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The biochemical diversity of life near and above 100 °C in marine environments

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1. SUMMARY

Hyperthermophilic micro-organisms grow at temperatures above 90 °C with a current upper limit of 113 °C. They are a recent discovery in the microbial world and have been isolated mainly from marine geothermal environments, which include both shallow and deep sea hydrothermal vents. By 16S rRNA analyses they are the most slowly evolving of all extant life forms, and all but two of the nearly 20 known genera are classified as *Archaea* (formerly *Archaebacteria*). Almost all hyperthermophiles are strict anaerobes. They include species of methanogens, iron-oxidizers and sulphate reducers, but the majority are obligate heterotrophs that depend upon the reduction of elemental sulphur (S°) to hydrogen sulphide for significant growth. The heterotrophs utilize proteinaceous materials as carbon and energy sources, although a few species are also saccharolytic. A scheme for electron flow during the oxidation of carbohydrates and peptides and the reduction of S° has been proposed. Two S°-reducing enzymes have been purified from the cytoplasm of one hyperthermophile (T_{opt} 100 °C) that is able to grow either with and without S°. However, the mechanisms by which S° reduction is coupled to energy conservation in this organism and in obligate S°-reducing hyperthermophiles is not known. In the heterotrophs, sugar fermentation is achieved by a novel glycolytic pathway involving unusual ADP-dependent kinases and ATP synthetases, and novel oxidoreductases that are ferredoxin- rather than NAD(P)-linked. Similarly, peptide fermentation involves several unusual ferredoxin-linked oxidoreductases not found in mesophilic organisms. Several of these oxidoreductases contain tungsten, an element that is rarely used in biological systems. Tungsten is present in exceedingly low concentrations in normal sea water, but hydrothermal systems contain much higher tungsten concentrations, more than sufficient to support hyperthermophilic life.

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2. INTRODUCTION

This review focuses on the physiology and metabolism of hyperthermophiles, which are micro-organisms that grow at temperatures of 90 °C and above. They are a relatively recent discovery in the field of microbiology. Most of the currently known species grow only under anoxic conditions and depend upon the reduction of elemental sulphur (S°) to H_2S for optimal growth. Another predominant metabolic mode amongst hyperthermophilic species is obligate heterotrophy wherein peptides serve as the primary carbon source. Only a few saccharolytic species are known. The majority of hyperthermophilic species at present are therefore strictly anaerobic, S°-reducing, heterotrophic organisms, and these will be the main focus of this review. The remaining hyperthermophiles include some methanogenic species, and some that gain energy by reducing nitrate or sulphate, or by oxidizing ferrous iron, and these will be briefly considered. No doubt the diversity of the hyperthermophiles will increase in the near future, as more of their known environments are examined and methods of isolation and identification become more sophisticated. The microbiology, physiology and biochemistry of the hyperthermophiles has been summarized in several recent reviews (see Blöchl *et al.* 1995; Adams 1996; Antranikian *et al.* 1996; Sunna *et al.* 1997; references therein). In the following only key or very recent papers are referred to.

Table 1 The hyperthermophilic genera: organisms that grow at 90 °C*

Genus	T_{max}†	Metabolism‡	Substrate§	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^+
Desulphurococcus (d/c)	90°	hetero	Pep	S°, H^+
Thermoproteus (c)	92°	hetero (auto)	Pep, CBH (H_2)	S°, H^+
Pyrodictium (d/m)	110°	hetero (auto)	Pep, CBH (H_2)	S°, H^+
Pyrococcus (d/m)	105°	hetero	Pep, CBH	±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				
Sulfophobococcus (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
Sulphate-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^-

* Data taken from Kelly and Adams (1994)c, Stetter (1996), Blöchl *et al.* (1997), Sako *et al.* (1996), Jochimsen *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 sulphur (S°) as electron donors. ¶ Indicates that species have been found in continental hot springs (c), shallow marine (m) and/or deep sea (d) environments.

3. HYPERTHERMOPHILIC MICRO-ORGANISMS

Hyperthermophiles will usually defined as organisms with an optimum growth temperature of at least 80 °C with a maximum growth temperature of 90 °C and above. Such organisms were discovered in the early 1980s through the pioneering efforts of Stetter and coworkers (Stetter 1996). At present more than 20 different genera of hyperthermophiles are known, and these are listed in Table 1. However, as indicated, only two of them, *Aquifex* and *Thermotoga*, are bacteria; the rest are classified as *Archaea* (formerly *Archaebacteria*). *Archaea* constitute the third domain of life and were first recognized as such in the early 1980s based on 16S rRNA analyses (Woese *et al.* 1990). The first genome sequence of an archaeon, that of *Methanococcus jannaschii* (Bult *et al.* 1996), contained genes encoding both bacterial- and eucaryotic-type proteins, in addition to those with no similarity in the databases, thus confirming that *Archaea* are indeed distinct from the other two domains. By 16S rRNA analyses, both the hyperthermophilic archaea and the two hyperthermophilic bacteria are the most slowly evolving organisms within their domains (Woese *et al.* 1990; Olsen *et al.* 1994; Stetter 1996), suggesting that the original organisms on this planet may have first evolved under conditions of high temperature. If true, then present-day hyperthermophiles could provide information on the evolution of enzymes and metabolic pathways. However, as will become evident, these organisms cannot be considered 'primitive' from a biochemical perspective, and the precise implications of their phylogenetic placement is really still unclear.

