mM and 5 mM amino acids were similar, therefore, 1mM amino acid mixture was used for the growth studies.

Figure 4.4 summarizes the results including the cell densities obtained during the various growth conditions and specific activity of the three aldehyde oxidoreductaes AOR, FOR and GAPOR, in these cells. Culture 4 grown in a medium containing maltose/yeast composition of 0.4%/0.05% did not show a significant change in cell density on addition of the amino acid mixture (Fig. 4.4). However, in such cells, the specific activity of AOR increased almost 2.3 fold and surprisingly, FOR activity decreased slightly while GAPOR activity remained the same.

Culture 5 on the other hand, did show a dramatic increase in the cell density on the addition of the mixture of amino acids. The final cell count went up over 2-fold. AOR specific activity in the cell-free extracts of these cells increased 2.5 fold (as compared to the control culture without any added amino acids) while FOR and GAPOR activity remained approximately the same.

Fig. 4.3.

Growth rates of *P. furiosus* cells on various substrates. Final cell densities for the first three transfers are indicated. Labels on the X-axis indicate the medium composition for each culture as % maltose (w/v) /% yeast extract (w/v).

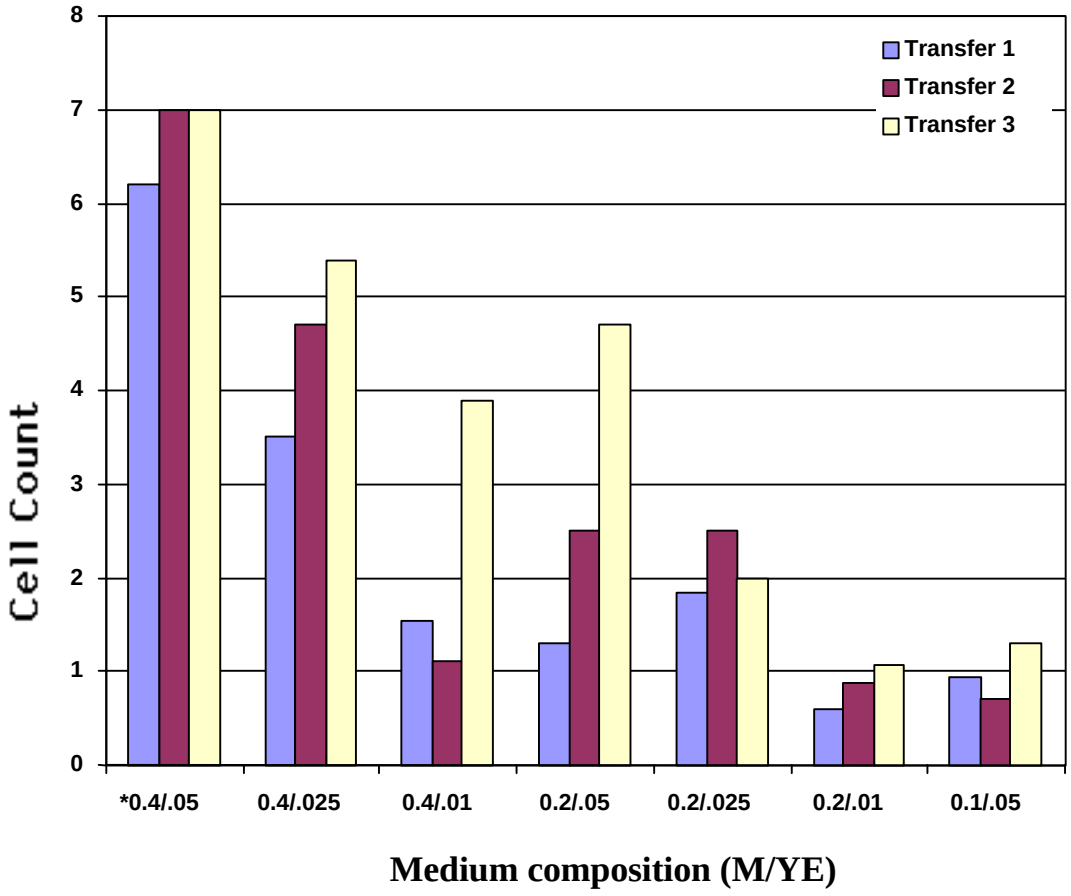
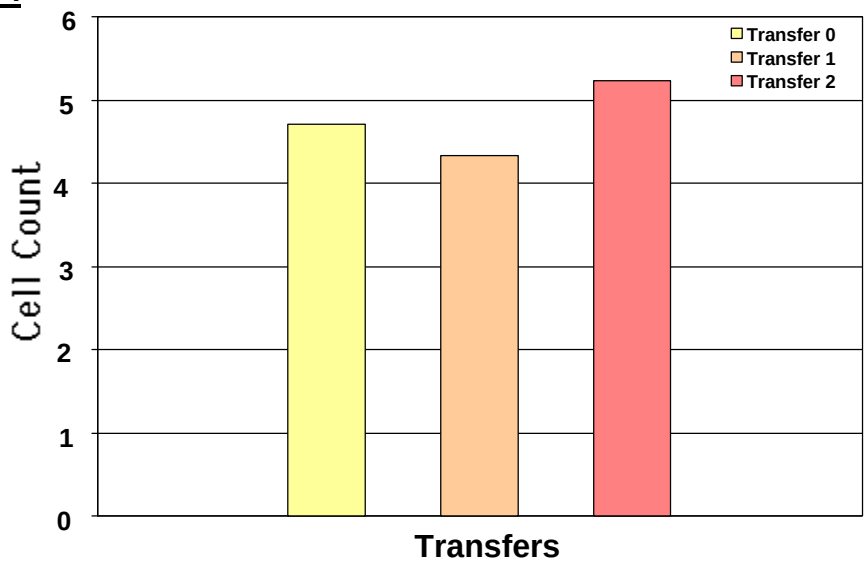

Transfer 1
Transfer 2
Transfer 3
Cell Count
0
1
2
3
4
5
6
7
8
*0.4/.05
0.4/.025
0.4/.01
0.2/.05
0.2/.025
0.2/.01
0.1/.05
Medium composition (M/YE)

Fig. 4.4.

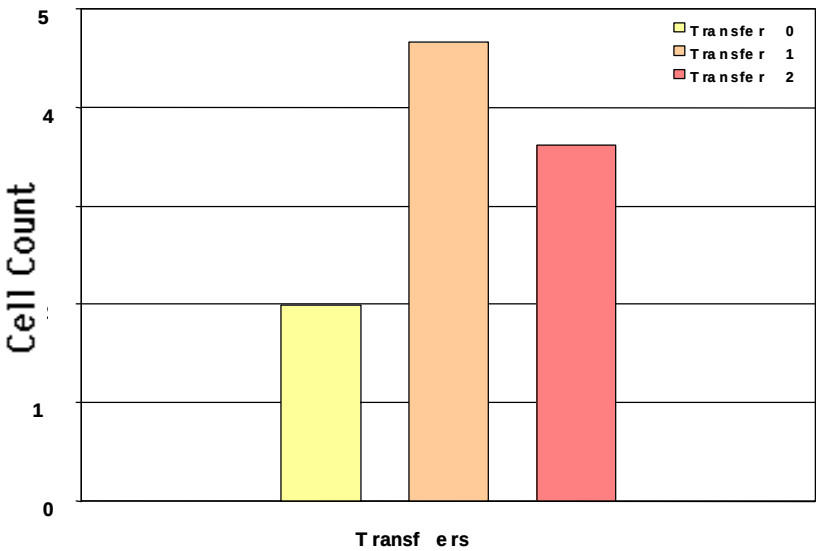
Effect of supplemented amino acids on the growth rate of *P. furiosus* cells, and the activities of AOR, FOR and GAPOR. The bar graph depicts maximum cell density (x 10^8 cells ml^{-1}) of cultures before (Transfer 0) and after (Transfers 1 and 2) addition of amino acid mixture. The tables display the specific activity of AOR, FOR and GAPOR in cells grown on the experimental medium with or without the addition of amino acids.

Culture 4



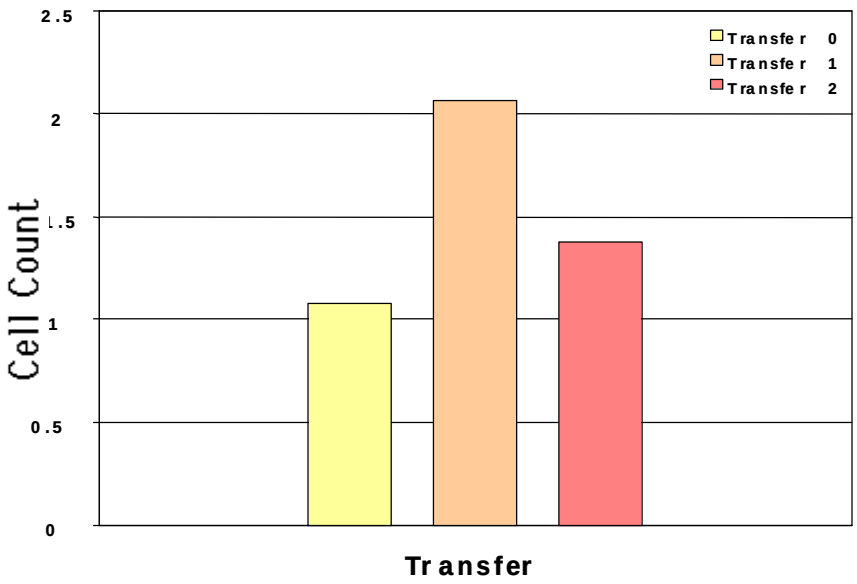
Growth conditions 4. 0.2% M/0.05% YE	Specific Activty (U/mg) AOR	FOR	GAPOR
No added amino acids	1.93	2.31	2.05
With 1mM amino acids	4.59	0.77	2.71

Culture 5



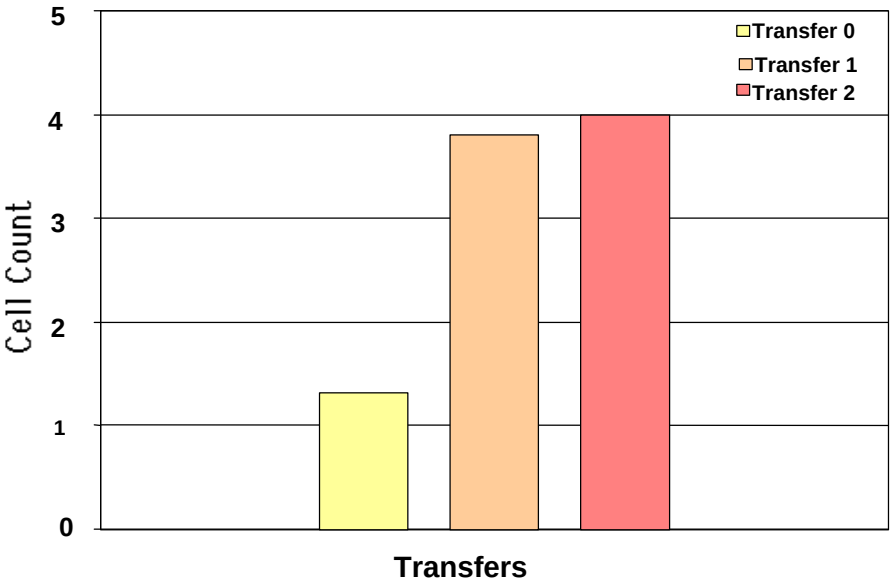
Growth conditions 5. 0.2% M/0.025% YE	Specific Activty (U/mg) AOR	FOR	GAPOR
No added amino acids	0.67	0.91	2.17
With 1mM amino acids	2.29	0.56	2.91

Culture 6



Growth conditions 6. 0.2% M/0.01% YE	Specific Activty (U/mg) AOR	FOR	GAPOR
No added amino acids	--	--	--
With 1mM amino acids	3.00	0.74	4.07

Culture 7



Growth conditions 7. 0.1% M/0.05% YE	Specific Activty (U/mg) AOR	FOR	GAPOR
No added amino acids	1.08	1.59	1.91
With 1mM amino acids	2.17	1.18	4.87

Culture 6 was grown in a medium containing a maltose/yeast extract composition of 0.2%/0.01%, showed very poor growth. In fact, the control cultures showed almost no growth and cells could not be harvested to prepare cell-free extract for enzyme assays. Growth was much better when the amino acid mixture was added, with a cell count of 2 x 10^8 ml^{-1} at late log-phase. The specific activities of AOR, FOR and GAPOR were measured in the cell-free extract, but these could not be directly compared with those of the control cells (no amino acids added) as these could not be assayed.

Culture 7, was grown using a maltose/yeast extract composition of 0.1%/0.05% but, displayed slow growth. The growth rate increased considerably when the amino acid mixture was added and the cell count increased almost three-fold. In this case, specific activity of AOR and GAPOR both increased two-fold, whereas FOR specific activity remained the same.

Discussion

Previous studies have shown that the glycolytic enzymes, GAPOR and GAPDH, are regulated in *P. furiosus* [van der Oost et al., 1998]. The activities of these enzymes increased five-fold and decreased seven-fold respectively, when *P. furiosus* was grown on cellobiose relative to growth on pyruvate. Expression of the GAPOR gene (*gor*) is regulated at the transcriptional level, while the activity of GAPDH appears to be regulated posttranslationally [Oost et al., 1998]. The results from our experiments seem to support GAPOR regulation. The specific activity of GAPOR is higher in cells grown on maltose and decreases when peptides were used as the sole carbon source. In contrast, AOR activity remained unchanged in cells grown on the various substrates. However, a slight decrease in AOR activity was observed when *P. furiosus* was grown on maltose

plus S° (Fig. 4.2). The specific activity of FOR remained about the same when the organism was grown on maltose or on the rich medium (Fig. 4.2). However, the specific activity increased approximately two-fold in cells grown either on the peptides plus S° or the rich plus S° medium. This supports the proposed role of this enzyme in the peptidolytic pathway of *P. furiosus* rather than – S° reduction, since FOR specific activity decreased considerably in cells grown on the maltose plus S° medium. The reason for this decrease which, is not seen in maltose only cells is not known at this point.

In the experiment designed to measure the effect of supplemented amino acids in the growth medium of *P. furiosus* on the specific activities of FOR, AOR and GAPOR, our initial goal was to establish limited growth conditions. With the growth medium used routinely in our laboratory (maltose or casein hydrolysate or yeast extract each at 0.5% (w/v) as the carbon source), *P. furiosus* cells grow rapidly to high cell densities (2.5×10^8 cells ml^{-1}). Under the usual conditions of growth, the cells have enough nutrients in the growth medium for robust growth. Of the various combinations of substrates used in the experimental media (Table 4.1), cultures 1 and 2 displayed growth properties closest to that seen with the medium used to routinely grow *P. furiosus*. Cultures 3 and 4 initially grew poorly, but the cells seemed to adapt to the medium and by the third transfer the final cell counts were high (Fig. 4.3). Cultures 5, 6 and 7, however, displayed noticeably slow growth rates and these remained unchanged on subsequent transfers. Of all the combination of substrates examined cultures 4, 5, 6 and 7 were selected to investigate the effects of additional amino acids. These cultures each had limited but consistent growth rates and any change in the growth rate in response to modification to the medium would be easily discernable. For all cultures, similar results were obtained when amino acid

mixture was added at a final concentration of either 1 mM or 5 mM. Therefore, for the final scaled up cultures (1L) amino acids were added to a final concentration of 1 mM. Addition of 0.1% (w/v) S° did not seem to effect the growth of any of the cultures.

With the exception of culture 4, all cultures showed moderate to dramatic increases in cell density and growth rates on the addition of the amino acid mixture (Fig. 4.4). The most dramatic increases (almost three-fold), were seen for cultures 5 and 7 and these were sustained in subsequent transfers, demonstrating that the change in growth was response to the addition of the amino acids.

The specific activity of AOR increased in all the cultures when the amino acids were added, and this agrees with the enzyme's proposed role in the peptide metabolism [Ma et al., 1997, Heider et al., 1995]. Although the increase in specific activity was modest (~ 2-fold), it was consistently higher in all cultures. Whether, this is in response to the addition of amino acids or to the increased rate of growth of the cells is not known. GAPOR activity remained constant for all the cultures. This is expected, since the primary carbon source for these cells is carbohydrate (maltose) and GAPOR plays a key role in the glycolytic pathway of *P. furiosus*.

Contrary to expectations, the specific activity of FOR did not change dramatically in response to the addition of amino acids to the medium and in fact, decreased slightly. Based on these results, FOR does not seem to play a role in the catabolism of these basic amino acids. However, there are certain unknown factors that preclude making this conclusion. One is the uptake of these amino acids by *P. furiosus*. The organism is routinely grown on peptides (casein hydrolysate, peptone or yeast extract) in the form of chemically or enzymatically hydrolyzed proteins. Extracellular proteases produced by *P.*

furiosus further break down these peptides into oligopeptides [Cowan et al., 1987]. The uptake of amino acids from the growth medium has been previously investigated for anaerobic hyperthermophilic archaea. These microorganisms actively transport low molecular weight organic compounds such as amino acids – and store them such that the intracellular concentration is 50 – 150 fold higher than outside [Usenko et al., 1993]. The rate of uptake of amino acids is independent of the carbon source. However, even though *P. furiosus* is capable of taking up free amino acids from the medium, growth of this organism solely on a mixture of 20 amino acids has been reported to be extremely weak [Raven and Sharp, 1997]. Enzyme activities from such cells grown on mixtures of individual amino acids have not been reported.

One possibility is that unlike GAPOR, FOR and AOR are not significantly regulated in response to the growth conditions. Instead of being specific for a single substrate, both enzymes display a much broader substrate specificity and may well be active in multiple metabolic pathways in the oxidation of a variety of aldehydes.

So, even though an increase in FOR activity in response to addition of basic amino acids was not observed, a role for this enzyme in the peptidolytic pathway cannot be completely ruled out. Future studies should attempt to investigate the regulation of these enzymes at the transcription level. Microarray analysis of mRNA expression levels of some of the key genes in the amino acid metabolic pathways (as well as FOR and AOR) under different growth conditions should provide clues to the physiological role of these enzymes in *P. furiosus*.

