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Metabolism of hyperthermophiles

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Hyperthermophiles are characterized by a temperature optimum for growth between 80 and 110°C. They are considered to represent the most ancient phenotype of living organisms and thus their metabolic design might reflect the situation at an early stage of evolution. Their modes of metabolism are diverse and include chemolithoautotrophic and chemoorganoheterotrophic. No extant phototrophic hyperthermophiles are known. Lithotrophic energy metabolism is mostly anaerobic or microaerophilic and based on the oxidation of H_2 or S coupled to the reduction of S, SO_4^{2-}, CO_2 and NO_3^- but rarely to O_2. The substrates are derived from volcanic activities in hyperthermophilic habitats. The lithotrophic energy metabolism of hyperthermophiles appears to be similar to that of mesophiles. Autotrophic CO_2 fixation proceeds via the reductive citric acid cycle, considered to be one of the first metabolic cycles, and via the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway. The Calvin cycle has not been found in hyperthermophiles (or any Archaea). Organotrophic metabolism mainly involves peptides and sugars as substrates, which are either oxidized to CO_2 by external electron acceptors or fermented to acetate and other products. Sugar catabolism in hyperthermophiles involves non-phosphorylated versions of the Entner-Doudoroff pathway and modified versions of the Embden-Meyerhof pathway. The 'classical' Embden-Meyerhof pathway is present in hyperthermophilic Bacteria (*Thermotoga*) but not in Archaea. All hyperthermophiles (and Archaea) tested so far utilize pyruvate:ferredoxin oxidoreductase for acetyl-CoA formation from pyruvate. Acetyl-CoA oxidation in anaerobic sulphur-reducing and aerobic hyperthermophiles proceeds via the citric acid cycle; in the hyperthermophilic sulphate-reducer *Archaeoglobus* an oxidative acetyl-CoA/carbon monoxide dehydrogenase pathway is operative. Acetate formation from acetyl-CoA in Archaea, including hyperthermophiles, is catalysed by acetyl-CoA synthetase (ADP-forming), a novel prokaryotic enzyme involved in energy conservation. In Bacteria, including the hyperthermophile *Thermotoga*, acetyl-CoA conversion to acetate involves two enzymes, phosphate acetyltransferase and acetate kinase.

Key words: Acetate formation, acetyl-CoA oxidation, Archaea, Bacteria, chemolithoautotroph, chemoorganoheterotroph, glycolytic pathways, hyperthermophiles, metabolic pathways, peptide metabolism, sugar metabolism.

Hyperthermophilic organisms — according to Stetter (Stetter *et al.* 1990; Stetter 1993; Blöchl *et al.* 1995) — have a temperature optimum for growth between 80 and 110°C. All hyperthermophiles known so far are prokaryotes. Most prokaryotes belong to the Archaeal domain (Woese *et al.* 1990) although some belong to two bacterial orders, the *Thermotogales* and the *Aquificales*. Hyperthermophiles represent the deepest branch-offs and shortest lineages close to the root of the phylogenetic tree and are thus considered to be the most ancient living organisms and closely related to the postulated "common ancestor" of all extant life, which is assumed to have been a hyperthermophile (Woese 1987; Woese *et al.* 1990; Kandler 1992; Zillig 1991; Figure 1). Analysis of the metabolism of hyperthermophiles might therefore give an idea of the metabolic design of phylogenetically ancient organisms and, by comparison with the established metabolism of mesophilic bacteria, provide information concerning the evolution of metabolic pathways. The metabolism of recent hyperthermophiles is diverse; it includes obligate or facultative chemolithoautotrophs and chemoorganoheterotrophs. No case of phototrophic hyperthermophile is known, indicating that, in evolution, chemolithoautotrophy preceded photoautotrophy as a process for primary production of organic matter (see Kandler 1993). It is proposed that the first lithoautotrophs grew on H_2 and S