All of the hyperthermophiles have been isolated from geo-

thermally heated ecosystems. As indicated in Table 1, some are found in continental hot springs but the majority are of marine origin. In fact, of the 22 genera listed, only four of them (*Thermoproteus, Thermofilum, Sulfophobococcus* and *Methanothermus*) have been found so far exclusively in freshwater habitats. The marine environments include shallow coastal waters at depths down to 100 m or so, and deep sea hydrothermal vents, located up to 4000 m below sea level. The deep sea vent waters can reach temperatures approaching 400 °C and although micro-organisms have not been found at such temperatures, they have been isolated both from the spreading smoker plumes and from inside the smoker 'chimneys' at temperatures well in excess of 100 °C. All of the marine hyperthermophiles require quite high salt concentrations to grow, hence one might not expect the same hyperthermophilic genus to be isolated from both freshwater (continental) and marine environments. However, some species of *Desulphurococcus* and *Pyrobaculum* have been isolated from both types of ecosystem. Similarly, representatives of some marine genera such as *Pyrococcus* and *Methanococcus* have been found in both shallow and deep sea vents. Another parameter to be considered in evaluating the physiology of hyperthermophiles is pressure. For example, in the ocean hydrostatic pressure increases with depth by $\approx$100 atmospheres/km. However, of the known hyperthermophiles, from the deep sea or elsewhere, none have been shown to be dramatically affected by growth under the high pressures of their natural environment, although some of the enzymes purified from them may show a pressure response (Michels *et al.* 1996; Canganella *et al.* 1997). An obligately barophilic (or extremely halophilic) hyperthermophile has yet to be isolated from any environment.

4. METABOLIC PROPERTIES OF HYPERTHERMOPHILES

The metabolic properties of the currently known hyperthermophilic genera are also summarized in Table 1, in which the organisms are organized mainly according to the electron acceptors that they utilise. The majority of them fall into the 'S°-dependent category' as they metabolize elemental sulphur, or S°, reducing it to H_2S. In fact, many of this group are able to obtain energy for growth only by S° respiration. The exceptions are some species of *Pyrococcus* and *Thermococcus*, which grow in the absence of S° by fermentative-type mechanisms, and a species of *Pyrobaculum, P. aerophilum*, which is a nitrate-reducing, microaerophile. In fact, the latter organism has several unusual properties. It appears to be a true denitrifier since N_2 is the primary product not nitrite, and it has an optimal O_2 concentration for growth of below 1% (v/v) with a toleration limit of about 5% (v/v). The only other genus in the S°-dependent group able to use O_2 is *Acidianus* which, remarkably, can grow anaerobically by reducing S° to H_2S, or aerobically, by oxidizing S° to sulphate.

Also included in Table 1 are three recently isolated genera which fall into the 'S°-independent' category as they are unable to utilize S° (and do not fall into any of the other categories). These include *Sulfophobococcus* (Hensel *et al.* 1997) which grows by fermentation, and *Aeropyrum* (Sako *et al.* 1996) which is an obligate aerobe. The third member is *Pyrolobus*, the type species of which, *P. fumarii*, obtains energy for growth by a respiratory metabolism in which H_2 is the electron donor and nitrate, thiosulphate or low concentrations of O_2 (up to 0·3%, v/v) are the electron acceptors. These are reduced to ammonia, sulphide and water, respectively (Blöchl *et al.* 1997). The upper growth temperature of this organism is 113 °C, the highest yet reported. Moreover, it does not grow below 85 °C, and a significant fraction of exponentially growing cultures survive autoclaving (121 °C for 1 h).

The other hyperthermophilic archaea known include three genera of methanogens which, like their mesophilic relatives, produce methane from H_2 and CO_2, and two closely related genera, the sulphate-reducing *Archaeoglobus* and the iron-oxidizing *Ferroglobus*. As electron acceptors the latter uses either thiosulphate and nitrate, which are reduced to sulphide and nitrite, respectively. As shown in Table 1, the two genera of hyperthermophilic bacteria differ considerably in their metabolic properties. Species of *Thermotoga* are all fermentative which reduce S° to H_2S but also grow well in its absence. In contrast, the type species of *Aquifex, A. pyrophilus*, like *P. aerophilum*, is a microaerophilic denitrifier. Optimal growth requires an optimal O_2 concentration below 1% (v/v), and it also reduces nitrate to N_2. *A. pyrophilus*, like *Acidianus*, will also oxidize S° to sulphate.