CHAPTER 5

PHYSIOLOGICAL SIGNIFICANCE OF SULFIDE-ACTIVATION OF TUNGSTEN-CONTAINING ALDEHYDE OXIDOREDUCTASES FROM THE HYPERTHERMOPHILIC ARCHAEON *Pyrococcus furiosus*[1]

[1]Roy, R., and M. W. W. Adams. To be submitted to *J. Bacteriol.*

Introduction

In the past two decades, numerous species of anaerobic hyperthermophiles have been isolated from solfataric fields and submarine hydrothermal systems [Blochl et al, 1995, Stetter, 1996]. These organisms exhibit optimal growth at temperatures of 90 °C or above, and most of them belong to the domain *Archaea* [Woese, 1990]. Virtually all of them are strict anaerobes, and most are heterotrophs. All of the heterotrophic microorganisms utilize peptides as a carbon source, although a few saccharolytic species are also known. A majority of the hyperthermophiles depend on the reduction of elemental sulfur (S°) to H_2S for significant growth and a number of them obtain energy for growth by S° respiration. S° is the most common sulfur species in terrestrial and marine volcanic environments, the habitats for the majority of these hyperthermophilic microorganisms [Bonch-Osmolovskaya, 1994]. Some species of *Pyrococcus* and *Thermococcus* can grow with or without S° by fermentative-type mechanisms. Most of these organisms only utilize peptide-related substrates as a carbon source, but *Pyrococcus furiosus*, *P. glycovorans* and *P. woesei* can metabolize polysaccharides as well as peptides [Fiala and Stetter, 1986, Barbier et al., 1999, Blamey et al., 1999]. *P. furiosus* (optimum growth temperature, 100 °C) is an anaerobic heterotroph that grows on peptides (casein, peptone, yeast extract) and also uses both simple (maltose, cellobiose) and complex (starch, glycogen) sugars. *P. furiosus* metabolizes carbohydrates by an unusual fermentation-type pathway producing acetate, CO_2, H_2 and alanine as end products [Kengen et al., 1994, Mukund and Adams, 1995]. When S° is present in the medium, it is reduced to H_2S, with a corresponding decrease in the amount of H_2 produced [Fiala and Stetter, 1986]. Peptide catabolism in *P. furiosus* is thought to

involve four distinct 2-keto acid oxidoreductases that convert transaminated amino acids into their corresponding coenzyme A (CoA) derivatives, and then transform them to organic acids, with concomitant substrate-level phosphorylation to form ATP [Heider et al., 1996, Mai and Adams, 1994, 1996, 1996a]. Recent growth studies of *P. furiosus* have indicated that this microorganism efficiently utilizes peptides only in the presence of S°, although growth on carbohydrates is unaffected by S° [Adams et al., 2001]. The precise mechanisms by which H_2 is evolved and S° is reduced are not yet clearly understood. *P. furiosus* contains two cytoplasmic, NAD(P)H-dependent hydrogenases, both of which can reduce S° *in vitro* [Bryant and Adams, 1989, Ma et al, 1993, Ma and Adams, 2000], and an H_2-evolving, membrane bound hydrogenase complex, which does not reduce S° to H_2S *in vitro* [Sapra et al., 2000].

S° is nearly insoluble in water [5 μg/l at 25 °C; Boulegue, 1978], even at 100 °C and it is unlikely that it is directly reduced to S^{2-} by these microorganisms. Therefore, S° must be converted to a more 'hydrophilic' or 'colloidal' form prior to reduction [Zophel et al., 1988, Fauque et al., 1991]. In anaerobic aqueous solutions polysulfides are formed from S° and sulfide according to Eq. 5.1 [Teder, 1971, Giggenbach, 1972]

$$nS^\circ + HS^- \longrightarrow S^{2-}_{n+1} + H^+ \qquad (5.1)$$

Polysulfides are composed of a sulfide dianion and a chain of sulfur atoms in the zero oxidation state. They are soluble in water and can serve as soluble intermediates for sulfur reduction by some bacterial species [Klimmeck et al., 1991]. Saturation concentrations of polysulfides can be obtained from S° in aqueous sulfide solutions at

neutral or basic pH and high temperatures (≥70 °C). The equilibrium concentration of polysulfide in sulfide solution is a function of pH and sulfide concentration, with decreasing pH equilibrium concentration of polysulfide drops drastically [Schauder and Kroger, 1993]. However, the equilibrium concentration increases with increasing temperatures. Thus, 0.1mM S° will dissolve at pH ≥ 6.7 and at 30 °C, while the same amount will dissolve in solution at pH 5.5 at 90 °C. The amount of S° that can maximally be dissolved in a sulfide solution at pH 8.0 and 37 °C is nearly equivalent to the sulfide concentration [Klimmeck et al., 1991]. The length of the polysulfide chain depends on the solution pH. At pH ≥ 7.0, tetrasulfides (S_4^{2-}) and pentasulfides (S_5^{2-}) appear to be the predominant species [Giggenbach, 1972].

Respiration with polysulfide (S°) as the terminal electron acceptor is carried out by several species of bacteria and archaea. These include members of the archaeal genera *Acidianus*, *Stygiolobus*, *Thermoproteus*, *Pyrobaculum* and *Pyrodictum*, and of the bacterial genera, *Wolinella* and *Desulfuromonas*, all of which gain energy by so-called lithotrophic S° respiration. In contrast, members of the archaeal genera *Desulfurococcus*, *Staphylothermus*, *Hyperthermus*, *Thermococcus* and *Pyrococcus*, and of the bacterial genera, *Thermotoga*, are strictly heterotrophic S° reducers [Stetter, 1996]. Early growth studies of *P. furiosus* have shown that polysulfide accumulates in the growth medium (when supplemented with S°) during growth and reaches concentrations of ~ 0.3 mM [Blumenthals et al., 1990]. However, as growth proceeds, the polysulfide concentration decreases considerably with a concomitant increase in H_2S in the head-space, indicating that polysulfide serves as the soluble intermediate of S° reduction for this microorganism. Further, uptake experiments with ^{35}S show that the elemental sulfur (S°) added to the

medium is not transported inside the cell, suggesting that sulfur reduction in *P. furiosus* probably occurs extracellularly and that it is a membrane-associated event [Blumenthals et al., 1990].

The growth of *P. furiosus* is stimulated by the trace mineral tungsten (W) [Bryant and Adams, 1989]. Three tungsten-containing aldehyde oxidizing enzymes, aldehyde ferredoxin oxidoreductase (AOR, Mukund and Adams, 1990, 1991), formaldehyde ferredoxin oxidoreductase (FOR, Mukund and Adams, 1993, Roy et al., 1999) and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR, Mukund and Adams, 1995) were isolated from this organism. AOR, FOR and GAPOR show high sequence similarity and are members of the 'AOR family' of tungstoenzymes. All of them consist of a single type of subunit that contains a mononuclear W coordinated by four dithiolene sulfur atoms from two pterin molecules, together with a single [4Fe-4S] cluster coordinated by four sulfur atoms from four cysteine residues [Chan et al., 1995, Hu et al., 1999]. However, the tertiary structures of these enzymes are very different, GAPOR is thought to be monomer whereas AOR is a dimer and FOR is tetrameric.

All three enzymes catalyze the oxidation of various types of aldehydes to the corresponding acid using ferredoxin (Fd) as the physiological electron acceptor. However, they differ in their substrate specificity. AOR oxidizes a wide range of both aliphatic and aromatic aldehydes, and it has the highest affinity for aldehyde substrates that are derived from amino acids during peptide fermentation [Heider et al., 1995]. FOR has a more limited substrate range and oxidizes C_5 – C_6 di- and semialdehydes and C_1 – C_3 aldehydes. FOR is proposed to function in the catabolism of certain amino acids such as Arg, Lys and Pro, since metabolic pathways of these amino acids have C_4 – C_6

semialdehyde intermediates [Bender, 1985, Gottschalk, 1986]. GAPOR, on the other hand, is extremely specific for its substrate glyceraldehyde-3-phosphate, which it oxidizes to yield 3-phosphoglycerate. GAPOR serves as a key enzyme in the sugar fermentation pathway of *P. furiosus* [Mukund and Adams, 1995].

The enzymes of the AOR family are extremely sensitive to oxygen inactivation, rapidly losing activity when exposed to air. In fact, during their purification from *P. furiosus*, a significant loss of activity is observed even when strictly anaerobic conditions are maintained. A preliminary study showed that FOR could be activated several-fold by treating it with sulfide under reducing conditions [Mukund, 1995, Roy et al., 1999]. Herein we report on the kinetics of sulfide-activation of *P. furiosus* FOR and show that AOR and GAPOR also undergo sulfide activation. In addition, we show that selenide can replace sulfide in the activation process. The question also arises as to whether sulfide-activation is a physiological process. This is very relevant to *P. furiosus* as the organism can grow both with and without the presence of S° in the medium. To address this issue, intracellular concentrations of various sulfur species such as acid-labile sulfide and polysulfide were estimated in *P. furiosus* cells grown with and without S°. The results are correlated with the specific activities of these three tungstoenzymes from the same cell types.

Material and Methods

Growth of organism: *P. furiosus* (DSM 3638) was grown in a 20-liter fermentor containing 15 liters of medium, prepared as described previously [Verhagen et al., 2000, Adams et al., 2001]. The medium composition was termed ‘rich’ as it consisted of 0.5%

(w/v) each of maltose, tryptone and yeast extract as the carbon sources. S° was added (0.1%, w/v) to give the rich-plus-sulfur medium. *P. furiosus* cells that had undergone at least four successive transfers in each medium (rich or rich-plus-sulfur) were used to innoculate the 20 liter fermentor. Cells were harvested in the late-logarithmic phase of growth (~ 4 x 10^8 cells per ml). The cultures were cooled to room temperature and then harvested by centrifugation. Cultures with S° were initially centrifuged at 1,000 x *g* for 10 min (Beckman J2-21 centrifuge, JA-10 rotor) to separate solid S° particles followed by a spin at 10,000 x g for 15 min. Harvested cells were washed three times in isotonic buffer (50mM Tris-HCl, pH 8.0 containing 100mM NaCl), frozen in liquid N_2 and stored at –80 °C. AOR, FOR and GAPOR were purified from frozen cells (500 g [wet weight]) under strictly anaerobic conditions at 23 °C as described [Roy et al., 2001]. *E. coli* (strain BL21 (DE3)) was grown aerobically in a medium containing Luria broth (LB) at 37 °C with constant shaking for ~12 hr. Cells were harvested, washed with isotonic buffer (50mM Tris-HCl, pH 8.0 containing 100 mM NaCl), frozen in liquid N_2 and stored at –80 °C. For growth under anaerobic conditions, the medium contained LB and 1 % glucose and the cells were grown at 37 °C for 12 hr without shaking. After growth the cells were harvested as described above.

Cell fractionation: All sample transfers and manipulations were carried out in an anaerobic chamber and all buffers were degassed and flushed with Ar. Sodium dithionite and dithiothreitol (2 mM each) were added to the cell-free extracts used for enzyme assays, but not for estimation of sulfide and polysulfide (see below). For assays the cells were suspended in buffer (50mM Tris-HCl, pH 8.0, 1g/3mls) containing deoxyribonuclease I (0.0002%, w/v). The cell suspension was incubated at room

temperature with shaking for 30 min. The cells were then disrupted under Ar by sonication for 30 min (Branson Sonifier 450) by placing the sample vial in an ice-water slurry with the sonicator probe. Cell lysis was verified using phase-contrast microscopy. Debris and unbroken cells were removed by centrifugation (10,000 x *g* for 15 min in a Beckman L8-M ultracentrifuge with a 60 Ti rotor) and the supernatant was termed the whole-cell extract (WCE). To prepare the cytoplasmic fraction, the WCE was centrifuged at 100,000 x *g* for 45 min, and the precipitate was discarded.

Enzyme assays: Activities are expressed in units where 1U is equivalent to 1 μmol of substrate transformed min^{-1} at 80 °C, unless otherwise stated. Protein concentrations were measured using the Bradford method [Bradford, 1975], with bovine serum albumin as a standard. Aldehyde oxidoreductase assays were carried out anaerobically in rubber stopper-sealed glass cuvettes that had been degassed and flushed with Ar. The buffer used was 50 mM *N*-(2-hydroxyethyl)piperazine-*N*'–3-propanesulfonic acid (EPPS) buffer (pH 8.4). AOR, FOR and GAPOR activities were determined by measuring the reduction of 3 mM benzyl viologen (BV) at 600 nm [ε = 7,400 $(M \cdot cm)^{-1}$] using 0.3 mM crotonaldehyde, 50 mM formaldehyde, or 0.4 mM glyceraldehyde-3-phosphate, respectively, as the substrate. GAPOR assays were carried out at 70 °C, as its substrate GAP is unstable at higher temperatures.

Sulfide activation: The enzymes (5 - 10 mg/ml) was incubated with buffer (50 mM Tris-HCl, pH 8.0) containing 20 mM sodium dithionite and 20 mM sodium sulfide under anaerobic conditions at 23 °C. At regular intervals aliquots were withdrawn and assayed for enzyme activity. For selenide activation, 5 mM sodium selenide replaced sodium sulfide but all other conditions were the same. Oxygen inactivated enzyme was prepared

by exposing a sample of enzyme (2 - 5 mg/ml) to air and measuring enzyme activity at regular intervals.

Stability studies: The oxygen sensitivity of the various forms of the enzymes was measured by exposing the appropriate sample to air (protein concentration ~ 5 mg/ml in 50mM Tris-HCl, pH 8.0). At regular intervals samples were withdrawn and assayed for activity as described above. For high temperature stability studies, enzyme samples were incubated in stopper-sealed vials in a 80 °C water bath.

Acid-labile sulfide estimation: To measure intracellular sulfide concentrations, cells were suspended in buffer (50 mM CAPS, pH 10.5) and disrupted as described above. Acid-labile sulfide was quantitatively determined by the method of King and Morris (1967) with slight modifications. The cell extract (10 -50 µl) was shaken with 300 µl of distilled water and 250 µl of 1% (w/v) zinc acetate for 1 min at room temperature. 125 µl of *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride in 5.5 M HCl (0.1%, w/v), was added, followed by 50 µl of 0.0468 M ferric chloride in 1.2 M HCl. The sample was mixed vigorously with each addition and then was shaken for a further 1 min and incubated at room temperature for 30 min. 425 µl of deionized distilled water was added the suspension was centrifuged for 1 min in an Ependorf microfuge at 10,000 x *g*, and the absorbance was measured at 670 nm. A calibration standard curve was prepared with a stock solution of 200 µM sodium sulfide in 10 mM NaOH to give 1 – 20 nmol per assay. Sulfide concentrations were measured in the culture medium of *P. furiosus* both before and after growth and also in uninnoculated media that had been incubated at 95 °C (the routine growth temperature of *P. furiosus*) overnight. Intracellular sulfide concentrations were measured in *Thermococcus* sp. ES4 cells (which grow in the presence of S°), *E. coli*

cells grown both anaerobically and aerobically on minimal medium and in recombinant *E. coli* cells that harbor genes encoding FeS-containing proteins from *P. furiosus* such as ferredoxin and pyruvate ferredoxin oxidoreductase (POR) δ subunit. Total acid-labile sulfide was estimated using the sodium sulfide standard curve and this value was normalized per mg protein and then converted to cell volume where 5 µl cell volume is equivalent to 1 mg protein [Ramakrishna et al, 1997].

Whole cell extracts of *P. furiosus* cells grown with and without S° were further separated into high and low molecular weight fractions by ultrafiltration. The extract (~ 4 ml, ~ 22 mg/ml) was loaded into an Amicon ultrafiltration cell fitted with PM-30 membrane, (exclusion size 30 kDa) and was washed with 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl. The pass-through (< 30 kDa, ~6 ml, ~ 0.2 mg/ml) and retained (> 30 kDa, ~ 2.5 ml, ~25 mg/ml) fractions were collected and stored under strictly anaerobic conditions. Ferredoxin concentration was estimated in the < 30 kDa fraction using an extinction coefficient of 17,000 M^{-1} cm^{-1} at 390 nm [Aono et al., 1989].

Polysulfide estimation: A stock solution of polysulfide was prepared by adding sodium tetrathionate (as an oxidant of sulfide) to a 2 – 5 mM solution of sodium sulfide in 100 mM CAPS pH 8.5 buffer [Schauder and Muller, 1993]. The content of S° in the form of both polysulfide and S° is adjusted by the amount of tetrathionate added to the sulfide solution [Klimmek et al., 1997]. Polysulfide is extremely unstable and was stored under anoxic condition and used within a few hours of preparation. Polysulfide concentrations were measured spectrophotometrically using an extinction coefficient of 365 M^{-1} cm^{-1} at 365 nm [Schauder and Muller, 1993]. To measure the intracellular concentration of polysulfide, cells were suspended in 100 mM CAPS buffer pH 9.0 and disrupted as

described above. Polysulfide concentration was determined by the cold cyanolysis method [Then and Truper, 1983]. In brief, 0.5 ml of 100 mM CAPS (pH 8.5) and 0.1 ml of 0.2 M sodium cyanide were added to the extract (0.05 – 0.10 ml) and incubated at 30 °C for 1 h. The reaction mixture was then cooled, 0.9 ml of 2.5% (w/v) zinc acetate was added and the precipitate formed was removed by centrifugation at 10,000 rpm for 3 min. Thiocyanate formation was determined by adding 0.1 ml of 0.75 M ferric nitrate in 20% (v/v) nitric acid to 0.9 ml of the reaction supernatant. The absorbance was measured at 460 nm and concentrations were calculated using a standard curve prepared with sodium thiocyanate (0 – 250 μM).

Results

Activation of enzymes with sulfide and selenide: When purified FOR was incubated with excess sodium sulfide (20 mM) and sodium dithionite (20 mM) at 23 °C (pH 8.0), there is almost a 5-fold increase in specific activity over a 5 hr period (Fig. 5.1a) [Roy et al., 1999]. No activation is observed if either dithionite or sulfide is omitted. The degree of activation varied from preparation to preparation, but all samples analyzed showed at least a three-fold increase in specific activity. The optimal conditions for sulfide activation required a concentrated protein sample (> 6 mg/ml) and a pH of 8 – 10, with the highest degree of activation at pH 8.0 (data not shown). The degree of activation by sulfide of the as purified form of FOR (at pH 8.0 and 23 °C) was not significantly affected by increasing the incubation temperature from 23 °C to 60 °C. After the enzyme had been activated with sulfide, the excess sulfide was removed (by gel filtration with 50 mM Tris-HCl, pH 8.0, containing 2 mM sodium dithionite, 2 mM DTT, and 10% (v/v)

glycerol), the specific activity of the resultant sulfide-free form of the activated enzyme decreased by ~20%. However, this activity remained stable even after a further 24 hr under anaerobic conditions (in the absence of sulfide but the presence of sodium dithionite).

The activation of the native form of FOR by sulfide prompted us to determine if sulfide had the same effect on the related *P. furiosus* enzymes, AOR and GAPOR. Like FOR, both enzymes are routinely purified anaerobically in the presence of 2 mM dithiothreitol. With AOR, all purification buffers also include sodium dithionite (2 mM) but GAPOR is inhibited by prolonged incubation with this reagent [Mukund and Adams, 1995]. As purified AOR responded to sulfide treatment in a fashion similar to that of FOR, with a 2 - 3 fold activation over a 4 hr period and an increase in specific activity from 20 to 50 units/mg (Fig. 5.1b). No activation occurred if sulfide or sodium dithionite were omitted from the reaction mixture. In addition, when AOR was partially inactivated by treatment with O_2 by a 12 hr exposure to air and was then treated with sodium sulfide and sodium dithionite, the original specific activity (prior to oxygen inactivation) was restored after a 6 hr incubation (data not shown).