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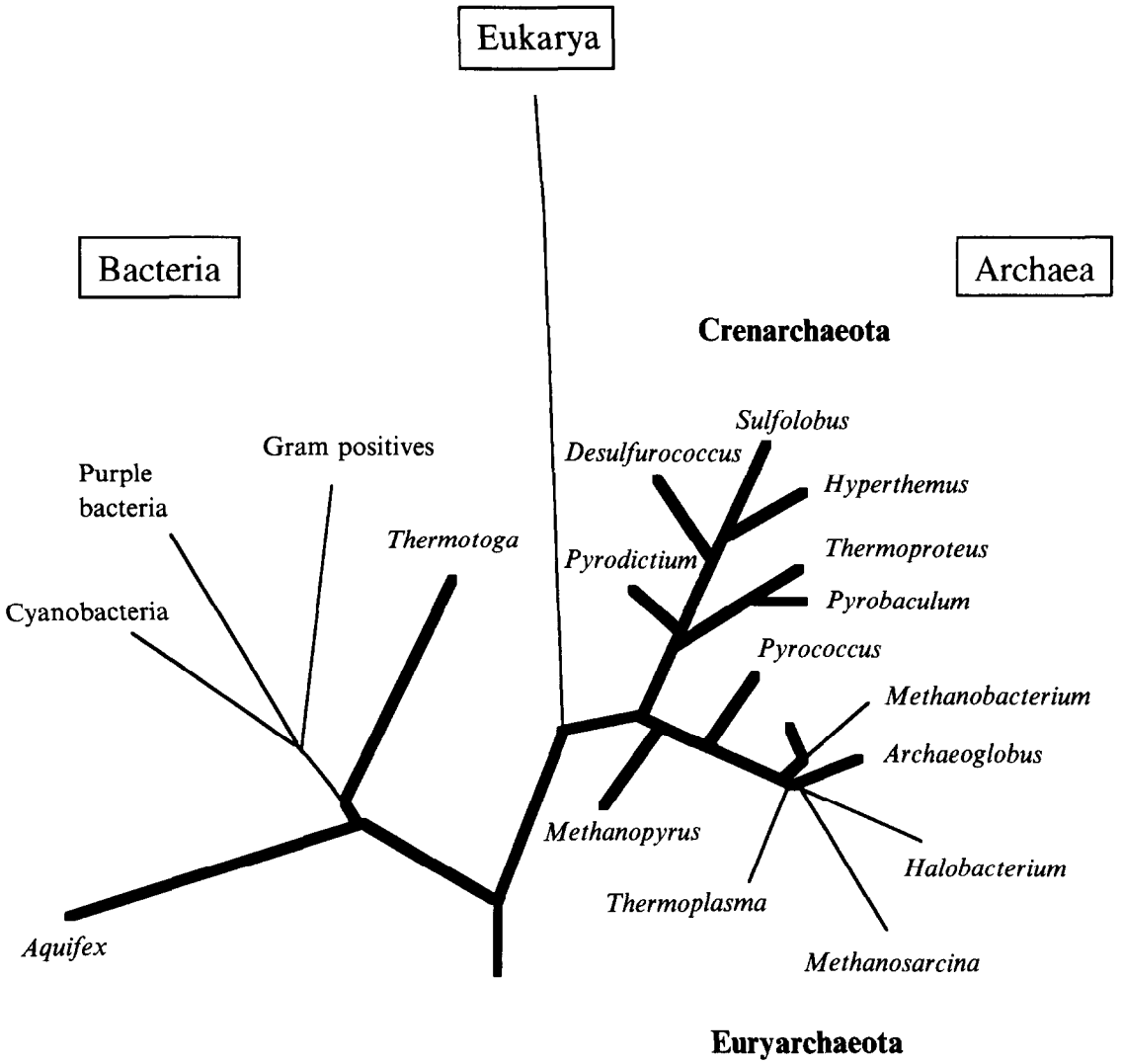