Thus, in considering electron acceptors, a predominant metabolic mode amongst the hyperthermophilic species is the use of S° as an electron acceptor with the production of H_2S. Consequently, of course, only a few aerobic hyperthermophiles are known. In fact, aerobic species are found in only five genera (*Acidianus, Pyrobaculum, Aeropyrum, Pyrolobus* and *Aquifex*) and three of them (*Pyrobaculum, Pyrolobus* and *Aquifex*) are microaerophiles. In fact, nitrate reduction is almost as prevalent as O_2 reduction within the hyperthermophilic world, with species of four genera (*Pyrobaculum, Pyrolobus, Ferroglobus* and *Aquifex*) having this capability. While the aerobic and nitrate-reducing species are, by definition, obtaining energy by respiratory-type metabolisms, this is not necessarily the case with the S°-reducing organisms, as discussed below.

As shown in Table 1, most of the hyperthermophilic species are obligate heterotrophs. For the few autotrophic species known, the range of primary electron donors that can be used is quite limited, and includes only S° (by *Acidianus* and

Aquifex), ferrous iron and sulphide (by *Ferroglobus*), and H_2 (by the methanogens and certain species of *Ferroglobus, Archaeoglobus, Pyrolobus, Acidianus, Thermoproteus* and *Pyrodictium*). In addition, the growth of some of the heterotrophs (*Hyperthermus* and *Stetteria*) is stimulated by H_2 although the precise reason for this is not clear. Moreover, while heterotrophy is a predominant metabolic mode at extreme temperatures, most of the organisms are obligately proteolytic. In other words, significant growth is obtained only on complex protein-based substrates such as yeast, bacterial or meat extracts, peptone or tryptone. Their true growth substrates are therefore not clear. Some species of *Pyrococcus, Thermococcus* and *Desulphurococcus* have been reported to grow on mixtures of amino acids but in general the highest cell yields and lowest doubling times required complex media, suggesting that peptone, tryptone, yeast extract, etc., contain additional factors not present in mixtures of amino acids and vitamins (Rinker and Kelly 1996).

While most of the heterotrophic species are obligately proteolytic, a few of them are also saccharolytic (Table 1) although they typically are able to utilize a limited substrate range. Most of them are able to use as primary carbon sources complex carbohydrates such as starch and glycogen, and disaccharides such as maltose and cellobiose. None of the hyperthermophilic archaea are able to utilise monosaccharides, with the exception of a species of *Thermoproteus*, which grows using glucose as the main carbon source. On the other hand, utilization of monosaccharides is a characteristic of species of the hyperthermophilic bacterium, *Thermotoga*. It is not clear at present why most of the hyperthermophilic archaea do not use monosaccharides while hyperthermophilic bacteria are able to do so. Other than proteinaceous substrates and certain sugars, pyruvate is the only other carbon source that has been reported to support the growth of some of the hyperthermophilic archaea, including some species of *Pyrococcus*. However, cell yields with pyruvate as the primary carbon source are much lower than those obtained when complex peptides or carbohydrates are the growth substrates.

It is therefore evident that most of the hyperthermophiles so far obtained in pure culture are strictly anaerobic heterotrophs that are either obligately or facultatively dependent upon the reduction of S° for optimum growth. Most of them are also proteolytic and some can also utilise carbohydrates. We now turn to what is known about the biochemistry of the pathways of peptide and sugar catabolism, as well as the enzymology of S° reduction. Most studies of these topics have been carried out with *P. furiosus*, which was isolated in 1986 from a shallow marine volcanic vent by Stetter and coworkers. It grows optimally at 100 °C by fermenting carbohydrates and peptides to organic acids, H_2 and CO_2 (Fiala and Stetter 1986).