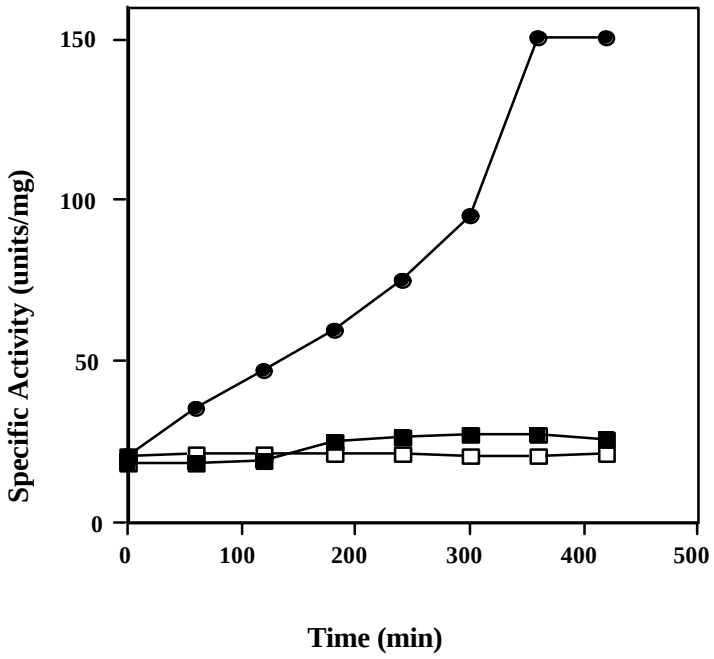
Similar activation experiments with GAPOR are complicated by the fact that this enzyme is inactivated by sodium dithionite. Therefore, titanium (III) citrate (10 mM) was used as the reductant for its activation process. Moreover, as shown in Fig. 5.1b, both the rate and extent of activation of GAPOR were greater than for either AOR or FOR. Thus all three aldehyde oxidoreductases from *P. furiosus* are activated by sulfide, although to different extents (Fig. 5.2). AOR showed the smallest increase upon sulfide treatment, whereas FOR and GAPOR were activated by up to 5- and 8–fold, respectively.

Fig. 5.1.

(a) Activation of *P. furiosus* FOR by sulfide. Enzyme (10 mg/ml in 50 mM Tris-HCl, pH 8.0) was incubated at room temperature with either sodium sulfide (20 mM, open squares), sodium dithionite (20 mM, filled squares), or both (filled circles). At the indicated times, samples were removed and the residual activities were determined with formaldehyde as the substrate under standard assay conditions (see Materials and Methods). Taken from Roy et al., 1999.

(b) Activation of *P. furiosus* AOR and GAPOR by sulfide. *P. furiosus* AOR (8 mg/ml) was incubated with sodium dithionite (20mM, open triangles) or sodium sulfide and sodium dithionite (20 mM each, filled triangles). *P. furiosus* GAPOR (12 mg/ml) was treated with reductant only (titanium (III) citrate, 10 mM, open diamonds) and sodium sulfide (20 mM) and reductant (filled diamonds).

(a)


150
100
50
0
Specific Activity (units/mg)
0
100
200
300
400
500
Time (min)

(b)

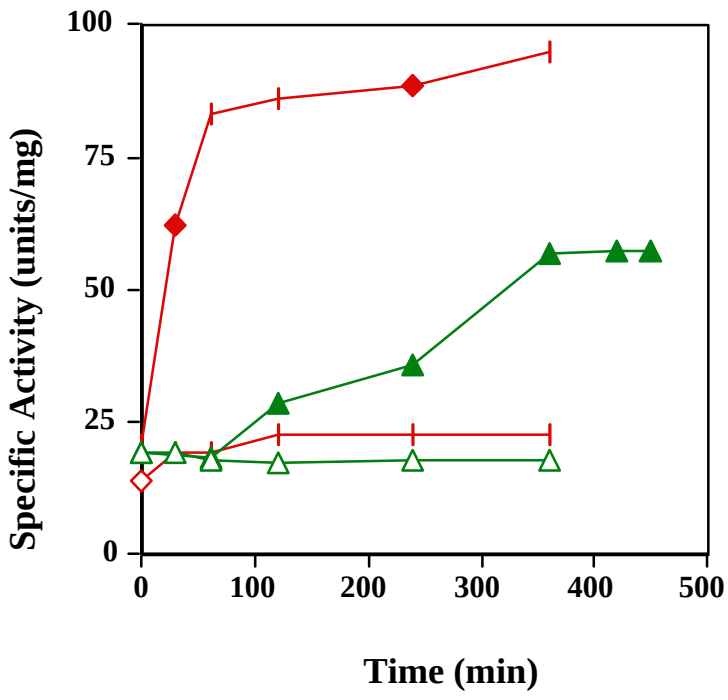
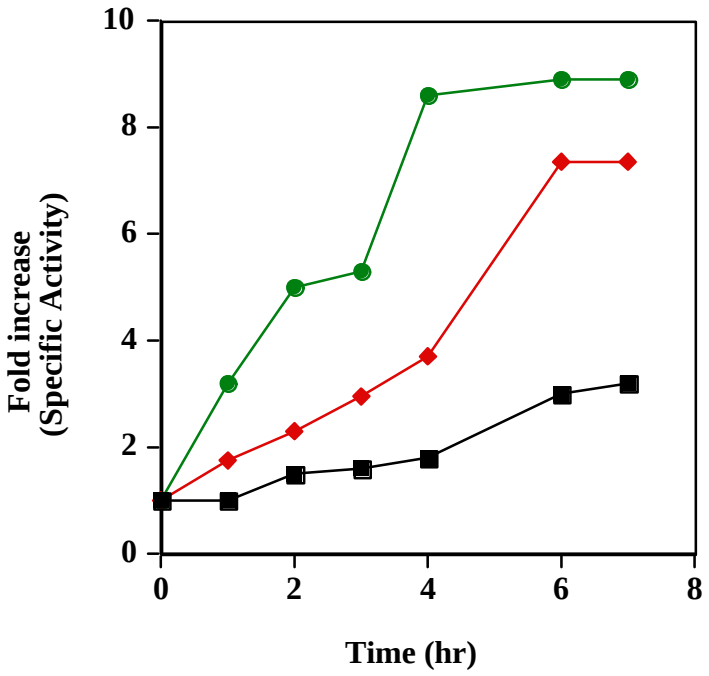

100
75
50
25
0
Specific Activity (units/mg)
0
100
200
300
400
500
Time (min)

Fig. 5.2.

Fold increase in specific activity of AOR, FOR and GAPOR on activation with sulfide. Sodium sulfide was added to the enzyme samples at time zero. The activities of AOR (squares), FOR (diamonds) and GAPOR (circles) were measured over a period of 6 hr and are expressed relative to the zero time values.


Fold increase
(Specific Activity)
10
8
6
4
2
0
0
2
4
6
8
Time (hr)

P. furiosus FOR as purified under standard conditions is oxygen sensitive, with the $t_{50\%}$ being about 12 hr when the enzyme is exposed to air (Fig. 5.3a) (no activity was lost under anaerobic conditions). The same extent of inactivation of the enzyme was found when formaldehyde or glutaric dialdehyde were used as the substrate to assay the enzyme. GAPOR has a $t_{50\%}$ value similar to that of FOR while AOR is much more sensitive to oxygen, with a $t_{50\%}$ value of about 30 min in air (data not shown). Treatment with sulfide (and dithionite or titanium (III) citrate) activated the oxygen-inactivated form of FOR. For example, when a sample of FOR (specific activity ~25 u/mg) was exposed to air for ~ 18 hr, approximately 90 % of the original specific activity was lost (Fig. 5.3a). When this inactivated sample (specific activity ~ 2 u/mg) was incubated with sodium sulfide under anaerobic conditions, the original specific activity of the enzyme was regained over a period of 6 hr (Fig. 5.3b). No subsequent increase was seen on further incubation. Therefore, the oxygen-inactivated form of the enzyme can be activated by sulfide to about the same extent (8 - 10 fold) as the native as purified form (see Fig. 5.1). The same is true for oxygen-inactivated samples of AOR and GAPOR (data not shown).

When the oxygen-inactivated form of FOR was incubated with sodium selenide (5 mM) as a replacement for sodium sulfide, along with reductant (20 mM sodium dithionite), a 2 to 3-fold increase in specific activity was observed (Fig. 5.3c). This phenomenon was termed selenide activation. When incubated under similar conditions with selenide, *P. furiosus* AOR displayed very little activation (≤ 1.5 fold), whereas GAPOR (with titanium (III) citrate as reductant) showed about a 2-fold increase in activity (data not shown).

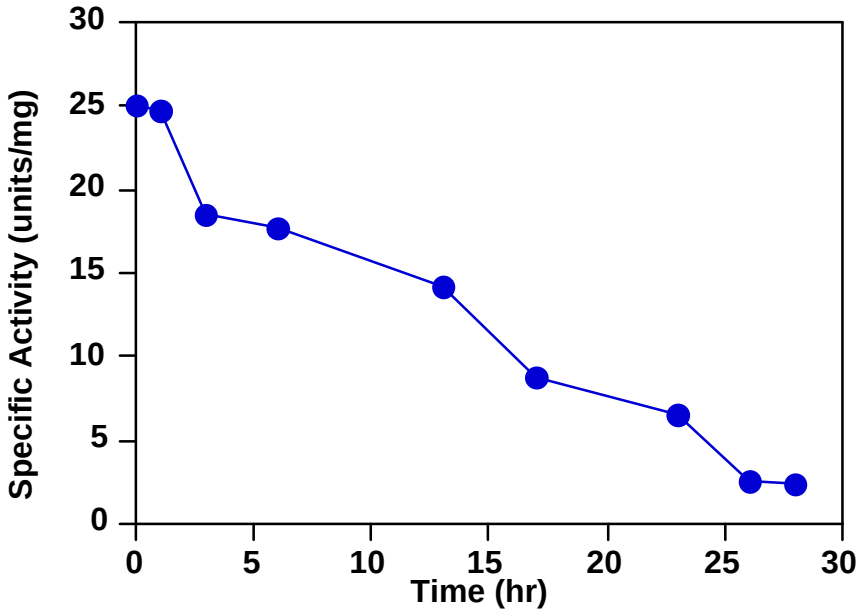
Fig. 5.3.

(a) Oxygen-inactivation of *P. furiosus* FOR. Enzyme (5 mg/ml) in buffer (50 mM Tris-HCl, pH 8.0) containing 2 mM dithiothreitol was exposed to air at 23 °C. Aliquots were withdrawn periodically and assayed under standard conditions.

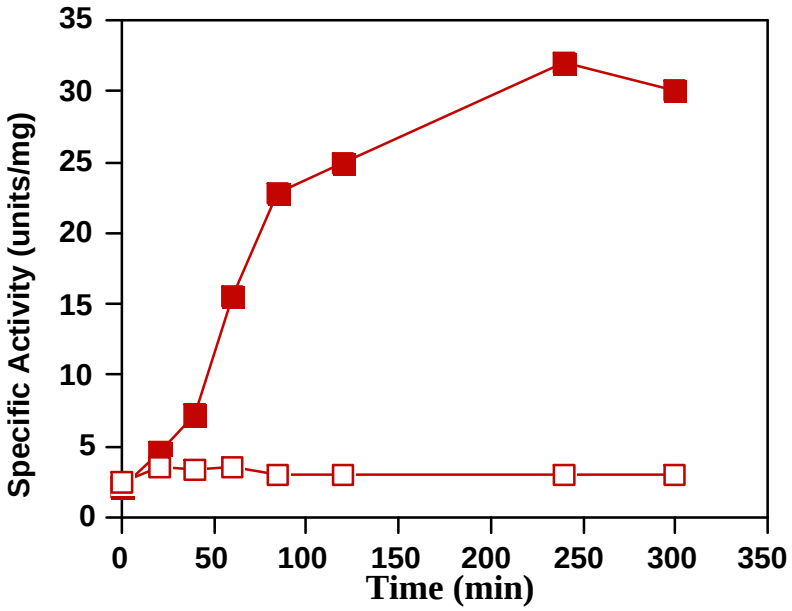
(b) Sulfide-activation of oxygen-inactivated FOR. A sample of oxygen-inactivated FOR (10 mg/ml in 50 mM Tris-HCl, pH 8.0, buffer) with 10% of the original (as purified) specific activity was incubated with either sodium dithionite (20 mM, open squares) or sodium sulfide (20 mM) and reductant sodium dithionite (filled squares). Aliquots were withdrawn at intervals and assayed for FOR activity.

(c) Selenide-activation of oxygen-inactivated FOR. A sample of oxygen-inactivated FOR (11.5 mg/ml in 50 mM Tris-HCl, pH 8.0, buffer) with 10% of the original (as purified) specific activity was incubated with either sodium dithionite (20 mM, open circles) or sodium selenide (5 mM) and reductant sodium dithionite (filled circles). Aliquots were withdrawn at intervals and assayed for FOR activity.

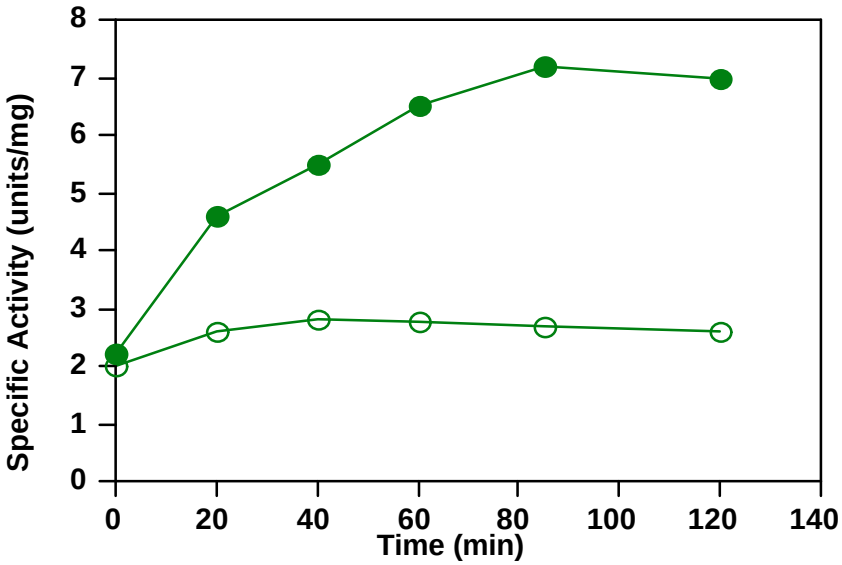
(a)


Specific Activity (units/mg)
Time (hr)
0
5
10
15
20
25
30

(b)


Specific Activity (units/mg)
Time (min)
0
5
10
15
20
25
30
35
0
50
100
150
200
250
300
350

(c)


Specific Activity (units/mg)
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
Time (min)

Properties of different forms of FOR: Experiments were carried out to compare the properties of the activated and inactivated forms of FOR with the 'as purified' form. For example, the sulfide-activated form of FOR was much more sensitive to inactivation by oxygen than the purified form, with a decrease in $t_{50\%}$ from 12 hr to approximately 4 hr (data not shown). In addition, in contrast to the sulfide-activated enzyme, the selenide-activated form of the enzyme was not stable. When the excess selenide was removed (by gel filtration) from the activated sample, the specific activity reverted to approximately the original level. On the other hand, the temperature optimum (for activity) was the same for the as purified, sulfide- and selenide-activated and oxygen-inactivated forms of FOR (data not shown).

The activity of as purified *P. furiosus* FOR was unaffected by incubation with a 40-fold excess of potassium cyanide (5 mM) for a period of four days (Fig. 5.4) but the sulfide-activated samples showed a pronounced cyanide inactivation. After the removal of excess sulfide, the activated sample had a specific activity of ~ 20 units/mg and this remained essentially unchanged upon incubation at room temperature for up to 24 h. However, when this sample was treated with excess sodium cyanide there was an approximately 60% loss of specific activity after a period of 20 h. Oxidation of the enzyme with thionine (2 mM) prior to cyanide treatment did not change the amount of inactivation. No further loss in activity was observed for the next four days under anaerobic conditions.

Substrate specificity studies: Kinetic constants for various substrates have been previously reported for the as purified and sulfide-activated forms of *P. furiosus* FOR [Roy et al., 1999].

Fig. 5.4.

Cyanide-inactivation of sulfide-activated *P. furiosus* FOR. After removal of excess sulfide and sodium dithionite by gel filtration, the enzyme (15 mg/ml) in 50 mM Tris-HCl, pH 8.0, was incubated at 23 °C with 2 mM thionine and 5 mM sodium cyanide (filled circle) or 5 mM cyanide alone (filled diamonds). A sample to which neither thionine or cyanide was added acted as control (filled squares). As purified FOR incubated with 5 mM cyanide is shown by the filled triangles.

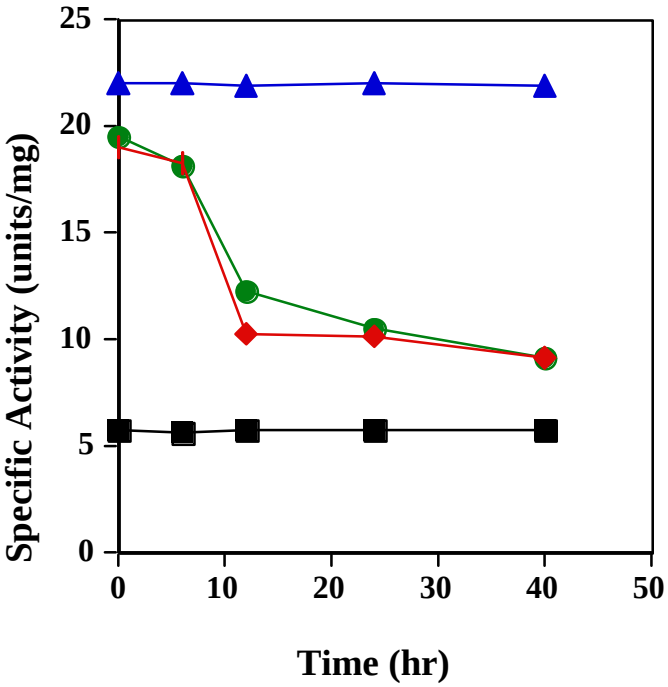

Specific Activity (units/mg)
25
20
15
10
5
0
0
10
20
30
40
50
Time (hr)

Table 5.1. Kinetics constants for various forms of FOR

Substrate	**Selenide-activated FOR**			**Oxygen-inactivated FOR**		
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Formaldehyde (0.5-100mM)	18.0	$1.92x10^4$	1.07	24	$1.20x10^4$	0.50
Glutaric dialdehyde (0.3-100mM)	20.0	$1.70x10^4$	0.85	15	$1.60x10^4$	1.06
Phenylpropionaldehye (1-70mM)	12.0	$2.50x10^4$	2.08	55	$1.25x10^4$	0.20

Substrate	**Sulfide-activated FOR**			**As-isolated FOR**		
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Formaldehyde (0.5-100mM)	15.0	$1.27x10^5$	8.46	25	$7.1x10^4$	3.00
Glutaric dialdehyde (0.3-100mM)	1.0	$5.7x10^4$	57.0	0.8	$4.2x10^4$	60.0
Phenylpropionaldehye (1-70mM)	10.0	$2.20x10^4$	2.22	15	$2.5x10^4$	1.7

Overall, while the sulfide-activated enzyme displayed catalytic efficiencies, as determined by the k_{cat}/K_m values, generally higher than those exhibited by the as purified enzyme, this was mainly due to lower K_m values with this form of the enzyme. Specifically, the activated enzyme showed a large increase in activity with formaldehyde (C_1) as the substrate under standard assay conditions, relative to the activity of the purified enzyme. However, this was not true for all the substrates that can be used by the enzyme. Notably, the kinetic values determined for glutaric dialdehyde (C_5), the best substrate found for FOR so far, were not significantly different for the two forms of enzyme [Roy et al., 1999].