Figure 1. Phylogenetic position of hyperthermophilic genera (thick lines) and a few moderate thermophilic and mesophilic archaeal genera (thin lines), the metabolism of which is discussed in this review. This phylogenetic tree is modified from Woese *et al.* (1990) and Stetter (1993).

or H_2 and CO_2 as energy sources. According to Wächtershäuser (Wächtershäuser 1988; Drobner *et al.* 1990), molecular hydrogen, the electron donor for both catabolism and autotrophic CO_2 fixation, originated at an early stage of life from a geochemical process considered to be quantitatively important, the exergonic formation of pyrite (FeS_2) from H_2S and FeS ($H_2S + FeS \rightarrow FeS_2 + H_2$; $\Delta G^{\circ\prime} = -41.9$ kJ/mol).

In this review the various modes of metabolism of hyperthermophiles are discussed. They include different types of lithotrophic energy metabolism and pathways of autotrophic CO_2 fixation. The organotrophic metabolism, in particular sugar catabolism, of hyperthermophiles is described in more detail. Figure 1 shows a phylogenetic tree (Woese *et al.* 1990; Stetter 1993) indicating the position of the hyperthermophilic genera discussed in this review. Various aspects of the metabolism of hyperthermophiles and Archaea, including ecology (Kristjánsson & Stetter 1992), distribution of different modes of metabolism within hyperthermophiles (Stetter *et al.* 1990; Stetter 1993), metabolic pathways (Danson 1988, 1993; Fuchs *et al.* 1992), energy transduction (in moderate thermophiles) (Konings *et al.* 1992), enzymes and proteins of hyperthermophiles (Adams 1990, 1993) and evolutionary aspects (e.g. Kandler 1992, 1993; Zillig 1991) have recently been reviewed. The isolation, taxonomy and phylogeny of hyperthermophiles is discussed in the article by Blöchl *et al.* (1995), in this volume.

Lithotrophic Metabolism

The modes of lithotrophic metabolism of hyperthermophiles can be deduced from the compounds present in natural habitats: H_2, CO_2, H_2S, elemental sulphur, various

Table 1. Modes of lithotrophic energy metabolism of hyperthermophiles.

Metabolism/Organism	References*
S-reduction: $H_2 + S \rightarrow H_2S$	
Pyrodictium occultum	Stetter (1982), Stetter *et al.* (1983), Parameswaran *et al.* (1987)
Pyrodictium brockii	Stetter *et al.* (1983), Pihl *et al.* (1992)
Pyrobaculum islandicum	Huber *et al.* (1987)
Thermoproteus neutrophilus	Zillig *et al.* (1981), Schäfer *et al.* (1986)
Thermoproteus tenax	Fischer *et al.* (1983), Hensel *et al.* (1987)
Desulfurolobus ambivalens	Zillig *et al.* (1986), Kletzin (1994)
Acidianus infernus	Segerer *et al.* (1985, 1986)
Acidianus brierley	Segerer *et al.* (1985, 1986)
Stygiolobus azoricus	Segerer *et al.* (1991)
Thermodiscus maritimus	Fischer *et al.* (1983)
SO_4^{2-}-reduction: $4 H_2 + SO_4^{2-} + 2 H^+ \rightarrow H_2S + 4 H_2O$	
Archaeoglobus fulgidus	Stetter *et al.* (1987), Stetter (1988), Dahl *et al.* (1993)
Archaeoglobus lithotrophicus	Stetter *et al.* (1993)
Archaeoglobus profundus	Burggraf *et al.* (1990b)
$S_2O_3^{2-}$-reduction: $4 H_2 + S_2O_3^{2-} + 2 H^+ \rightarrow 2 H_2S + 3 H_2O$	
Archaeoglobus fulgidus	Stetter *et al.* (1987), Stetter (1988)
Pyrodictium occultum	König *et al.* (1988)
Archaeoglobus profundus	Burggraf *et al.* (1990b)
SO_3^{2-}-reduction: $3 H_2 + SO_3^{2-} + 2 H^+ \rightarrow H_2S + 3 H_2O$	
Pyrodictium brockii	Pley *et al.* (1991)
Archaeoglobus profundus	Burggraf *et al.* (1990b)
CO_2 (methanogenesis): $4 H_2 + CO_2 \rightarrow CH_4 + 2 H_2O$	
Methanopyrus kandleri	Huber *et al.* (1989b), Kurr *et al.* (1991), Rospert *et al.* (1991) Klein *et al.* (1993a)
Methanococcus jannaschii	Jones *et al.* (1983), Sprott *et al.* (1993)
Methanococcus igneus	Burggraf *et al.* (1990a)
Methanococcus spec.	Zhao *et al.* (1988)
Methanothermus fervidus	Stetter *et al.* (1981), Fabry *et al.* (1988)
Methanothermus sociabilis	Lauerer *et al.* (1986)