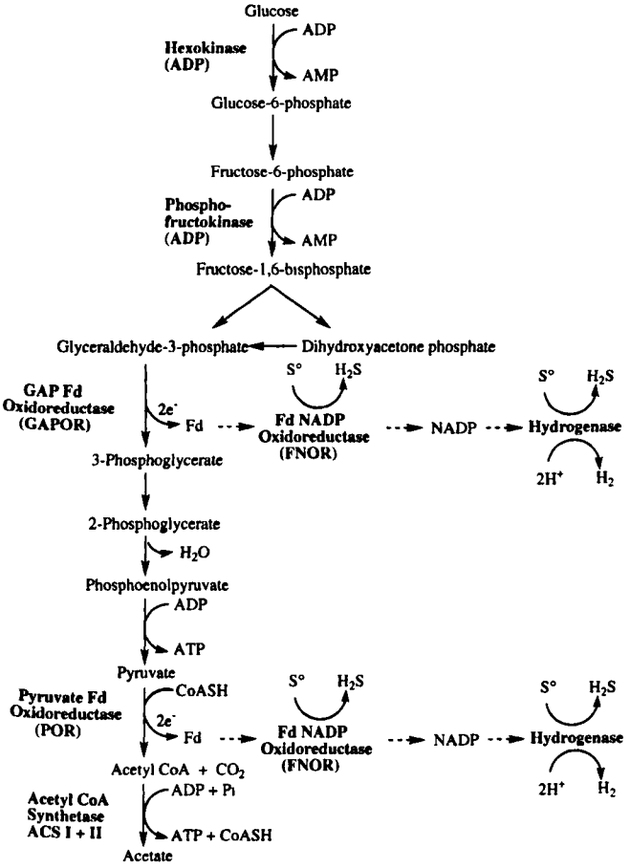

Fig. 1 Proposed glycolytic pathway in *Pyrococcus furiosus* and production of H_2 and H_2S. Unusual enzymes are indicated. Fd represents ferredoxin. Modified from Mukund and Adams (1995).

5. FERMENTATIVE PATHWAYS

5.1. Saccharolytic

Pyrococcus furiosus grows on starch, glycogen, maltose and cellobiose, but not monosaccharides, as its primary carbon source, and several of the enzymes involved in the degradation of these compounds to glucose have been characterized (Sunna *et al.* 1997). The route of sugar catabolism in *P. furiosus* was established primarily from the results of two recent investigations. In the first (Kengen *et al.* 1994), it was demonstrated using ^{13}C NMR spectroscopy that the predominant route for sugar fermentation in this organism is an unusual Embden–Meyerhof rather than the modified Entner–Doudoroff type pathway that had been proposed earlier based on enzymatic analyses (Mukund and Adams 1991; Schäfer and Schönheit 1992). However, a puzzling aspect of the this Embden–Meyerhof pathway was the presence in cell-free extracts of *P. furiosus* of an extremely low activity of the conventional glycolytic enzyme, gly-

ceraldehyde-3-phosphate dehydrogenase (GAPDH). The second investigation solved this problem by showing that *P. furiosus* contained a new enzyme, glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR: Mukund and Adams 1995). As shown in Fig. 1, GAPOR was proposed to replace GAPDH in the Embden–Meyerhof fermentation pathway. Note that GAPOR differs from GAPDH in two ways. First, it uses ferredoxin rather than NAD as the electron carrier. Second, it is proposed to produce 3-phosphoglycerate rather than 1,3-bisphosphoglycerate (which is the product of the GAPDH reaction). Hence, this pathway lacks one of the energy conservation steps found in the conventional glycolytic pathway, which is consistent with the reported growth yield data from *P. furiosus*.

In addition to GAPOR, the proposed conversion of glucose to acetate by *P. furiosus* involves three other unusual enzymes (Fig. 1). These are hexokinase and phosphofructokinase, which are ADP-rather than ATP-dependent enzymes (Kengen *et al.* 1994), and acetate-producing acetyl CoA synthetase (Schäfer *et al.* 1993). The hexokinase has been purified (Kengen *et al.* 1996) and does not utilize GTP or ADP. Acetyl CoA synthetase has also been characterized from *P. furiosus and* is present as two isoenzyme forms, termed ACS I and ACS II (Mai and Adams 1996a). These enzymes also function in peptide catabolism and their properties are described below. While GAPOR catalyses the first oxidation step in the conversion of glucose to acetate, the second oxidation step involves pyruvate ferredoxin oxidoreductase (POR), which produces acetyl CoA from pyruvate and, like GAPOR, uses ferredoxin as its electron acceptor (Fig. 1). The properties of POR, which is one of four 2-ketoacid oxidoreductases that have been purified from *P. furiosus*, are also discussed below.

An unusual feature of the Embden-Meyerhof-type pathway in *P. furiosus* is that the excess reductant is generated in the form of reduced ferredoxin rather than as NAD(P)H. The oxidation of this redox protein must be coupled ultimately to the production of H_2 or, if S° is present, to the production of H_2S. The H_2-evolving hydrogenase of *P. furiosus* does not use ferredoxin as an electron carrier, rather, NADPH is the proposed electron donor (Ma *et al.* 1994). As shown in Fig. 1, the reduction of NADP using ferredoxin as the electron donor is catalysed by the enzyme ferredoxin NADP oxidoreductase (FNOR: Ma and Adams 1994). The question therefore arizes as to the nature of the enzyme that reduces S° in *P. furiosus*. Surprisingly, it is the hydrogenase that catalyses this reaction (Ma *et al.* 1993), and the term 'sulfhydrogenase', derived from sulphur reductase hydrogenase, was coined to reflect the dual catalytic activities of this enzyme. The pathway of electron flow from the glycolysis to S° is complicated by finding that FNOR also catalyses S° reduction to H_2S using NADPH as the electron donor (Ma and Adams 1994). Thus, as summarized in Fig. 1, electrons from the oxidation reactions catalysed by GAPOR and POR are used to produce H_2 via FNOR, NADP and hydrogenase, but when S° is present, FNOR and hydrogenase catalyse the reduction of S° to H_2S.