Kinetic analyses were also conducted on the selenide-activated and oxygen-inactivated forms of FOR, and results are shown in Table 5.1. For two substrates, formaldehyde and glutaric dialdehyde, the selenide-activated form has kinetic values very similar to that of the oxygen-inactivated form. For phenylpropionaldehyde, on the other hand, the selenide form of enzyme has a K_{cat} value similar to that of the sulfide-activated or as purified enzyme (Table 5.1). The turnover rates for formaldehyde by the selenide-activated and oxygen-inactivated forms of FOR are ~ 10-fold lower than that of the sulfide-activated form, whereas for glutaric dialdehyde they are approximately 5-fold lower.

Cellular sulfide concentrations: The ability of sulfide to activate the purified forms of not only FOR, but also of GAPOR and AOR from *P. furiosus*, prompted an evaluation of the possible physiological significance of this reaction. The intracellular concentrations of sulfide (determined as acid-labile sulfide) of intact, washed cells grown with (5 g/l) and without S° were 7.2 ± 1.0 mM and 2.4 ± 1.0 mM, respectively (from ten independent

determinations) (Table 5.2). These values are much higher than the extracellular sulfide concentrations, which were 1.1 ± 0.2 mM and 0.014 ± 0.003 mM, respectively, in the spent media from which cells were obtained. The sulfide concentrations of the freshly-prepared, uninnoculated media (± S°) were beyond detection (< 5 μM), and when these same media were incubated for 14 hr (the same as the cultures) at 95 °C, the one without S° still lacked detectable sulfide while the sulfide concentration in the medium with S° increased to 0.036 ± 0.002 mM. Previous studies have shown that abiotic S° reduction to sulfide can occur at temperatures above 80 °C. In fact, the rates of sulfide formation increase with increasing temperature and increasing pH [Belkin et al., 1985]. Our data suggests that *P. furiosus* cells accumulate acid-labile sulfide, with S°-grown cells containing about three times as much as those grown without S°. It should be noted that the medium used to grow *P. furiosus* contains 1.2 mM methionine and 0.46 mM cysteine (from yeast extract). These are assumed to be the sources of sulfur for biosynthesis, independent of S° (which is not used for biosynthesis: Blumenthals et al, 1990).

An indication of the chemical nature of the acid-labile sulfide in *P. furiosus* cells came from the results of similar analyses with *E. coli* cells (Table 5.2). When grown aerobically either on a minimal medium or on one containing yeast extract and tryptone (Sambrook et al, 1998), the intracellular sulfide concentration was 1.9 ± 0.5 mM, whereas anaerobically-grown *E. coli* contained 0.7 ± 0.2 mM sulfide inside the cell. The extracellular concentration of sulfide was (aerobic; rich medium) comparable to that found with *P. furiosus* grown without S° (Table 5.2). Presumably, virtually all of the intracellular sulfide in *E. coli* cells is protein-bound in the form of FeS clusters.

Table 5.2. Acid-labile sulfide concentrations inside and outside cells of various types

Cell type/growth condition	Acid-labile sulfide concentration (mM)	
	Intracellular[a]	Extracellular[b]
P. furiosus		
plus sulfur cells	7.2 ± 1.0	1.1 ± 0.2
minus sulfur cells	2.5 ± 1.0	0.014 ± .003
Thermococcus sp. ES4		
plus sulfur cells	7.1	ND[d]
E. coli[c]		
aerobically grown	1.9 ± 0.5	0.008
anaerobically grown	0.7 ± 0.2	0.005
cells with POR gene (aerobic)	4.5 ± 0.3	ND
cells without POR gene (aerobic)	1.0 ± 0.5	ND
cells with Fd gene (aerobic)	1.45 ± 0.5	ND
cells without Fd gene (aerobic)	0.6 ± 0.02	0.010

[a]Indicates sulfide levels inside the cell. [b]Indicates sulfide levels in the medium after growth. No sulfide was detected in *P. furiosus* medium as prepared before innoculation. [c]*E. coli* media did not contain any added S°, see Material and Methods for details. [d]Not determined.

This was demonstrated by analyzing recombinant cells that produce high cellular amounts of FeS proteins from *P. furiosus*, including the δ subunit of POR [Menon et al., 1998] or Fd [Heltzel et al., 1994]. In both cases, the sulfide content of cells expressing the *P. furiosus* gene was more than twice that of the same cells lacking the genes (Table 5.2). The sulfide concentration of *Thermococcus*ES4 cells, an organism that is obligately dependent on S° for growth, was comparable to that of *P. furiosus* cells grown with S° and more than 3-times that found in *E. coli* (Table 5.2).

The question arises as to how much "free" sulfide is present in S°-grown *P. furiosus* cells? For example, the intracellular concentration of the [4Fe-4S] cluster containing ferredoxin in *P. furiosus* is approximately 0.7 mM [Aono et al., 1989], which is equivalent to 2.8 mM sulfide. Similarly, the subunits of AOR, FOR and GAPOR each contain a [4Fe-4S] cluster. Clearly, considering the various other iron-sulfur proteins that *P. furiosus* contains, these could account for most if not all of the measured intracellular sulfide. To investigate this, the cytoplasmic fractions of extracts from *P. furiosus* cells grown with and without S° (which contained 7.5 and 2.5 mM sulfide respectively) were passed through an ultrafiltration membrane (exclusion size, 30 kDa). The filtrate (nominally < 30 kDa) and the retained fraction (> 30 kDa) of the extract were analyzed for sulfide and also for Fe (Table 5.3). A small amount (< 10 % of the total) of labile sulfide was detected in the < 30 kDa fraction, but this originated largely from ferredoxin (which passed through the membrane). Further, the amount of the total acid-labile sulfide associated with the < 30 kDa fraction (0.1 - 0.3 %) was the same for both types of cells. Therefore, most surprisingly, most of the sulfide was associated with the > 30 kDa fraction of the whole cell extract of cells grown both with and without S°.

Table 5.3. Acid-labile sulfide concentration in low (< 30 kDa) and high (> 30 kDa) molecular weight fractions of *P. furiosus* cell-free extract.

Sample	Total protein (mgs)	Total sulfide (nmoles)	Total Fe[a] (nmoles)	Total ferredoxin[b] (nmoles)	Total polysulfide (nmoles)
***P. furiosus* minus S° cells**					
whole cell extract[c]	90	3,876	20,520	----	1,077
PM 30 pass through	2	70	560	42	----
PM 30 retained[d]	82	2,080	13,000	----	890
***P. furiosus* plus S° cells**					
whole cell extract	82	17,535	17,745	----	3,413
PM 30 pass through	2	1,500	1,000	75	390

PM 30 retained	78	14,090	14,850	----	2,200

[a]Fe concentrations were estimated by colorimetric analysis.

[b]ferredoxin concentration was estimated using its extinction coefficient. See Materials and Methods.

[c]whole cell extract includes soluble fraction as well as membranes.

[d]retained portion included high molecular weight (> 30 kDa) fraction as well as membranes.

Moreover, the total Fe amounts in the two types of *P. furiosus* are approximately the same (Table 5.3). In fact, the Fe content of non-S° cells is at least 5-times the sulfide-content, suggesting that a major part of the Fe is not part of FeS clusters. Since *P. furiosus* is not known to contain heme, much of this Fe is presumably in a storage form, and the amount is independent of whether the cells are grown on S°. In any event, the concentration of FeS clusters, and the amount of acid labile sulfide in this cluster form (not derived from S°) must also be about the same. Concentrations of other metals known to form complexes with sulfide such as Mo and Cu were also similar in the two cell types (data not shown). Thus, the > 4-fold higher sulfide content of S°-grown cells is associated with the > 30 kDa molecular weight fraction and appears not to be in the from of a metal-sulfide cluster.

The next question concerns the nature of the 3 - 4 fold excess sulfide detected in cells grown with S°. To investigate this, we looked at other S° species that might be present within the cell. Polysulfide could serve as a possible source of sulfide inside the cell. Polysulfides are known to form in the medium during growth of *P. furiosus* on S° [Blumenthal et al., 1990] and these compounds might freely permeate through the membrane. *P. furiosus* cells grown with and without S° had intracellular polysulfide concentrations of 9.6 mM and 5.4 mM respectively. This compares with sulfide concentrations of 7.2 and 2.5 mM, respectively (Table 5.2). Similar results are presented in Table 5.3. Polysulfide concentrations were measured by the cold cyanolysis method, however, this method does not measure the exact concentration of polysulfides in the solution. It is also not possible to determine the precise length distribution of these molecules, although at solution pH 8.5 the highest proportion of molecules are penta- or

tetrasulfides [Giggenbach, 1972]. Under these conditions only polysulfides reacted to form thiocyanate, and sulfides, cysteine, cystine or elemental or colloidal sulfur do not interfere with the assay [Blumenthals et al., 1990].

Thus, acid-labile sulfide levels appear to be about 3 - 5 fold higher in *P. furiosus* cells grown with S° whereas polysulfide levels are about 2-fold higher compared to non-S° cells. These results therefore indicate that S°-grown cells contain very high amounts of sulfide compared to non-S° cells and the same is true for polysulfide. Although sulfide is present in non-S° cells, it is probably all in the form of FeS clusters. Therefore, the concentrations of "free" sulfide in S°-cells is about 5 mM (after substracting that in the form of clusters: Table 5.2) and a comparable concentration of polysulfide is also present. However, neither form of sulfur appears to be freely accessible as only a very small fraction is present in the < 30 kDa wash. A significant amount of polysulfide was also detected in non-S° cells, although the significance of this is not clear.

Cell-free extracts from cells grown with and without S° were also assayed for AOR, FOR and GAPOR but their specific activities were not significantly different in the two types of cells. When oxygen-inactivated samples of AOR and FOR (with 10 % of original activity) were incubated (for ~ 5 h at 23 °C) with freshly prepared extracts from *P. furiosus* cells (grown with S°) and sodium dithionite (20 mM) as the reductant, no increase in specific activity was observed. Thus, although an estimated 2.5 mM sulfide is available in cell-free extracts of S°-grown cells, it has little effect on the activities of the three tungstoenzymes of *P. furiosus*.

Discussion

All three tungsten-containing aldehyde oxidoreductases in *P. furiosus* are activated by incubation with sulfide under reducing conditions. Loss of sulfide (due to inadvertent exposure to oxygen) during purification of these enzymes could explain the loss in specific activity. However, the nature of this 'restored' sulfide is not clear at this point. Presumably, sulfide is lost from the active site of these enzymes and incubation with sodium sulfide replaces the lost sulfide at the catalytic site and restores activity [Mukund, 1995, Roy et al., 1999]. Incubation with sodium selenide resulted in partial activation of FOR, however, selenide-activation is not very efficient and a very small fraction of the enzyme becomes selenide-activated. Furthermore, this is not a stable form and upon exposure to oxygen the enzyme immediately loses any Se bound to the active site and reverts back to the oxygen-inactivated state. Therefore, the selenide-form of the enzyme does not seem to mimic the sulfide-activated form. In addition, the selenide form of the enzyme has substrate specificity and kinetic properties similar to that of the oxygen-inactivated enzyme rather than the as purified or the sulfide-activated forms.

Structural analyses indicates that both AOR [Chan et al., 1995] and FOR [Hu et al., 1999] contain a tungstobispterin site where the W atom is coordinated in part by four S atoms from dithiolene groups of two pterin molecules, with water, hydroxide, and/or a terminal oxo group completing the coordination spheres. Interestingly, mononuclear molybdopterin-containing enzymes such as xanthine oxidase lose sulfur as thiocyanate when the oxidized forms are treated with cyanide [Hille, 1996, Wahl and Rajagopalan, 1982]. The resulting "desulfo" enzymes are activated by treatment with sulfide, which is

accomplished by conversion of a terminal M=O species to M=S. It is important to note that the sulfide-activated form of FOR is more sensitive to inactivation by oxygen and cyanide, suggesting that both may cause a W=S to W=O conversion. Similar sulfide activation and cyanide inactivation phenomenon have also been observed for *T. litoralis* FOR [Dhawan et al., 2000]. Preliminary EXAFS analysis [George et al., personal communication] on the different forms of FOR show that the oxygen-inactivated and as purified forms have a W(VI) oxidation state and a coordination sphere involving two W=O, one W-O and approximately three W-S bonds. The sulfide-activated form of FOR, on the other hand appears to have only one W=O and four or five W-S bonds. Crystallographic data shows that the W atom at the active site of these enzymes is coordinated by two pairs of dithiolene sulfurs from two pterin cofactors in addition to two oxo ligands to give a distorted trigonal prismatic arrangement, but there is no evidence for a W=S bond. Presumably, exposure to oxygen causes a decrease in W-S coordination and an increase in W=O bonds with a concomitant decrease in specific activity of the enzymes. Incubation with inorganic sulfide appears to reverse this effect, restoring the S coordination of the W atom as well as the catalytic activity of the enzyme. However, it is not clear whether the S loss is due to displacement of a dithiolene S or replacement of a terminal S (either as W=S or W-SH). Further spectroscopic and structural studies of the W active site are required to clarify this point.

To address the issue of whether sulfide activation is a physiologically-relevant process, we measured levels of various sulfur species in *P. furiosus* cells grown with and without S°. Cells grown with S° appear to have 3 – 5 fold higher intracellular concentrations of sulfide and approximately 2-fold higher polysulfide than those grown

without S°. However, the additional sulfide and polysulfide in S°-grown cells are associated with the > 30 kDa fraction of the cell-free extract and they are not present in a 'free' form. Polysulfide serves as the soluble intermediate of S° reduction by *P. furiosus*, and this reduction presumably takes place on the outer surface of the membrane (although the enzyme responsible for this reaction is not yet known). Therefore, it is probable that the excess polysulfide is actually associated with the membranes of *P. furiosus* that does not get washed off very easily. Furthermore, under the highly acidic conditions of the acid labile sulfide assay, this polysulfide becomes reduced to individual S^{2-} atoms and accounts for the excess labile sulfide measured in the cells grown with S°.

The result that neither the sulfide nor polysulfide are in a free form, along with the fact that specific activities of aldehyde oxidoreductases remain unchanged in cells grown with or without S°, strongly indicate that sulfide-activation is not a physiological reaction. Loss of sulfide from the catalytic sites of the enzymes appears to be an artifact of the purification process, which can be reversed *in vitro* by incubation with high concentrations of sulfide and reductant. Although the intracellular environment of *P. furiosus* is expected to be highly reducing, high concentrations of 'free' sulfide are not available inside the cells for the activation of aldehyde oxidoreductases.

CHAPTER 6

PHYLOGENETIC ANALYSIS OF KNOWN AND PUTATIVE TUNGSTOENZYMES OF THE AOR FAMILY[1]

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[1]Roy, R., S. Mukund, G. Schut, D. M. Dunn, R. Weiss and M. W. W. Adams. 1999 *J. Bacteriol*. **181**, 1171-1180. Reprinted here with permission of publisher.

Introduction

About a decade ago it was reported that the growth of the hyperthermophilic archaeon *Pyrococcus furiosus* was stimulated by the addition of the trace element tungsten [Bryant and Adams, 1989]. Subsequently, three tungsten-containing, aldehyde-oxidizing enzymes, aldehyde ferredoxin oxidoreductase (AOR), glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) and formaldehyde ferredoxin oxidoreductase (FOR) have been purified from this and related organisms. AOR has been isolated from *P. furiosus* [Mukund and Adams, 1990, 1991], *Pyrococcus* strain ES-4 [Mukund, 1995], and *Thermococcus* strain ES-1 [Heider et al., 1995], FOR has been purified from *Thermococcus litoralis* [Mukund and Adams, 1993] and *P. furiosu*s [Mukund, 1995, Roy et al, 1999], whereas GAPOR has been obtained so far only from *P. furiosus* [Mukund and Adams, 1995]. AOR, FOR and GAPOR are members of one of three distinct groups of tungsten-containing enzyme known as the "AOR family". The three tungstoenzymes of *P. furiosus* contain a single type of subunit of approximately ~ 67 kDa in size, and their amino acid sequences display high similarity. AOR and FOR share ~ 61% overall sequence similarity, whereas GAPOR is slightly distantly related with ~ 50% similarity to AOR/FOR. The crystal structures of both AOR and FOR from *P. furiosus* have been determined in the last few years. Both enzymes have very similar tertiary structures [Chan et al., 1995, Hu et al., 1999], where each subunit comprises a mononuclear tungsten coordinated by four dithiolene sulfur atoms from two pterin molecules, together with a single [4Fe-4S] cluster coordinated by four sulfur atoms from four cysteine residues. The amino acid residues involved in binding the pterins and the four cysteines coordinating the iron-sulfur cluster are conserved in all three of the tungstoenzymes.

Based on this, GAPOR is proposed to contain a tungstobispterin cofactor and a single [4Fe-4S] cluster, even though it has not been structurally characterized yet. The three enzymes differ in their quaternary structures. GAPOR is though to be monomeric, AOR is dimeric and FOR exists as a tetramer.

As their names imply, all three enzymes catalyze the oxidation of various types of aldehyde substrates to the corresponding acid, using ferredoxin as the physiological electron acceptor. However, they do differ in their substrate specificity. GAPOR is very specific for its substrate glyceraldehyde-3-phosphate, which it oxidizes to 3-phosphoglycerate [Mukund and Adams, 1995]. This is a key enzyme in the sugar fermentation pathway of *P. furiosus*. AOR can oxidize a wide range of aliphatic and aromatic aldehydes. It has a high affinity for aldehydes that are generated by the decarboxylation of 2-keto acids derived from amino acids [Heider et al., 1995, Ma et al., 1997]. Thus this enzyme is proposed to play a key role in peptide fermentation. FOR on the other hand, has a more limited substrate range and oxidizes C_5-C_6 di- and semialdehydes and C_1-C_3 aldehydes [Roy et al., 1999]. Various C_4-C_6 semialdehydes are involved in the metabolism of some amino acids such as Arg, Pro and Lys [Bender, 1985, Gottschalk, 1986], suggesting that FOR may possibly function in the catabolism of one or more of these amino acids.