Hence, in *P. furiosus*, there are two cytoplasmic enzymes that reduce S° to H_2S-hydrogenase (sulfhydrogenase) and FNOR. Obviously, this is unlikely to be the case in hyperthermophiles that grow by S° respiration, as one would expect a S°-reducing enzyme to be part of a membrane-bound electron transfer chain where peptides, in the case of the heterotrophic species, and H_2, in the case of the autotrophs, serve as the electron donor, and energy is conserved by electron transport linked phosphorylation. However, as yet, such a system has yet to be characterized from a hyperthermophilic organism. A further complication in understanding the metabolism of S° by hyperthermophiles is that S° reduction by *P. furiosus* does, in fact, lead to the conservation of energy. For example, the yield of cells grown with maltose as the primary carbon source in the presence of S° is about twice that obtained in the absence of S° (Schicho *et al.* 1993). In light of the cytoplasmic location of the S°-reducing enzymes in this organism, the precise mechanism of S°-dependent energy conservation is unclear at present. Similarly, it is not known why most of the heterotrophic hyperthermophiles do not grow to any extent in the absence of S°, while some, such as *P. furiosus*, grow to comparable densities with and without this compound. Does this reflect a fundamental difference between the two types of organism in the mechanisms by which ATP is synthesized? Clearly, much is to be learnt about microbial S° reduction at temperatures near 100 °C.

5.2. Proteolytic

Protein-based substrates can be utilized by virtually all of the hyperthermophilic archaea as their sole sources of carbon and nitrogen (see Table 1) so it is perhaps not surprising that several of them have been found to contain high intracellular and in some cases extracellular protease activities, some of which have been characterized (Bauer *et al.* 1996). Such enzymes are assumed to generate various amino acids, the primary steps in the utilization of which in *P. furiosus* appear to be aminotransferase-rather than dehydrogenase-type reactions. In mesophilic, anaerobic bacteria, the resulting 2-keto acids are converted via decarboxylation to the aldehyde, and then oxidation to the acid via nicotinamide-linked dehydrogenases In contrast, *P. furiosus* contains four distinct 2-keto acid oxidoreductases to convert transaminated amino acids to their corresponding acyl CoA derivative. These are termed IOR (where I represents indolepyruvate) which utilizes aromatic 2-keto acids, VOR (where V represents 2-ketoisovalerate) which oxidizes branched chain 2-keto acids, and KGOR (where KG represents 2-ketoglutarate, the only substrate for this enzyme), in addition to the POR enzyme described above, which oxidizes primarily pyruvate (see

Adams and Kletzin 1996; Heider *et al.* 1996; Mai and Adams 1996b). Thus, VOR shows the highest catalytic efficiency with 2-ketoisovalerate, 2-ketoisocaproate and 2-keto-3-methylvalerate, which are derived from valine, leucine and isoleucine, respectively, while IOR oxidizes phenylpyruvate, p-hydroxyphenylpyruvate and indolepyruvate, which are generated from phenylalanine, tyrosine and tryptophan, respectively. Note that all four of the oxidoreductase reactions are coupled to ferredoxin reduction, as shown in Fig. 2. In each case, it is assumed that ferredoxin is re-oxidized by FNOR with the subsequent production of H_2 or H_2S, as shown in Fig. 1.

The question arizes as to the fate of the acyl CoA derivatives generated by the 2-keto acid oxidoreductases. As mentioned above, *P. furiosus* contains two isoenzyme forms of ACS and these convert the CoA derivative, ADP and phosphate to the corresponding acid and ATP. ACS I uses acetyl CoA and isobutyryl CoA as substrates, which are the products of the POR and VOR reactions, respectively, but it does not use phenyl-or indoleacetyl CoA, which would be produced by IOR. On the other hand, ACS II, utilizes all four substrates, but neither enzyme oxidized succinyl CoA, which would be produced by KGOR. Presumably, succinyl CoA is used for biosynthesis rather than for energy conservation.

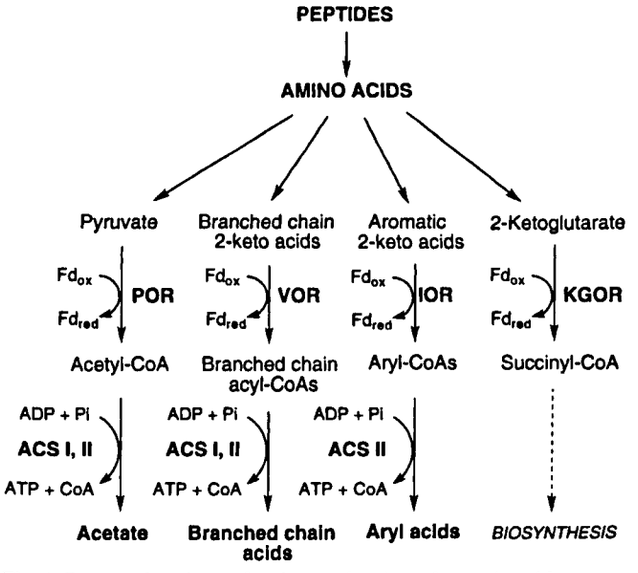

Fig. 2 Proposed pathway for the production of organic acids from peptides in *Pyrococcus furiosus*. IOR, POR, KGOR and VOR represent ferredoxin-dependent oxidoreductases that utilize indolepyruvate, pyruvate, 2-ketoglutarate and 2-ketoisovalerate, respectively. The other abbreviations are: ACS, acyl CoA synthetase; and Fd_{ox} and Fd_{red}, the oxidized and reduced forms of ferredoxin. Modified from Mai and Adams (1996a).

Organisms such as *P. furiosus* are known to generate organic acids such as isovalerate, isobutyrate and phenylacetate, as well as acetate, during growth on peptides.

Hence, as shown in Fig. 2, both ACS I and II are proposed to produce acetate and the branched chain organic acids from the POR and VOR activities, but only ACS II yields aromatic acids (from the IOR reaction). Both ACS enzymes were also shown to catalyse the ATP-dependent conversion of the acids to their CoA derivatives, suggesting that they might also function to provide carbon skeletons for biosynthesis under nutrient limited conditions. Interestingly, ACS I, which is the predominant isoenzyme, exhibited twice the catalytic efficiency with guanine nucleotides as it did with adenine nucleotides, suggesting perhaps that GTP synthesis is also used as a means of energy conservation. ACS I and II were the first ATP-dependent enzymes to be purified from hyperthermophiles, and ACS II is the first enzyme of the ACS type to utilize aromatic CoA derivatives.

Our understanding of the pathways of amino acid metabolism in *P. furiosus* was recently complicated by the finding that POR not only catalyses the oxidative decarboxylation of pyruvate to acetyl CoA, it also functions as a pyruvate decarboxylase and generates acetaldehyde (Ma *et al.* 1997). Initially this was thought to be an artifact of the *in-vitro* assay but the decarboxylase activity requires CoASH (this has a structural role as it can be replaced with desulfo-CoASH) and acetyl CoA and acetaldehyde are produced simultaneously, suggesting aldehyde production is an intrinsic part of the mechanism. Moreover, aldehyde production *in vivo* explains the physiological function of two aldehyde-utilizing enzymes that are found in *P. furiosus* and related heterotrophic hyperthermophiles, alcohol dehydrogenase (ADH) and aldehyde ferredoxin oxidoreductase (AOR). ADH uses NADPH as an electron donor and reduces aldehydes to the corresponding alcohol, whereas AOR oxidizes aldehydes to the corresponding acid using ferredoxin as the electron acceptor. Kinetic analyses have shown that the most efficient aldehyde substrates for both enzymes are those that correspond to the aldehyde derivatives of the substrates of POR, VOR and IOR, namely, acetaldehyde, isovalerylaldehyde and phenylacetaldehyde, respectively (see Adams and Kletzin 1996). Thus, it was proposed that aldehyde production by the 2-keto acid oxidoreductases occurs *in vivo* and that the physiological roles of AOR and ADH is to remove them, as shown in Fig. 3. How electron transfer between these competing reactions is controlled remains to be determined but it should be noted that AOR oxidizes the aldehydes and therefore generates reductant that must be disposed, while ADH reduces aldehydes to alcohols, which could in turn be excreted. Hence, how the aldehydes from the 2-keto acid oxidoreductase reactions are disposed of might depend on the redox status of the cell. However, it should be pointed out that metabolic labelling analyses have yet to be carried out to confirm these