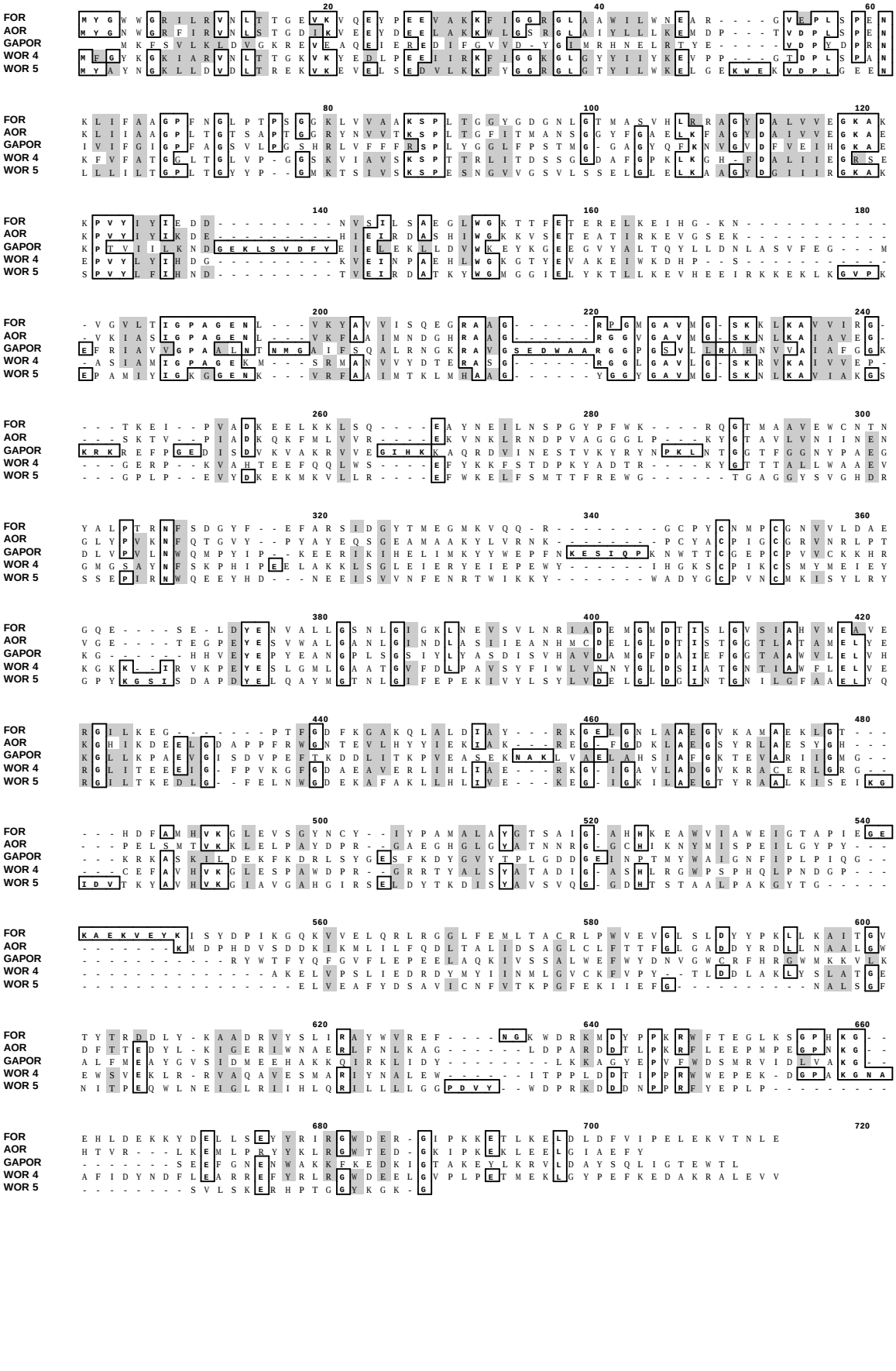
Homologs in the *P. furiosus* genome

The genome of *P. furiosus* has recently been sequenced in its entirety [Borges et al., 1996, Robb et al., 2001, http://comb5-156.umbi.umd.edu/genemate/]. The genes encoding FOR (*for*), AOR (*aor*) and GAPOR (*gor*) are spatially separated on the *P.*

furiosus genome, and except for the previously identified cofactor-modifying (*cmo*) gene adjacent to AOR [Kletzin et al., 1995], none of the ORFs immediately adjacent to the *aor*, *for* and *gor* genes appear to have a role in the synthesis or function of these three tungstoenzymes in *P. furiosus*. A search of the *P. furiosus* genome database with the complete amino acid sequences of AOR, FOR and GAPOR revealed the presence of two additional ORFs, and these appear to encode the fourth and fifth members of this enzyme family. Tentatively termed *wor4* and *wor5* (to depict genes encoding putative oxidoreductases within the tungstoenzyme family), these genes are also spatially separated from those encoding the other three tungstoenzymes and adjacent genes appear to be unrelated (although obviously the functions of these putative enzymes are as yet unknown). The *wor4* and *wor5* genes encode 622 and 582 codons, corresponding to proteins with molecular weights of 69,363 and 64,889 respectively. Thus the two putative tungstoenzymes in *P. furiosus*, have subunit molecular weights comparable to those (range 66,000 to 73,000) of the other members of the AOR family. Fig. 6.1 shows the alignment of complete amino acid sequences of the known tungstoenzymes FOR, AOR and GAPOR along with the putative tungsten enzymes WOR 4 and WOR 5. The similarity (identity) of the sequences of the WOR 4 and WOR 5 proteins to those of FOR, AOR and GAPOR are 57 (36), 58 (37) and 49 (25), and 56 (33), 58 (36) and 49 (25), respectively. Hence, both WOR 4 and WOR 5 appear to be more closely related to AOR and FOR, than they are to GAPOR. In addition to the overall sequence homology with the three known members of the AOR family, WOR 4 and WOR 5 also have the conserved motifs that coordinate the metal cofactors in these enzymes.

Fig. 6.1.

Alignment of the sequences FOR, AOR, and GAPOR and the putative gene products WOR 4 and WOR 5 from *P. furiosus*. Identical residues are boxed and similar residues are shaded. Taken from Roy et al., 1999.

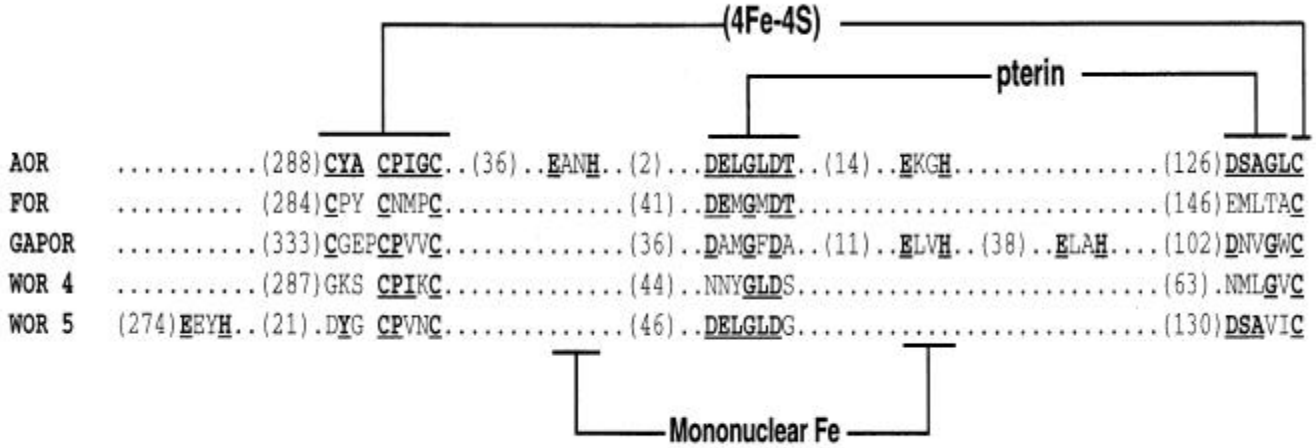

FOR
AOR
GAPOR
WOR 4
WOR 5

From crystallographic analysis, both AOR and FOR are known to contain one [4Fe-4S] cluster and one mononuclear tungstopterin cofactor per subunit [Chan et al., 1995, Hu et al., 1999]. Two pterin-binding motifs and all four of the Cys residues that coordinate the [4Fe-4S] cluster are conserved in AOR, FOR as well as GAPOR (Fig. 6.2). AOR also contains two EXXH motifs, which coordinate a mononuclear metal site, most likely iron, which bridges the two subunits. FOR and GAPOR lack these motifs and presumably do not have these subunit-bridging metal ions. The two putative tungstoenzymes in *P. furiosus*, WOR 4 and WOR 5, each contains motifs, separated by the appropriate number of residues, that are proposed to bind a single [4Fe-4S] cluster and a bispterin site (Fig. 6.2). These enzymes also contain EXXH motifs but they are not in the same location as those of AOR, suggesting that WOR 4 and WOR 5 lack the subunit-bridging metal ion found in AOR. In any event, these two putative tungstoenzymes are clearly closely related both in cofactor content and in structure to the three tungsteoenzymes that have been purified from *P. furiosus*.

The reaction catalyzed by the putative tungstoenzymes WOR 4 and WOR 5 and thereby their function in the physiology of *P. furiosus*, is not known. Although AOR, FOR and GAPOR are closely related enzymes, *P. furiosus* utilizes them for very different purposes, namely AOR (and possibly FOR) in peptide metabolism and GAPOR in glycolysis. Thus it is difficult to predict the function of these putative tungstoenzymes based merely on their similarity to the other members of the AOR family. Isolation and purification of these new tungstoenzymes from the cell-free extracts of *P. furiosus*, in the absence of a known assay, is a challenging task (see Chapter 7).

Fig. 6.2.

Alignment of the cofactor-binding motifs of FOR, AOR, and GAPOR and of the putative gene products WOR 4 and WOR 5 from *P. furiosus*. The numbers in parentheses indicate the number of residues between the indicated motifs. The motifs that bind the pterin cofactor, FeS cluster and mononuclear iron (in AOR) are indicated. Taken from Roy et al., 1999.


(4Fe-4S)
pterin
AOR .....(288)CYA CPIGC..(36)..EANH..(2)...DELGLDT..(14)..EKGH.....(126)DSAGLC
FOR ..... (284)CPY CNMPC.....(41)..DEMGMDT.....(146)EMLTAC
GAPOR .....(333)CGEPCPVVC.....(36)..DAMGFDA..(11)..ELVH..(38)..ELAH....(102)DNVGWC
WOR 4 .....(287)GKS CPIKC.....(44)..NNYGLDS.....(63).NMLGVC
WOR 5 (274)EEYH..(21).DYG CPVNC.....(46)..DELGLDG.....(130)DSAVIC
Mononuclear Fe

Homologs in other hyperthermophilic archaea

The complete genome sequences of several hyperthermophilic archaea are now available, and these were searched for relatives of AOR and other members of the tungstoenzyme family found in *P. furiosus*. These include the genomes of *P. horikoshii* [Gonzalez et al., 1998, Kawarabayasi et al., 1998], *Pyrobaculum aerophilum* [Fitz-Gibbon et al., 1997], *Methanococus jannaschii* [Bult et al., 1996], *Aeropyrum pernix* [Kawarabayasi et al., 1999] and *Archaeoglobus fulgidus* [Klenk et al., 1997]. All of them contain putative genes that would encode proteins with significant sequence similarity to the *P. furiosus* tungstoenzymes. In addition, homologs of the AOR family were also found in the genome sequences of some moderately thermophilic archaea, and in genomes of three mesophilic bacteria. Table 6.1 lists the various microorganisms and accession numbers of the genes thought to encode relatives of the AOR family. Surprisingly, not all are anaerobic hyperthermophilic archaea. *A. pernix* is a strict aerobe although it is a hyperthermophilic arhcaeon. However, *Thermoplasma acidophilum* and *T. volcanium* are aerobic thermophiles (optimum growth temperature ≤ 60 °C), and *Clostridium acetobutylicum* and *Eubacterium acidaminophilum* are obligately anaerobic, gram-positive mesophilic bacteria. Remarkably, the *E. coli* genome also contains a homolog of the AOR family although it is currently annotated as hypothetical protein. This putative gene encodes a protein with a molecular weight of ~ 77,870 which has an overall amino acid sequence similarity with members of the AOR family of about ~ 30%. Interestingly, the motifs that coordinate the bispterin site and the [4Fe-4S] cluster in the structurally characterized tungstoenzymes AOR and FOR appear to be conserved in the putative *E. coli* protein.

Table 6.1. List of putative relatives of tungsten-containing enzymes of *P. furiosus* in the genomic databases of various microorganisms.

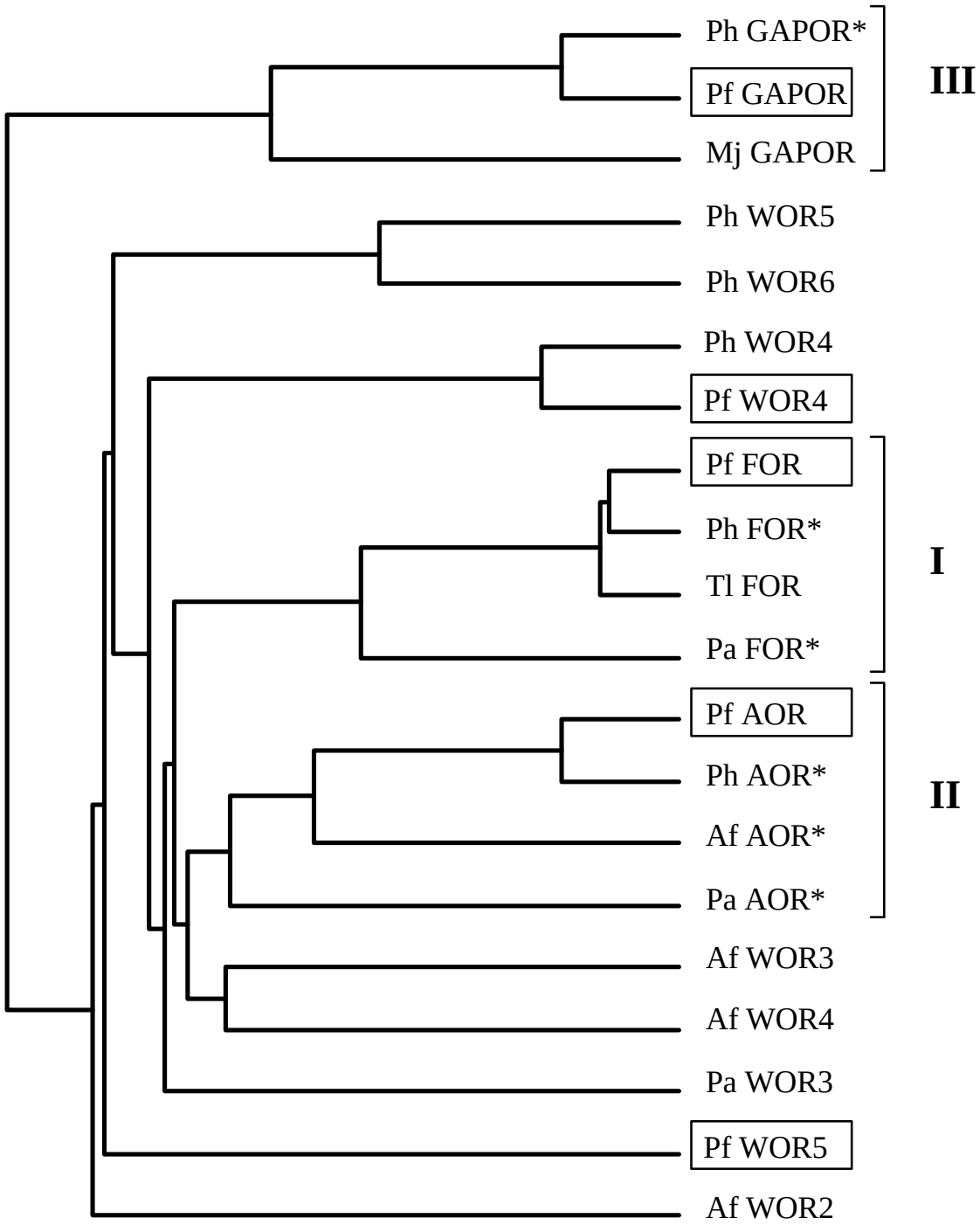
Source	Putative tungstoenzymes (GenBank accession number)	Reference
Archaeal species		
Pyrococcus furiosus	AF101432(WOR4), AF101433(WOR5)	Borges et al., 1996
P. horikoshii	BAA30116(AOR), BAA30377(FOR), BAA29096(WOR4), BAA29985, BAA29986, BAA29543(GAPOR)	Kawarabayasi et al., 1998
Methanococcus jannaschii	U67559(GAPOR)	Bult et al, 1996
P. abyssi	CAB49318(AOR), CAB50110(FOR), CAB48956(WOR4), CAB50484 (GAPOR), CAB49871	Faguy et al., 1999
Pyrobaculum aerophilum	Not available	Fitz-Gibbon et al., 1997
Thermoplasma volcanium	BAB59951(FOR), BAB60405	Kawashima et al., 2000
Thermococcus litoralis	X83963(FOR)	Kletzin et al., 1995
Aeropyrum pernix	BAA79181	Kawarabayasi et al., 1999
Thermoplasma acidophilum	CAC11939(AOR), CAC11963, CAC12268(WOR5)	Ruepp et al., 2000
Archeoglobus fulgidus	AE000947, AE01105(AOR), AE001081, AE001101	Klenk et al., 1997
Bacterial species		
Clostridium acetobutylicum	AE007705	Nolling et al., 2001
Eubacterium acidaminophilum	AJ318790(FOR)	Rauh, 2000
Escherchia coli	AE002558	Perna et al., 2001

Although *E. coli* possesses several molybdenum-containing enzymes, a tungsten-containing protein has not been isolated from this microorganism so far. In fact, tungsten has an antagonist effect on the uptake and incorporation of molybdenum in this organism and typically inhibits growth.

The phylogenetic relationship between the amino acid sequences of the five tungstoenzymes in *P. furiosus* and their homologs in the other genome sequences from various archaea is depicted in Fig. 6.3. From the dendogram three distinct groups are apparent, and these correspond to the three types of enzyme that have been purified and characterized so far. Group I or the FOR group has homologous genes in *P. horikoshii* and *Pm. aerophilum* in addition to the previously characterized genes in *P. furiosus* and *T. litoralis.* Group II enzymes have representatives in *P. horikoshii*, *A. fulgidus* and *Pm. aerophilum* in addition to *P. furiosus,* while Group III is the GAPOR group and this enzyme, while so far only purified from *P. furiosus,* has close homologs in *P. horikoshii* and *M. jannaschii*. It should be noted that these sequences have been placed in their respective groups based solely on similarity at the amino acid level, but the corresponding enzymes are predicted to have the same function as the previously characterized enzymes. As indicated in Fig. 6.3, there are also several sequences that do not fall into any of the three groups, including WOR 4 and WOR 5 of *P. furiosus,* and any existing relationship among them will have to await elucidation of function through biochemical or genetic analyses.

Fig. 6.3.

Phylogenetic relation of the amino acid sequences of tungstoenzymes from *P. furiosus* and their homologs found in genome databases. Sequences demarcated by brackets and Roman numerals indicate groups. Asterisk denote amino acid sequence homologous to a tungstoenzyme of known function, though the relevant enzyme activity has not yet been determined in these organisms. Length of the line represents distance in arbitrary units. Abbreviations: Ph, *P. horikoshii*; Pf, *P. furiosus*; Mj, *M. jannaschii*; Pa, *Pyrobaculum aerophilum*; Af, *A. fulgidus*. See Table 6.1 for the list of GenBank accession numbers of amino acid sequences.


Ph GAPOR*
Pf GAPOR
Mj GAPOR
III
Ph WOR5
Ph WOR6
Ph WOR4
Pf WOR4
Pf FOR
Ph FOR*
Tl FOR
Pa FOR*
I
Pf AOR
Ph AOR*
Af AOR*
Pa AOR*
II
Af WOR3
Af WOR4
Pa WOR3
Pf WOR5
Af WOR2

Conclusion

With the availability of the complete genome sequences of numerous microorganisms, it is evident that tungstoenzymes of the AOR family are more widespread among the hyperthermophlic archaea (and some bacteria) than previously thought. However, these ORFs have been included in the AOR family based solely on sequence homology. Whether any or all of these genes actually encode proteins that contain tungsten (or molybdenum) and iron-sulfur clusters, and what their functions are cannot be answered until attempts have been made to characterize the corresponding enzymes.