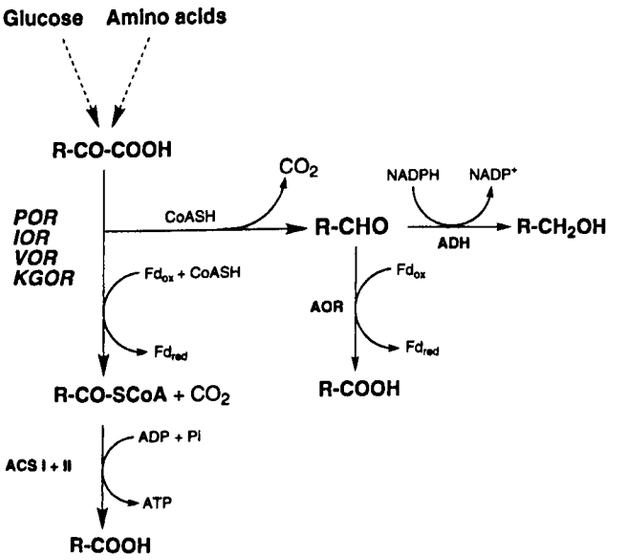

Fig. 3 Proposed function of 2-ketoacid oxidoreductases as decarboxylases. The abbreviations are: ADH, alcohol dehdyrogenase; and AOR, aldehyde ferredoxin oxidoreductase. Other abbreviations are as in the legend to Fig. 2. Modified from Ma *et al.* (1997).

proposed pathways (Figs 2, 3) for amino acid fermentation by *P. furiosus*.

6. ROLE OF TUNGSTEN-BASED OXIDOREDUCTASE-TYPE ENZYMES

An indication that *P. furiosus* might utilize unusual fermentative pathways came with the finding that it requires tungsten (symbol W) for optimal growth (Bryant and Adams 1989). This element is rarely used in biological systems, indeed, at that time, only one tungstoenzyme had been characterized (see Johnson *et al.* 1996). In contrast, the essential role of molybdenum (Mo), the chemically analogous element to W, in biology had been known for decades and a large number of molybdoenzymes have been extensively characterized (Hille 1996). Indeed, virtually all life forms that have been examined have been found to harbour molybdoenzymes, for example, *Escherichia coli* contains six varieties (Hille 1996). Hence, the obvious questions are, what does *P. furiosus* utilize W for, and why does it use W rather than Mo?

To date three different tungstoenzymes have been purified from *P. furiosus*. Two of them have already been described above, and these are GAPOR and AOR. The third variety is termed formaldehyde ferredoxin oxidoreductase, or FOR. In contrast to AOR and GAPOR, the physiological substrate for and the function of FOR are as yet unknown (see Adams and Kletzin 1996). FOR was purified by its ability to oxidize formaldehyde, and of the more than 20 unsubstituted monoaldehydes tested, only formaldehyde, acetaldehyde, propionaldehyde and phenylpropionaldehyde were oxidized at significant rates. These are unlikely to be the physiological substrates, however, because of the high K_m values (>20 mM). On the other hand, FOR does oxidize glutaric dialdehyde with high efficiency (K_m value, 0·8 mM) and although this compound does not lie on a known biochemical pathway various C4–C6 semialdehydes are involved in the metabolism of certain amino acids such as Arg, Lys and Pro. Thus, like AOR, FOR is thought to be involved in amino acid metabolism, although this has yet to be proven.

FOR, AOR and GAPOR all catalyse the oxidation of various aldehydes to the corresponding acid (eqn 1), a reaction of very low potential:

$$RCHO + H_2O \leftrightarrow RCOO^- + 3H^+ + 2e^- \qquad \text{(eqn 1)}$$

(acetaldehyde/acetate, $E_0' = -580$ mV) and all three enzymes use the redox protein ferredoxin as the electron carrier. Moreover, although FOR (α_4), AOR (α_2) and GAPOR (α) have different quaternary structures, their subunit molecular weights are comparable (≈65 kDa) and the close relationship between the three enzymes was first indicated by the similarity in their N-terminal sequences (Kletzin *et al.* 1995). This has now been confirmed by the availability of their complete amino acid sequences from the on-going project to sequence the genome of *P. furiosus* at the University of Utah (R. Weiss, unpublished data). Thus, 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). Moreover, the genomic database has revealed two additional ORFs and these appear to encode the fourth and fifth members of this enzyme family. Termed *worA* and *worB* (to depict genes encoding putative oxidoreductases within the tungstoenzyme family), these two genes encode proteins of molecular weight 64 and 69 kDa. The similarity (identity) of the sequences of the WOR A and WOR B 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 A and WOR B appear to be more closely related to AOR and FOR, than they are to GAPOR.

The five (putative) tungstoenzymes of *P. furiosus* also appear to be closely related structurally. The crystal structure of AOR, obtained to 2·3 Å resolution (Chan *et al.* 1995), showed that the W atom in its subunit is coordinated in part by four sulphur atoms originating from the dithiolene groups of two organic pterin cofactors, with a [4Fe-4S] cluster situated 8 Å from the W site. Sequence analyses show that all five enzymes contain the motifs that bind the two pterins and the FeS cluster, suggesting that FOR, GAPOR, WOR A and WOR B all contain the same cofactors as AOR. Spectroscopic analyses have also established that the W site in AOR under-

goes a two electron redox reaction, consistent with aldehyde oxidation (eqn 1), in which the W cycles between the IV, V and VI oxidation states. Interestingly, the amino acid sequences of the tungstoenzymes of *P. furiosus* show no similarity whatsoever to any of the more than 30 amino acid sequences available for molybdoenzymes (Kletzin *et al.* 1995). This is very surprising, since all molybdoenzymes known, with the notable exception of nitrogenase, also contain a pterin cofactor, and most also contain one or more iron-sulphur clusters. Therefore, it appears that while the structures of the catalytic sites in W and Mo enzymes are similar, the types of enzyme must have diverged very early on the evolutionary time scale, such that present-day versions show no detectable sequence similarity.