CHAPTER 7

A FOURTH TUNGSTEN-CONTAINING ENZYME FROM THE HYPERTHERMOPHILIC ARCHAEON *Pyrococcus furiosus*.[1]

[1]Roy R. and M. W. W. Adams. To be submitted to *J. Biol. Chem.*

Abstract

P. furiosus is a hyperthermophilic archaeon which grows optimally near 100°C by fermenting peptides and sugars to produce organic acids, CO_2, and H_2. Its growth requires tungsten, an element rarely used in biology, and three different types of tungsten-containing enzymes, aldehyde ferredoxin oxidoreductase (AOR), formaldehyde ferredoxin oxidoreductase (FOR) and glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR), have been previously purified from this organism. AOR and FOR are thought to function in peptide metabolism whereas GAPOR has a role in the glycolytic pathway of *P. furiosus*. The recently completed genome sequence of this organism reveals the presence of two additional genes termed *wor4* and *wor5*, proposed to encode putative tungstoenzymes WOR 4 and WOR 5. One of these tungstoproteins WOR 4 has now been purified from the cell-free extracts of *P. furiosus*. The N-terminal sequence of purified WOR 4 and the approximate size of its subunit determined electrophoretically (69,000) both correspond to what is predicted by the *wor4* sequence. WOR 4 is a homodimer and contains approximately 0.7 W atom, 2.7 Fe atoms and 0.8 Ca atom per subunit. Based on the metal content this enzyme is proposed to have 1 W and a [4Fe-4S] cluster per subunit. UV-Vis spectra of the oxidized and reduced enzyme indicate the presence of a FeS chromophore. WOR 4 does not oxidize short or long chain aldehydes and hydroxyacids nor does it reduce ketoacids. The reaction catalyzed by this enzyme is not known at present but WOR 4 could not be purified from cells grown in the absence of S°, suggesting that it may have a role in S° metabolism.

Introduction

Pyrococcus furiosus is one of the most extensively studied examples of anaerobic, hyperthermophilic archaea. This anaerobic heterotroph (optimum growth temperature, 100°C (Stetter et al., 1990) grows on peptides (casein, peptone, yeast extract) and can utilize both simple (maltose, cellobiose) and complex (starch, glycogen) sugars but monosaccharides (glucose, fructose) do not support growth. The carbohydrates are metabolized by an unusual fermentation-type pathway, yielding acetate, H_2 and CO_2 as end-products (Kengen and Stams, 1994, Kengen et al., 1994). The growth of *P. furiosus* is dependent upon the presence of the trace element tungsten in the medium (Bryant and Adams, 1989). Three tungsten-containing, aldehyde-oxidizing enzymes, aldehyde ferredoxin oxidoreductase (AOR; Mukund and Adams, 1991 and 1993), glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR; Mukund and Adams, 1995) and formaldehyde ferredoxin oxidoreductase (FOR; Roy et al., 1999) have been previously purified from this organism. AOR has a broad substrate specificity but is most active with aldehydes derived from amino acids (via transamination and decarboxylation (Heider et al., 1995, Ma et al., 1997). This enzyme is thought to play a key role in peptide fermentation by oxidizing aldehydes generated by the four types of 2-keto acid oxidoreductase present in this organism. FOR can oxidize short chain (C_1-C_3) aldehydes, however, it has the highest affinity for acid-substituted C_4-C_6 aldehyde substrates (Roy et al., 1999). FOR too is proposed to play a role in the metabolism of peptides. In contrast, GAPOR uses only glyceraldehyde-3-phosphate as a substrate and it functions in the unusual glycolytic pathway that is present in *P. furiosus,* replacing the expected glyceraldehyde-3-phosphate dehydrogenase (Mukund and Adams, 1995, Oost et al.,

1998). However, despite the difference in substrate specificity, all three enzymes have very similar molecular properties and are closely related phylogenetically, making up the so-called 'AOR family' of tungstoenzymes. The members of this family of enzymes are distinguished by the presence of one [4Fe-4S] cluster and a mononuclear tungsten atom coordinated by two pterin cofactors at the active site. These enzymes are termed 'true tungstoenzymes', as Mo-homologs for these enzymes have not yet been characterized (Mukund and Adams, 1996, Johnson et al., 1996).

Based on amino acid sequence similarity with AOR and FOR, two additional (non-linked) genes have been identified in the genomic database of *P. furiosus*. Termed *wor4* and *wor5*, these two genes are proposed to encode putative tungstoenzymes WOR 4 and WOR 5. Both WOR 4 and WOR 5 show high sequence similarity to AOR and FOR. The calculated molecular weights of these enzymes are comparable to those of AOR, FOR and GAPOR, and in its sequence each contains motifs that are proposed to bind a single [4Fe-4S] cluster and a bispterin site. Analysis of three-dimensional structure of *P. furiosus* AOR and FOR has shown that the tungstopterin site and [4Fe-4S] cluster are coordinated by similar motifs in these enzymes. Therefore, WOR 4 and WOR 5 represent two novel enzymes of the AOR family. However, in the absence of a known enzyme assay it is a challenge to isolate these enzymes. We have used a combination of column chromatography steps and tungsten analysis to isolate one of these putative tungstoenzymes, WOR 4 from the cell-free extracts of *P. furiosus*. This paper describes the purification and preliminary characterization of a novel tungstoprotein WOR 4 from *P. furiosus*.

Materials and Methods

Growth of the organism – P. furiosus (DSM 3638) was grown in both small (16L) and large (500L) scale cultures based on previous protocol (Bryant and Adams, 1989, Verhagen et al., 2001). The composition of the medium included 0.5% (w/v) each of maltose and tryptone as carbon source, trace minerals, salts and 0.1% sulfur. The cultures were grown under pH-controlled conditions (pH 6.8) until a cell-density of ~ 4 x 10^8 per ml was reached. The cells were then harvested, rapidly frozen using liquid nitrogen and stored at –80°C until use.

Purification of protein WOR 4 was purified from 200 g (wet weight) of frozen cells under strictly anaerobic conditions at 23 °C. In all chromatography steps, the columns were washed with at least 2 column volumes of buffer before the adsorbed proteins were eluted. *P. furiosus* AOR , FOR and GAPOR were purified according to previously established protocol (Roy et al., 2001). In the absence of a known assay, purification of WOR 4 was accomplished by following tungsten content in the fractions obtained in the subsequent chromatography steps. The techniques and procedures were the same as those used to purify other *P. furiosus* aldehyde oxidoreductases (Roy et al, 2001), up to and including the first chromatography step, except that sodium dithionite was used at 1 mM concentration and glycerol was omitted from all buffers. WOR 4 eluted from the first column of (5.0 x 18.0 cm) DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech Inc.) when 0.30 M NaCl in buffer A (50 mM Tris/HCl, pH 8.0, containing 2 mM dithiothreitol and 1 mM dithionite) was applied. Fractions containing tungsten (350 nmoles, 0.186 nmoles/mg protein) but no discernable AOR, FOR or GAPOR activity were applied to a column (2.5 x 10 cm) of Hydroxyapatite (Bio-Rad) previously

equilibrated with buffer A at 4 ml/min. Adsorbed proteins were eluted with a linear gradient (2000 ml) from 0 to 0.5 M potassium phosphate in buffer A at 2ml/min. Tungsten containing protein fractions (318 nmoles, 1.250 nmoles/mg protein) eluted as 0.15 – 0.22 M potassium phosphate was applied. These fractions were combined and loaded on to a Hi-TraP Q (Amersham Pharmacia Inc.) column (three 5 ml columns connected in series) at 50% with buffer B (50 mM BisTris, pH 6.5, containing 2 mM DTT) at 2 ml/min. Adsorbed proteins were eluted with a linear gradient (200 ml) from 0 to 0.5 M NaCl in buffer B at 2 ml/min. Fractions with high specific tungsten (14.22 nmoles/mg protein) eluted when 0.17 - 0.25 M NaCl was applied to the column. Purity of fractions was judged by SDS polyacrylamide gel electrophoresis. Pure samples were combined, concentrated by ultrafiltration using a PM-30 membrane (Amicon, Bedford, MA) and stored as pellets in liquid nitrogen.

Enzyme Assays – Assays were performed under strictly anaerobic conditions in serum-stoppered cuvettes. All buffers, substrates, reducing agents etc. were degassed thoroughly and flushed with argon. The assays were carried out in a Spectronic 500 (Fisher Scientific, Atlanta, GA) spectrophotometer equipped with a thermostated cuvette holder and a thermoinsulated cell compartment. The activities of AOR (Mukund and Adams, 1993) and FOR (Roy et al., 1999) were determined as described previously. Enzyme activity was measured at 80 °C with benzyl viologen (3 mM) as the electron acceptor and crotonaldehyde (AOR) or formaldehyde (FOR) as the substrate. GAPOR (Mukund and Adams, 1995) activity was determined at 70 °C using glyceraldehyde-3-phosphate as substrate. Reduction of benzyl viologen was measured at 600 nm (molar absorbance 7,800 M^{-1} cm^{-1}). Results are expressed as units per milligram of protein

where 1 U equals the oxidation of 1 μmol of substrate/min/mg of protein. Hydroxy/Oxoacid oxidoreductase assays were carried out at 80 °C in 100 mM EPPS (pH 8.4) with either 2-hydroxyacids as substrates and oxidized benzyl viologen (3 mM) as the electron acceptor or 2-keto acids as substrates and reduced benzyl viologen (2 mM) as electron donor (Trautwein et al., 1994). Alternately, the same assay was also done at 80 °C in 100 mM potassium phosphate buffer (pH 7.0) with $NADP^+$/NADPH as the electron mediator respectively (Bayer et al., 1994). NADPH oxidation was measured at 340 nm. Aldehyde/Aldose reductase assays were carried out at 75 °C in 100 mM EPPS buffer (pH 8.4) with various sugars and aldehydes as substrates (0.5 – 2 mM) and NADPH as the electron donor (Hayman and Kinoshita, 1964, Tani et al., 2000).

Other methods – Colorimetric iron determination (Lovenberg et al., 1963), protein determination (Bradford, 1975) and N-terminal amino acid sequence analysis were all carried out as previously described. Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis using 4-12% Bis-Tris acrylamide gel (NuPage, Invitrogen Corp.) Holoenzyme size was determined by gel-filtration chromatography on Superose 6 equilibrated with 50 mM Tris buffer containing 0.2 M NaCl. The column was calibrated with bovine serum albumin (66,000), phosphorylase b (99,700), alcohol dehydrogenase (150,000), catalase (200,000), apoferritin (443,000) and thyroglobulin (669,000). Western blot analysis was performed as described previously (Sambrook et al., 1989), protein samples (0.1 mg/ml) were first run on a 10% (w/v) acrylamide gel, then blotted onto nitrocellulose membrane. The membrane was then treated with protein specific antibodies and Anti rabbit alkaline phosphatase conjugated IgG (Promega). Immunospecific protein bands were detected by enzymatic immunoassay using alkaline

phosphatase. A complete metal analysis (31 elements including tungsten and iron) of protein fractions was performed using Inductively coupled plasma emission spectroscopy (ICP)(detection range~ppm) and ICP-MS (~ppb). Amino acid sequences were analyzed with MacVector (International Biotechnologies, Inc., New Haven, Conn.). EPR spectra were recorded on a Bruker ER 300E spectrometer equipped with an Oxford Instruments ITC flow cryostat and interfaced to an ESP 3220 computer. Amino acid sequences were analyzed with MacVector (International Biotechnologies, Inc., New Haven, Conn.).

Results

Purification of WOR 4. The presence of tungsten in fractions from chromatographic columns that did not contain AOR, FOR or GAPOR activities was used to identify and purify WOR 4. The specific activity and tungsten profile of the first step during purification (DEAE Sepharose) shows that peak activity fractions of the three previously characterized tungsten-containing enzymes AOR, FOR and GAPOR can be separated from each other (Fig. 7.1). GAPOR, FOR and AOR eluted as 100-160, 160-200 and 190-260 mM NaCl respectively was applied to the column. The activity in each of these fractions coincides with tungsten content. However, fractions that eluted as 300 mM NaCl was applied to the column contained ~350 nmoles tungsten but no discernable AOR, FOR or GAPOR activity. These fractions were combined and purified further. After two more steps (Hydroxyapatite and HiTrap Q) (Table 7.1) fractions with high tungsten content and judged homogenous by gel electrophoresis were obtained. The N-terminal amino acid sequence of the obtained protein was analyzed and when compared with the *P. furiosus* genome database, was identified as WOR 4.

Figure 7.1.

DEAE-Sepharose column profile during purification of WOR 4 from *P. furiosus* cell-free extracts. The peaks denote enzyme activity and total tungsten content in the fractions respectively. (-○-) indicates tungsten levels. Specific activity of the three enzymes are denoted as follows: GAPOR (●), FOR (■) and AOR (▲). The diagonal line across the graph indicates the salt gradient (0-50% of 1 M NaCl) applied to the column.

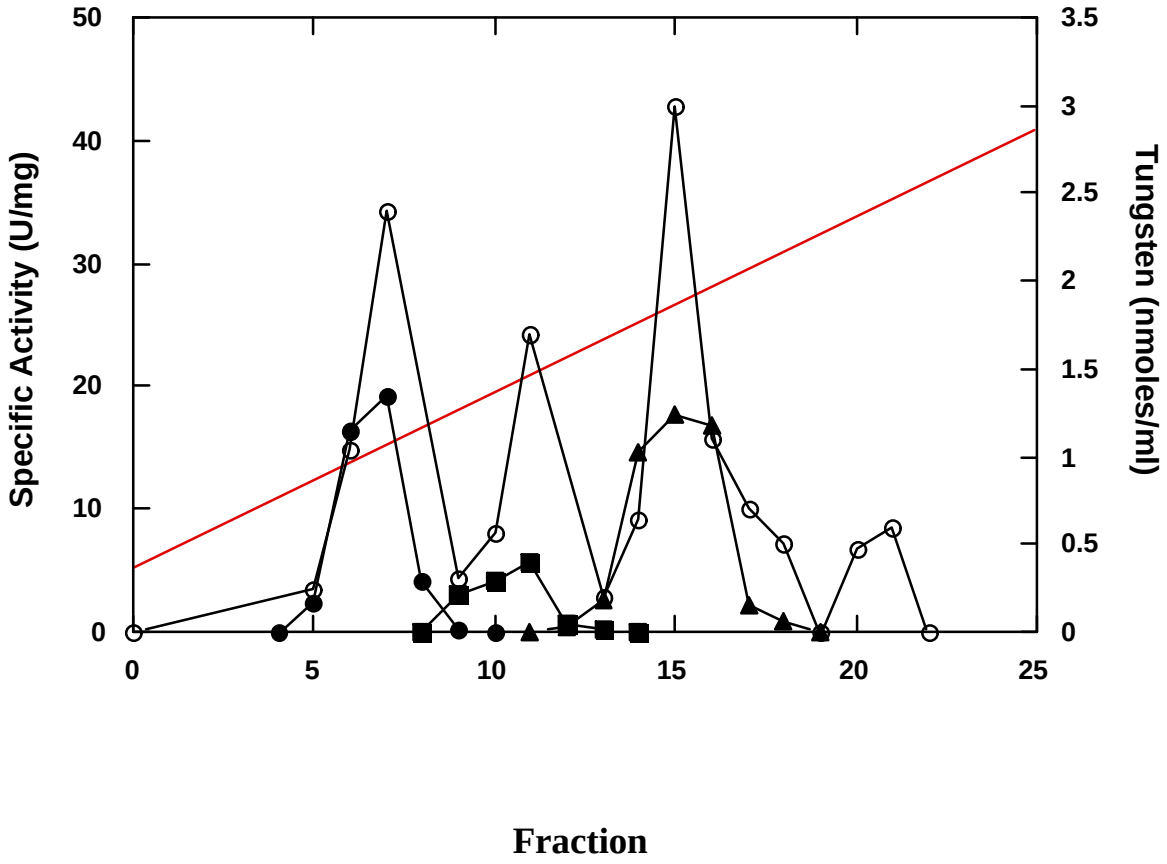
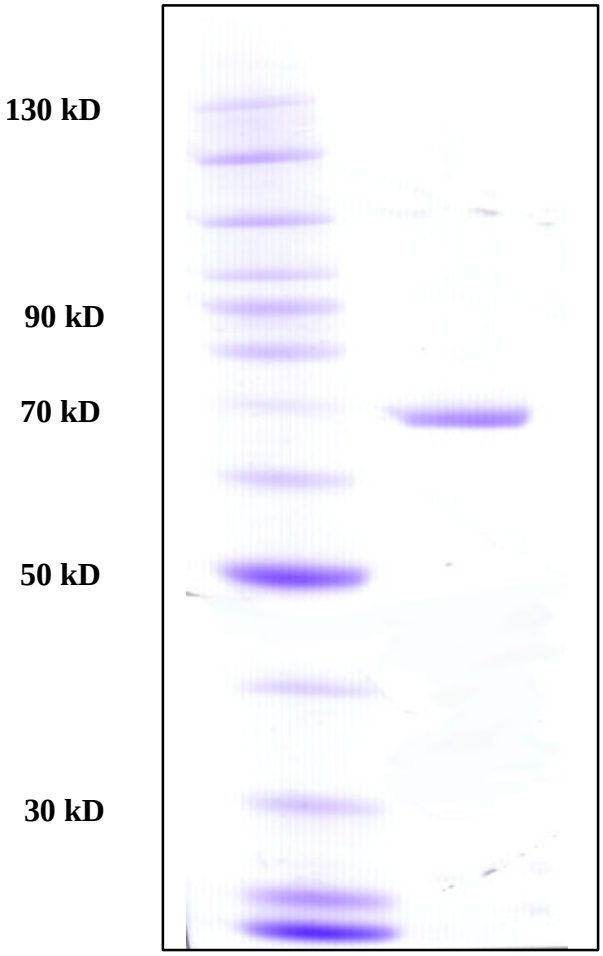

Specific Activity (U/mg)
Tungsten (nmoles/ml)
Fraction
0
10
20
30
40
50
0
0.5
1
1.5
2
2.5
3
3.5
0
5
10
15
20
25

Table 7.1. Purification of WOR 4 from *P. furiosus* cell-free extract.

Step	***Volume (ml)***	***Protein (mg)***	***Tungsten (nmoles)***	***Specific tungsten (nmoles/mg)***	***Purification fold***
Crude extract	847	21,300	2541	0.12	1.0
DeaeFF	630	1,877	350	0.19	1.3
HAP	310	254	318	1.25	10.5
HiTrap Q	4.5	12.3	175	14.22	119.3

Figure 7.2.

SDS gel electrophoresis analysis of purified *P. furiosus* WOR4. Sample of WOR 4 (2 mg/ml) was incubated with an equal volume of SDS (1%, w/v) at 100 °C for 10 min (lane 2) on a 4-12% gradient acrylamide gel. Lane 1 contained marker proteins (Gibco BRL 10 kD ladder) with the indicated molecular masses (in kilodaltons).


130 kD
90 kD
70 kD
50 kD
30 kD

The fractions with pure WOR 4, were then combined, concentrated by ultrafiltration using a PM-30 membrane (Amicon, Bedford, MA), and stored as pellets in liquid nitrogen. Approximately 12 mg of pure WOR 4 was obtained from 200 gm (wet weight) of *P. furiosus* cells (Table 7.1). In comparison yields of AOR, FOR and GAPOR from the same cells mass are ~70, 40 and 30 mgs respectively (Roy et al., 1999).

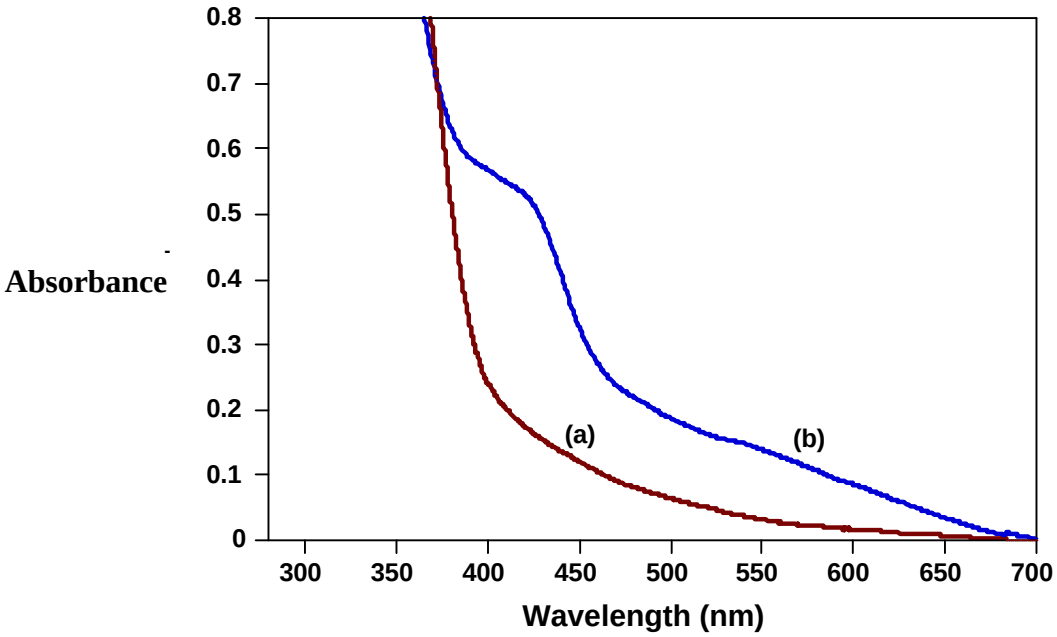
Molecular properties of WOR 4. As shown in Fig. 7.2, purified WOR 4 sample prepared by heating at 100 °C for 10 min with SDS (1.0% w/v) gave rise to a single protein band after SDS-electrophoresis (10%, w/v, acrylamide). This band had an approximate M_r value of 69,000 ± 2000 D. Analysis of WOR 4 by gel-filtration on Superose 6 (in the presence of 0.5 M NaCl) gave a value of 129,000 ± 12,000 D. These data suggest that the holoenzyme is a homodimer. The presence of a single subunit was confirmed by N-terminal amino acid sequence analysis of pure WOR 4 that gave rise to a single sequence of M F G Y K G K I A R. This N-terminal amino acid sequence shows homology to those of *P. furiosus* AOR and FOR respectively (see Chapter 6). Further, when this was used to search the genomic database of *P. furiosus*, the N-terminal matched exactly the 5' end of one open reading frame (ORF), termed aldehyde tungsten oxidoreductase *wor4*, which contained 623 codons corresponding to a protein with a molecular weight of 69,363, which is in good agreement with that (69,000 ± 2000) estimated by biochemical analyses. Although the translated sequence of *wor4* shows at least 49% amino acid sequence similarity with AOR, FOR and GAPOR of *P. furiosus*, antibodies generated to any one of the latter three tungstoproteins did not show any significant cross-reactivity with WOR 4 (data not shown). WOR 4 contained 0.71 ± 0.05 tungsten, 2.7 ± 0.4 iron, 0.80 calcium and 0.33 ± 0.005 zinc g atoms/mol when analyzed by plasma emission

spectroscopy and 2.0 ± 0.3 iron g atoms/mol by colorimetric analysis (each value is the average of at least two determinations using two different enzyme preparations). No other metals were present in significant amounts (>0.1 g atom/subuint). WOR 4 as purified gave a broad visible absorption peak around 420 nm, the intensity of which increased considerably on treatment with potassium ferricyanide (Fig. 7.3). The visible absorption decreased by about 50% upon addition of sodium dithionite, characteristic of an iron-sulfur chromophore. However, a dithionite-reduced WOR 4 (~ 80 μM protein) did not display any significant EPR resonance in either the g ~ 2 or g ~ 4 region of the spectrum (recorded at 4 K, 10 mW power: data not shown).

Catalytic properties of WOR 4. Since the reaction catalyzed by this enzyme is not known, it could not be assayed during purification. However, based on the similarity at the amino acid sequence level (see above) with the other tungsten-containing oxidoreductases from *P. furiosus*, the ability of WOR 4 to oxidize aldehydes to acids and *vice versa* was tested. The enzyme was assayed at 80 °C, using a wide range of aliphatic and aromatic aldehydes (formaldehyde (C_1), glyceraldehyde (C_2), propionaldehyde (C_3), crotonaldehyde (C_4), glutaraldehyde (C_5), salicaldehyde, benzaldehyde, phenylpropionaldehyde, isovaleraldehyde and glyceraldehyde-3-phosphate as substrates (0.5 – 10 mM concentration range) and benzyl (or methyl) viologen as electron acceptor. In the reverse reaction acids (formate, acetate and glutarate) were used as substrates (0.5 – 5 mM) and reduced viologen as the electron donor. WOR 4 did not display either activity. The N-terminal sequence of members of the AOR family of tungstoenzymes, to which the novel member WOR 4 is proposed to belong to, have been previously reported to have similarity with that of the molybdoenzyme HVOR (Johnson et al., 1996).

Figure 7.3.

UV-Vis spectra of WOR 4 from *P. furiosus*. The enzyme sample (5 mg/ml) was reduced by the addition of 2 mM sodium dithionite, just prior to recording the reduced spectrum (a). Oxidation was achieved by the gradual addition of aliquots of potassium ferricyanide to the enzyme sample (b). The oxidation was completely reversible on the addition of sodium dithionite (data not shown).


Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)
(a)
(b)

HVOR, which has been isolated from *Proteus vulgaris* (Trautwein et al., 1994) and *Clostridium tyrobutyricum* (Bayer et al., 1994), has broad substrate specificity and catalyzes the reversible oxidation of 2-hydroxycarboxylic acids with benzyl viologen or NADPH as the electron carrier. However, when assayed under similar conditions WOR 4 did not oxidize lactate, 2-hydroxybutyrate, 2-hydroxycaproate or 1-ethyl-2-hydroxycaproate nor did it reduce the oxoacids pyruvate, 2-ketobutyrate, 2-ketocaproate or 2-ketovalerate. Pure WOR 4 samples were assayed for aldehyde/aldose reductase type activity. The enzyme does not reduce aldehydes such as glyceraldehyde, propionaldehyde, benzaldehyde or sugars like glucose and galactose (1-10 mM concentration range) using NAD(P)H (or reduced benzyl viologen) as the electron donor (see Discussion). Further, none of these activities were detected in the cell-free extracts of *P. furiosus*. Thus so far, we have been unable to ascertain the reaction catalyzed and thereby the physiological function of this new member of the *P. furiosus* tungstoenzyme family.

Discussion

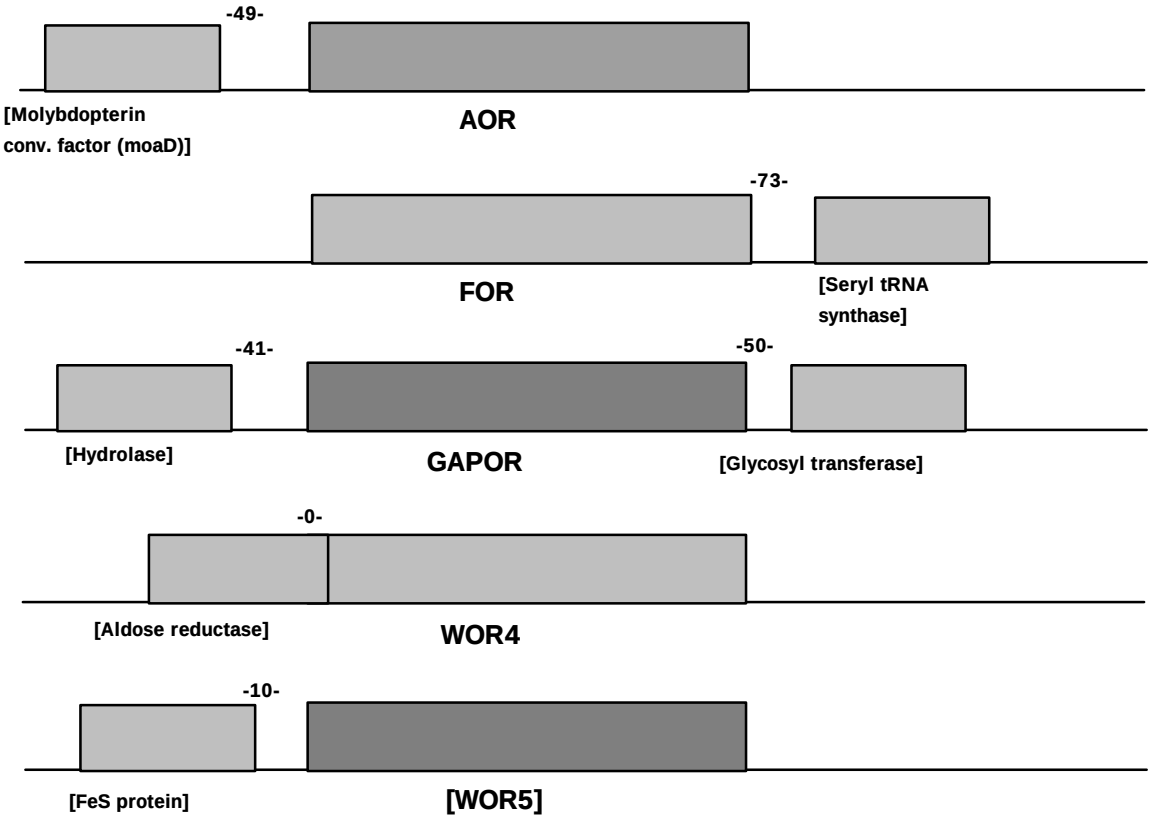
The tungstoprotein WOR 4 has been purified from cell-free extracts of *P. furiosus* and its size and N-terminal sequence correspond to that encoded by the ORF previously designated *wor4* in the genome. The subunit size of this homodimeric protein (69.3 kDa) is comparable to those of homodimeric AOR (66.3 kDa), homotetrameric FOR (69.0 kDa) and monomeric GAPOR (73.0 kDa). WOR 4 shows 58% (37%), 57% (36%) and 49% (25%) amino acid sequence similarity (identity), respectively, with these enzymes. Structural studies show that AOR and FOR contain one [4Fe-4S] cluster and a

mononuclear tungsten site per subunit [Chan et al., 1995, Hu et al., 1999]. While WOR 4 also seems to contain a single W atom/subunit, its Fe content (< 3 Fe atoms/subunit) is lower than expected. Moreover, although the visible absorption properties of WOR 4 indicate a redox active FeS cluster, this was not evident from EPR analysis. Data from other spectroscopic techniques such as resonance Raman and magnetic circular dichroism are clearly needed to ascertain the nature of what appears to be an unusual FeS center. WOR 4 also seems to contain one Ca atom/subunit. This is also the case with FOR (but not AOR or GAPOR), although crystallographic analyses indicate that Ca has a structural rather than catalytic role.

WOR 4 was unable to oxidize any of the aliphatic or aromatic aldehydes, 2-hydroxycarboxylic acids or sugar aldehydes that were tested using viologen dyes or nicotinamide nucleotides as mediators. Nevertheless, analysis of the *P. furiosus* genome suggests that WOR 4 may have a role in aldehyde conversions. Unlike the genes encoding AOR, FOR and GAPOR, which are non-linked and are separated from neighboring ORFs by at least 40 nucleotides, the start codon of *wor4* is directly adjacent to the stop codon of a putative ORF (Pf 1811080) that is annotated as a aldose/aldehyde reductase (Fig. 7.4) (http://comb5-156.umbi.umd.edu/genemate/). The close proximity of these genes suggests that the encoded proteins might be transcribed and translated together and probably are closely related in enzyme activity or physiological function. It is possible that WOR 4 uses or produces such aldehydes/aldoses.

Figure 7.4.

Operon organization of tungsten oxidoreductase genes in the *P. furiosus* genome. The five genes shown in the figure are non-linked. The numericals indicate the actual number of nucleotides between the start and stop sites of these genes. Names in square brackets indicate putative enzymes/proteins, annotated on the basis of similarity to characterized proteins.


-49-
[Molybdopterin
conv. factor (moaD)]
AOR
-73-
FOR
[Seryl tRNA
synthase]
-41-
-50-
[Hydrolase]
GAPOR
[Glycosyl transferase]
-0-
[Aldose reductase]
WOR4
-10-
[FeS protein]
[WOR5]

However, the putative reductase would be a member of a ubiquitous family of enzymes which catalyze the NADPH-dependent reduction of a diverse range of aldehydes and/or sugars to alcohols [Tani et al., 2000, Welle et al., 1991, Cao et al., 1998, Akashi et al., 1996] and identifying the reaction catalyzed by the putative *P. furiosus* enzyme is difficult. For example, the protein encoded by this ORF shows ~70% sequence similarity to the 6'-deoxychalcone synthase of soybean [Welle et al., 1991], the aldo/keto reductase isoform 1 of human [Cao et al., 1998], the polyketide reductase of *Glycerrhia echinata,* [Barski et al., 1996] and the aldehyde reductase of *Acinetobacter sp.* M-1 [Tani et al., 2000].

Insight into the function of WOR 4 is also not evident from an examination of other microbial genomes. Homologs of WOR 4 are present in other hyperthermophilic archaea such as the anaerobes *P. horikoshii* [Kawarabayasi et al., 1998] and *Archaeoglobus fulgidus* [Klenk et al, 1997], and the microaerophile *Pyrobaculum aerophilum* [Fitz-Gibbon et al., 1997], as well as in the moderately thermophilic aerobes *Thermoplasma volcanium* [Kawashima et al., 2000] and *T. acidophilum* [Ruepp et al., 2000]. A common link between these organisms is not obvious, other than the fact that they are all archaea. It may be significant that the *P. furiosus* cells used to purify WOR 4 were grown on peptides and maltose in the presence of S°, and we were unable to purify this protein by the same methods from cells grown in the absence of S°. Preliminary microarray analyses show that expression of *wor4* is almost three-fold higher in maltose-grown *P. furiosus* when S° is present [Schut et al., 2001]. Comparable data using cells grown on peptides and S° are not yet available. The availability of the pure WOR 4

protein will enable such studies to be complemented with immunological analyses, and these are in progress.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The essential role of molybdenum (Mo) in biological systems has been recognized for many decades. In comparison, a positive role for the analogous metal tungsten (W) has emerged very recently. Historically, W has been regarded as an antagonist of the biological function of Mo. However, about 30 years ago the stimulatory effect of W on the growth of various microorganisms was reported. Subsequently, a dozen or so tungsten-containing enzymes were isolated and characterized and these can be classified into three separate families. Interestingly, the majority of these enzymes have been found in hyperthermophilic archaea. Three members of the 'AOR' family of tungstoenzymes have previously been purified from the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus*. The focus of the research project described herein was the further molecular, biochemical and kinetic characterization of the members of this family of enzymes and attempt to elucidate the role they play in the physiology of *P. furiosus*.

Although *P. furiosus* AOR had been extensively characterized by detailed molecular, structural and spectroscopic studies, very little was known about the related enzyme, FOR, when this project began. Although formaldehyde was routinely used as a substrate for FOR, the high K_m value suggested that it was not the true substrate for this enzyme. The detailed substrate kinetic studies presented herein indicate that FOR oxidizes short chain (C_1 – C_3) aldehydes but shows very little activity with longer chain

aldehydes (≥ C_5) or with aromatic aldehydes, suggesting that the catalytic site of this enzyme is probably only accessible to short-chain aldehyde substrates. This enzyme can also use C_4 – C_6 acid-substituted aldehydes and dialdehydes. In fact, the enzyme has higher affinity for such substrates than it does for C_1 – C_3 aldehydes. For example, FOR can rapidly oxidize succinic semialdehyde (C_4) and glutaric dialdehyde (C_5) whereas similarly sized, unsubstituted aldehydes are very poor substrates. Some C_4 to C_6 semialdehydes are involved in the metabolism of certain amino acids such as Arg, Pro and Lys. Since the organisms in which FOR has been found, such as *P. furiosus* and *T. litoralis*, grow using proteins as a primary source, FOR may have a role in amino acid metabolism, although this has yet to be conclusively established.

Support for the proposal that a C_4 – C_6 substituted aldehyde might be the physiological substrate for FOR also came from crystallographic analysis of this enzyme. The crystal structure of FOR from *P. furiosus* was solved at 1.8 Å resolution by Dr. Rees's group at Caltech. As expected from the high degree of sequence similarity between AOR and FOR, these two enzymes are structurally related. Indeed, the overall tertiary folding of the FOR subunit is virtually superimposable on that of AOR. However, their quaternary structures are distinct: AOR is a dimer whereas FOR is tetrameric. The four subunits of FOR are arranged around a channel of ~ 27 Å diameter that passes through the center of the molecule (Fig. 8.1). Like AOR, each subunit in FOR has one tungsten center coordinated by the dithiolene sulfurs of two pterin molecules and a single [4Fe-4S] cluster located about 10 Å away (Fig. 8.2a). The two pterins are linked to each other by their phosphate groups via a magnesium ion. In contrast to AOR, in

FOR a calcium ion is located close to one of the pterin cofactors and coordinates a ring keto group, but this is proposed to have a structural rather catalytic role.

In FOR a cavity provides access for the substrates to the W active site. This cavity has two distinctive parts: a large chamber at the bottom and a narrower channel leading to the protein surface. The bottom chamber is lined with amino acids with bulky side chains such as tyrosine, arginine, leucine and valine, whereas the channel is lined with hydrophobic residues. FOR crystals were soaked with the dicarboxylic acid, glutarate, which is the oxidation product of glutaric dialdehyde oxidation. Glutaric dialdehyde was chosen since it is the substrate that has the lowest K_m of any known so far for FOR. In the crystal structure, the glutarate molecule can be clearly seen bound at the active site of FOR (Fig 8.2b). One carboxylate group of the glutarate is located near the W site, stabilized by hydrogen bond interactions with side chain carboxylate group of Glu 308, Tyr 416 and His 437. The second carboxylate group of glutarate is bound to the protein through electrostatic interactions with the side chains of Arg 481 and Arg 492. This structure indicates that a $C_4 - C_5$ aldehyde molecule would fit into the active site cavity of this enzyme much better than one with a shorter or longer chain.