So, why has *P. furiosus* 'chosen' to use W rather than Mo to function at the catalytic site of at least three and probably five different oxidoreductase-type enzymes? One reason could be availability, that is, in the environments in which hyperthermophiles are found, W is much more prevalent than Mo (Kletzin and Adams 1996). In the earth's crust, W and Mo are of comparable abundance but in freshwater environments the concentration of W rarely exceeds 0·5 nM, which is typically several orders of magnitude lower than that of Mo ($\approx 2\ \mu g/kg$). The difference between the two elements is even greater in sea water where the concentration of W is exceedingly low and about 500 000-fold less that than of Mo ($\approx 10\ \mu g/kg$). However, the situation appears to be reversed in deep sea hydrothermal systems, at least based on the analysis of one black smoker system (see Kletzin and Adams 1996). In the vent fluids, Mo was not detectable but the W concentration was significant (0·4 $\mu g/kg$), and in vent flanges, a geological formation known to harbour heterotrophic hyperthermophilic archaea, the concentration of W was about an order of magnitude greater than that of Mo (50 $\mu g/kg$). Hence, at least in one high temperature environment, there is more than sufficient W to support the growth of hyperthermophiles. In fact, in the one study of the W requirement of a hyperthermophile (*P. furiosus*), the W concentration required to support growth was ≈ 15 nM (Mukund and Adams 1996). Thus, the amount of W present in open sea water would probably be growth limiting to heterotrophic hyperthermophiles.

A second reason for the utilization of W rather than Mo by *P. furiosus* could be that W is much more suited than Mo to participate in the required chemical reactions, namely, aldehyde oxidation at temperatures near 100 °C. The properties of analogous W and Mo complexes was recently examined with this aspect in mind (Johnson *et al.* 1996). However, the chemistry of mononuclear W complexes is much less developed than that of Mo, which is in part due to the thermodynamic instability of oxo-W(IV) complexes compared to the equivalent oxo-Mo(IV) complex. On the other hand, W(VI/V) complexes with four sulphur ligands can be reduced to the W(IV) state, but such complexes are very O_2-sensitive compared to the equivalent Mo complexes, indicating that such a W site within an enzyme would likely function only under anaerobic conditions. Such W complexes also exhibit strongly enhanced thermal stability compared to the corresponding Mo complex, suggesting that only the former would be stable enough to be utilized at temperatures near the normal boiling point. This enhanced bond strength of W complexes may also account for the observation that such complexes are generally kinetically slower than the equivalent Mo complexes. Thus, one would expect that higher temperatures might be a requirement for W centres to catalyse reactions at high rates, reactions that could be performed at similar rates by Mo centres at much lower temperatures. So, if W was utilized in biology, the properties of the synthetic complexes would suggest that W would only catalyse low potential reactions under anaerobic conditions, and then significant catalytic rates would be observed only at high temperatures. Conversely, Mo complexes would be unstable at high temperatures but at lower temperatures they would be catalytically competent over the whole biological range of potentials under both aerobic and anaerobic conditions (Johnson *et al.* 1996).

Remarkably, these conclusions from the properties of synthetic W/Mo complexes fit very well with our current understanding of the utilization of W and Mo. So far, the only known organisms whose growth appear to be absolutely dependent upon W are the heterotrophic hyperthermophiles represented by *P. furiosus* (Mukund & Adams 1996). It appears that its tungstoenzymes are carrying out reactions near the limits of biological systems–very low potential reactions at extreme temperatures–a feat that can be accomplished by W but not by Mo. Interestingly, in mesophilic methanogens, the enzyme that activates CO_2 is a Mo-containing enzyme, but the growth of the hyperthermophilic methanogen, *Methanopyrus kandleri*, which grows up to 110 °C, was reported to be stimulated by W but not by Mo (Vorholt *et al.* 1997). It therefore appears that its CO_2-activating enzyme is W- rather than Mo-dependent, although the enzyme has yet to be purified. Thus, it will be intriguing to see if the growth of other types of hyperthermophile, in addition to the heterotrophs and the methanogens, are dependent upon W and whether they are incapable of utilizing Mo. As yet, a hyperthermophilic molybdoenzyme has yet to be characterized.

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