Physiological and enzymatic studies were carried out to test the hypothesis that FOR might be involved in amino acid metabolism. *P. furiosus* was grown in medium supplemented with amino acids such as Arg, Pro and Lys and the effect on cell growth and activity of FOR was measured. The specific activity of FOR did not change significantly in response to the addition of amino acids in the growth medium suggesting that this enzyme might not have a role in the catabolism of these basic amino acids, contrary to the conclusion from kinetic analysis.

Fig. 8.1.

Schematic representation of the FOR tetramer. The four subunits shown with the associated metal centers. Domains 1, 2 and 3 of each subunit are colored red, cyan and blue, respectively, while the metallocenters are represented as CPK models. Adapted from Hu et al., 1999

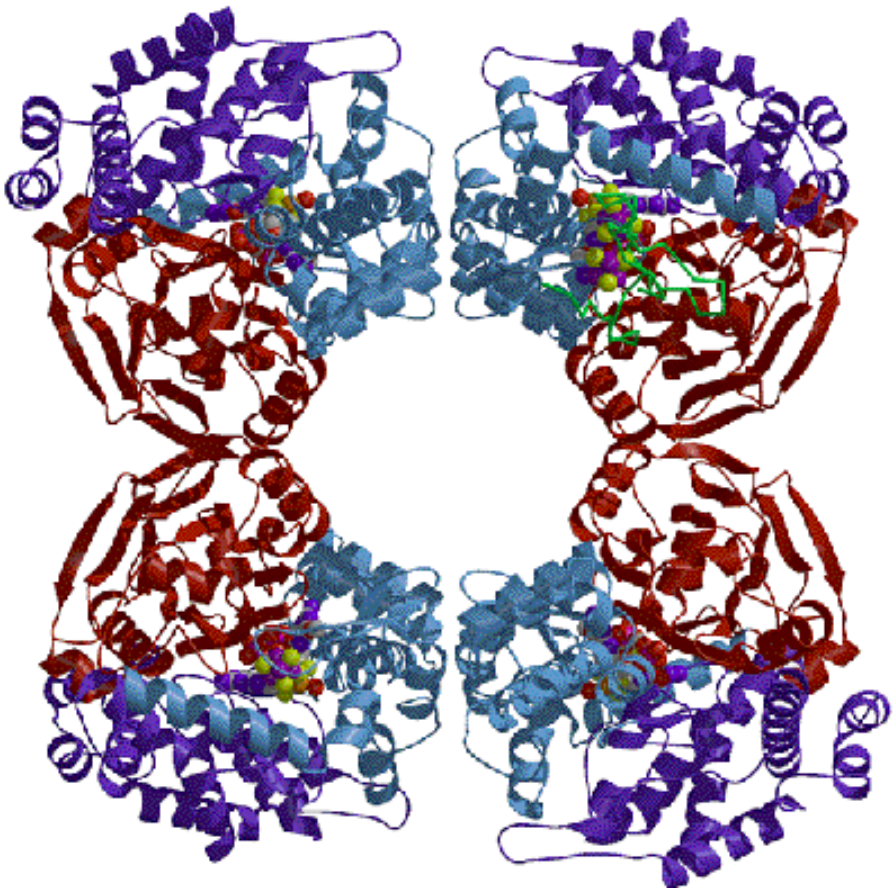
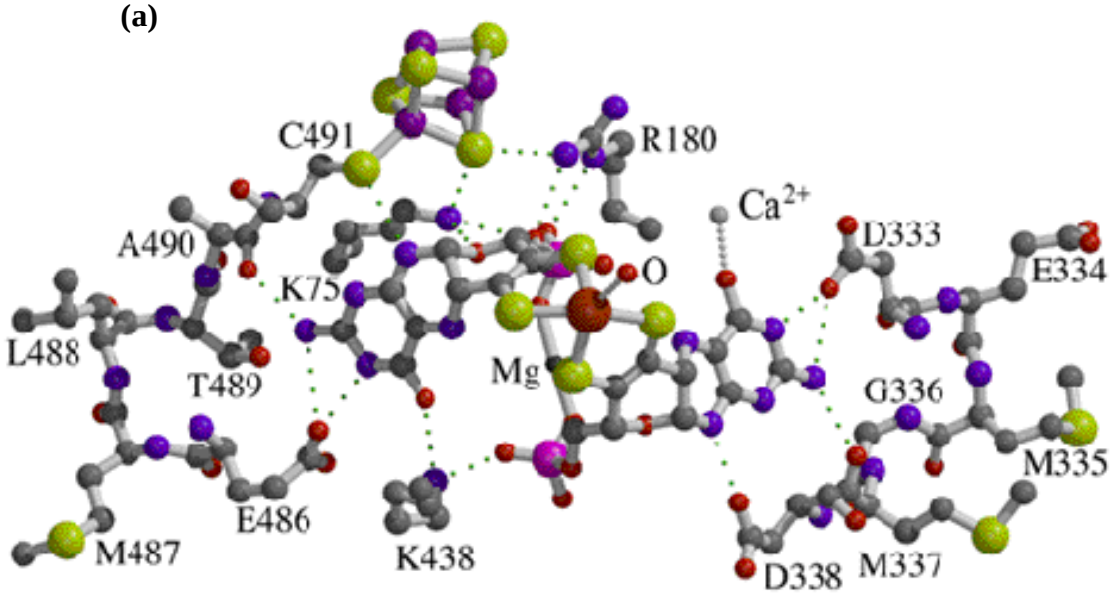


Fig. 8.2.

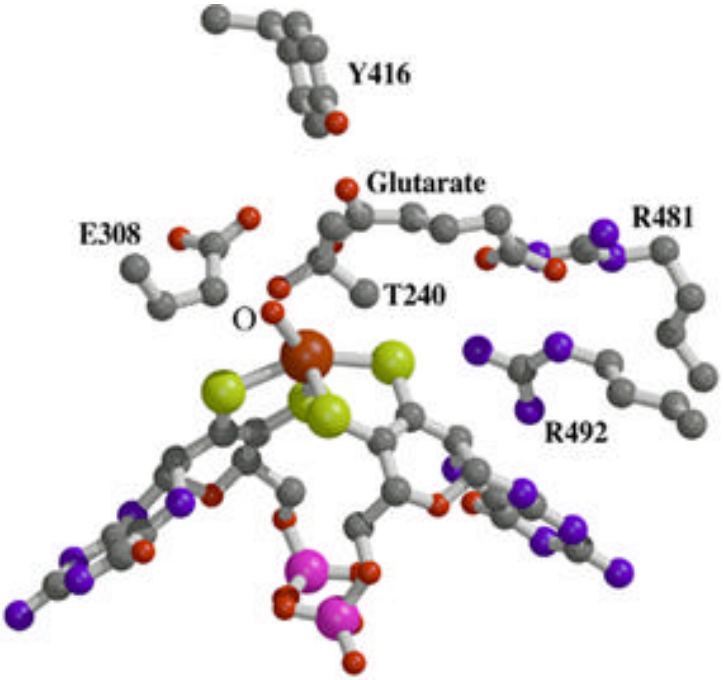
The structure of the tungsten center in the active site of FOR together with the pterin binding residues. The two pterin binding motifs are on the right and left sides of this figure. Hydrogen bond interactions are represented as green dotted lines.

Stereoview of the active site of FOR, including nearby residues and bound glutarate. The oxygen ligand coordinated to the W in the native FOR structure is shown. Adapted from Hu et al., 1999.

(a)


C491
R180
Ca2+
A490
D333
E334
K75
O
L488
Mg
T489
G336
M335
E486
M487
K438
D338
M337

(b)


Y416
Glutarate
R481
E308
T240
O
R492

However, a role for this enzyme in the peptidolytic pathway of *P. furiosus* cannot be completely ruled out based on this preliminary study. A more detailed analysis including the measurement of mRNA expression levels and specific activity of key enzymes in amino acid metabolic pathways (and FOR) under different growth conditions should provide clues to the physiological role of this tungstoenzyme.

All three aldehyde oxidoreductases from the 'AOR' family are inactivated *in vitro* on exposure to oxygen. The oxygen-inactivated enzyme can be reactivated to their original activity by incubating them with sodium sulfide under reducing conditions. With FOR incubation with sodium selenide has a similar effect although the degree of activation is much lower. Kinetic analyses of the various forms of FOR using a wide array of aldehyde substrates revealed that the catalytic efficiencies of the sulfide-activated enzyme are generally higher than those exhibited with the as purified enzyme. However, this result is mainly due to the lower K_m values displayed by the activated form of the enzyme. While the sulfide-activated enzyme did show a large increase (~ 5-fold) in activity with formaldehyde (routinely used as substrate for FOR), relative to the as purified enzyme, this was not the case for all the substrates that can be utilized by FOR. For example, similar kinetic values were obtained for the two forms of enzyme with the best substrate found so far, glutaric dialdehyde. It is not clear why the degree of activation depends on the substrate that is used.

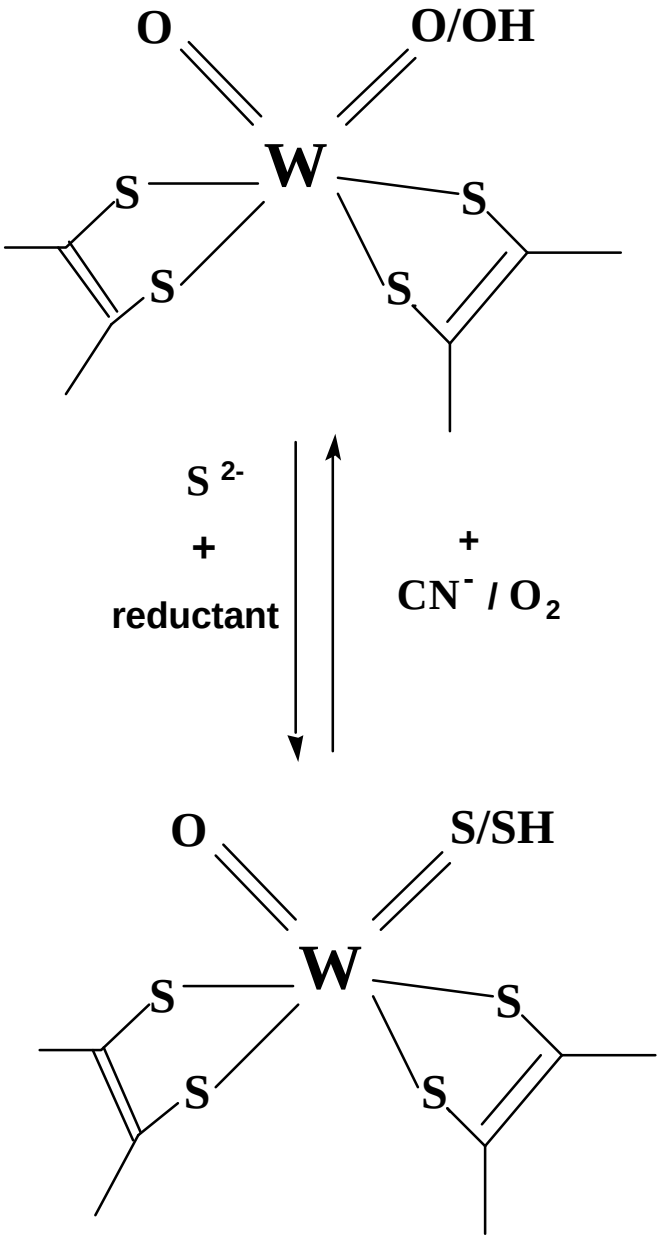
The phenomenon of sulfide-activation raises the issue as to the exact nature of the sulfide (presumably lost on exposure to oxygen) that is restored to the enzyme. Crystallographic and EXAFS data from both *P. furiosus* AOR and FOR has confirmed that the W atom at their active sites is coordinated by two pairs of dithiolene sulfurs from

a bispterin cofactor. In the oxidized W(VI) state, the W coordination sphere also includes two oxo ligands to yield an overall distorted trigonal prismatic arrangement. Therefore, the loss of sulfide caused by oxygen inactivation of the enzymes in their reduced W(V) states could either be due to a) displacement of a dithiolene S, or b) displacement of a terminal S (W=S or W-SH), with the formation of W=O species. Although crystallographic data obtained for FOR and AOR does not reveal a terminal S, the valence state of the W atom is not clear. However, the fact that the sulfide-activated form of FOR is susceptible to cyanide inactivation suggests the presence of a labile terminal S rather than a thiolene one. Therefore, by analogy with xanthine oxidase, the increase in specific activity of *P. furiosus* FOR and AOR on incubation with sulfide, can be interpreted in terms of replacement of a terminal oxo group by a terminal sulfido at the W active site (see Fig. 8.3). However, this is a tentative result and further crystallographic and spectroscopic analyses of the sulfide-activated and oxygen-inactivated forms of enzyme are necessary to more precisely define the W coordination sphere.

Herein the question was also addressed as to whether sulfide-activation is a physiologically relevant reaction? *P. furiosus* cells grown with S° appear to have a 3 – 5 fold higher intracellular concentration of acid labile sulfide and an approximately 2-fold higher polysulfide concentration than those grown without S°. While most of the acid-labile sulfide in the cell is expected to be associated with metals in the form of clusters, particularly of the FeS type, the intracellular levels of Fe and several other metals are the same in the two types of cell. Furthermore, this excess sulfide is not present in the ‘free’ form, rather, it is associated with the high molecular weight fraction of the whole cell extract (which includes the membranes).

Fig. 8.3.

Proposed model for W coordination sphere in oxygen-inactivated and sulfide-activated forms of enzyme.


O
O/OH
W
S
S
S
S
S 2-
+
reductant
+
CN- / O2
O
S/SH
W
S
S
S
S

Similarly, the polysulfide present in the cells grown with S° is associated mainly with the insoluble portion after the whole cell extract has been fractionated by centrifugation.

Therefore, it is possible that the polysulfide is associated with the outer surface of *P. furiosus* membranes rather than inside the cell. Polysulfides are generated in the growth medium and serve as the soluble intermediate for S° reduction by *P. furiosus*. This polysulfide is reduced to S^{2-} under the acidic conditions of the acid-labile sulfide assay and accounts for the excess sulfide measured in cells grown with S°.

Specific activities of the AOR, FOR and GAPOR remain unchanged in cells grown with and without S°. Based on these results, it seems improbable that sulfide-activation is a physiological reaction. More likey, exposure to oxygen during the purification process causes loss of sulfide from the enzymes active sites, and this can be reversed by incubation with high concentrations of sulfide and reductant. However, inside the cell the enzymes are unlikely to encounter oxygen and be inactivated by it. In addition, although the intracellular environment is highly reducing, high concentrations of 'free' sulfide are not available for the activation of aldehyde oxidoreductases as observed *in vitro.*

With the availability of the complete genome sequence of *P. furiosus* it was possible to search for ORFs that might encode other enzymes of the AOR family. The genes encoding AOR (*aor*), FOR (*for*) and GAPOR (*gor*) are spatially separated on the genome and, except for the ORFs adjacent to the previously identified cofactor-modifying (*cmo*) gene, none of the ORFs immediately adjacent to the *for*, *aor* and *gor* genes appeared to have a role in the synthesis or function of these three tungstoenzymes in *P. furiosus*. A search of the *P. furiosus* genome database with the

complete amino acid sequences of FOR, AOR and GAPOR revealed the presence of two additional genes, *wor4* and *wor5*, proposed to encode putative tungstoenzymes WOR4 and WOR5. Both WOR4 and WOR5 show high sequence similarity to AOR and FOR, and the calculated molecular weights of these enzymes are comparable to those of the previously isolated aldehyde oxidoreductases from *P. furiosus*. Analysis of the three-dimensional structures of *P. furiosus* AOR and FOR has shown that the tungstopterin site and [4Fe-4S] cluster are coordinated by conserved motifs present in the sequence of each enzyme. WOR 4 and WOR 5 appear to contain such motifs in their sequences. Therefore, these two putative enzymes appear to represent two novel enzymes of the AOR family.

Using a combination of column chromatography steps and tungsten analysis, one of these putative tungstoproteins WOR 4 has now been purified from the cell-free extracts of *P. furiosus*. WOR 4 is a dimeric protein of approximately 129 kDa. This enzyme is proposed to have 1 W and a [4Fe-4S] cluster per subunit. UV-Vis spectra of the oxidized and reduced enzyme indicate the presence of a FeS chromophore. This enzyme does not oxidize short or long chain aldehydes and hydroxyacids nor does it reduce ketoacids. The reaction catalyzed by the fourth member of the AOR family, WOR 4, is not known at present. WOR 4 could only be purified from *P. furiosus* cells grown with S°, although it does not rule out the fact that the enzyme is present in non-sulfur grown cells. Sulfur metabolism in *P. furiosus* is not well understood and whether WOR 4 expression changes in response to the presence of S° in the growth medium is not known. Future research will focus on the isolation of an active form of WOR 4 possibly using antibodies to the purified protein. Using microarray techniques, mRNA expression

levels can be compared in cells grown under different growth conditions to provide clues into the role played by this enzyme in *P. furiosus.*

The complete genome sequences of several hyperthermophilic archaea are now available. A search of these sequence databases revealed that certain hyperthermophilic archaea such as *Pyrococcus horikoshii*, *Pyrobaculum aerophilum*, *Methanococcus jannaschii*, *Aeropyrum pernix* and *Archeoglobus fulgidus*, all contain putative genes that would encode protein with significant sequence similarity to the AOR family of tungstoenzymes. In addition, homologs were also found in the genome sequences of some moderately thermophilic archaea and in genomes of three mesophilic bacteria. Therefore, the tungstoenzymes of the AOR family appear to be more widespread among the hyperthermophilic archaea (and some bacteria) than previously thought. However, it is important to note that these putative genes have been included in the AOR family based solely on sequence homology. It remains to be seen whether any or all of these genes actually encode proteins that contain W (or Mo) and iron-sulfur clusters. Isolation and characterization of these putative enzymes will provide clues to their phylogeny and function in the future.

The possibility that W can play an active role in biological systems has become clear in the last few decades. W is a relatively scarce on this planet. However, this element is enriched in certain ecological niches such as hot springs, brine lakes and hydrothermal vent fluids, and to microbial populations inhabiting these ecosystems W is abundantly available. Although, W salts are not highly reactive, its oxoacids are similar in structure to sulfate, phosphate and molybdate, compounds that are ubiquitous in nature. In fact, at present, all the known tungstoenzymes catalyze reactions involving

oxo atom transfer and coupled electron proton transfer, very similar to reactions catalyzed by Mo enzymes. Substitution of W into the active site of molybdoenzymes can produce active enzymes in some cases. For example, some can synthesize both molybdoenzyme and a genetically distinct W version of the same enzyme to catalyze the same reaction. However, the hyperthermophilic archaea are an exception as in the enzymes of the AOR family, W cannot be substituted with Mo. Thus, the members of this family are refered to as 'true' tungstoenzymes, as they only utilize W and they are phylogenetically distinct from the major classes of molybdoenzyme. It remains to be seen whether any or all of the AOR family homologs identified in the genome databases are 'true' tungstoenzymes. Future research in this field should not only attempt to isolate and characterize novel members of this unique class of tungstoenzymes but also focus on mechanism of action of these enzymes, their biosynthesis, and on the uptake, transport and storage of W within the cell.

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