(*continued* p. 29)

oxosulphur compounds (sulphate, sulphite, thiosulphate), but only trace amounts of oxygen (see Stetter 1993). Thus, the biotopes are mainly anaerobic containing microaerophilic niches. In accordance, most hyperthermophilic lithoautotrophs (and also organoheterotrophs) are anaerobes but some are microaerophilic and adapted to low O_2 tensions.

Energy Metabolism

The following modes of lithotrophic energy metabolism have been reported for hyperthermophiles (Table 1): (1) reduction of sulphur with H_2 to H_2S (dissimilatory sulphur reduction, sulphur respiration); (2) reduction of sulphate and other oxosulphur compounds (thiosulphate, sulphite) with H_2 to H_2S (dissimilatory sulphate reduction, sulphate respiration); (3) reduction of CO_2 with H_2 to CH_4 (methanogenesis); (4) reduction of oxygen to H_2O with either H_2 (Knallgas reaction), or sulphur, H_2S, and $S_2O_3^{2-}$ as electron donors (aerobic respiration); and (5) reduction of NO_3^- with H_2, S or $S_2O_3^{2-}$ to N_2 (denitrification).

Lithotrophic energy metabolism is coupled with ATP synthesis via the mechanism of electron transport phosphorylation. In general this mechanism implicates (e.g. with H_2 as electron donor): (1) H_2 oxidation via a membrane associated hydrogenase; (2) electron flow along an electron transport chain to the terminal inorganic electron acceptors (S, SO_4^{2-}, CO_2, O_2, NO_3^-), which is coupled with the generation of an electrochemical ion (mostly proton) potential; and (3) chemiosmotic ATP synthesis via a membrane-bound H^+-(ion)-translocating ATP synthase. The ATP yield depends on the redox potential difference of the electron donor and the electron acceptor. The ATP yields of anaerobic chemolithotrophs growing at the expense of the redox couples H_2/S, H_2/SO_4^{2-}, and H_2/CO_2 are lower than 1 ATP/reaction under physiological conditions due to their low redox potential differences and the low H_2 concentrations present in anaerobic habitats (see Thauer *et al.* 1977; Thauer & Morris 1984; Schink 1992; Fuchs *et al.* 1992).

So far, the enzymes, electron transport components and ATP synthases involved in lithotrophic metabolism in

Table 1—*continued*

Metabolism/Organism	References*
O_2-reduction:	
(1) H_2 as electron donor (Knallgas reaction): $2\,H_2 + O_2 \rightarrow 2\,H_2O$	
Aquifex pyrophilus	Huber *et al.* (1992b), Beh *et al.* (1993)
Sulfolobus spp.	Huber *et al.* (1992a)
Acidianus spp.	Huber *et al.* (1992a)
Metallosphaera sedula	Huber *et al.* (1992a)
Pyrobaculum aerophilum	Völkl *et al.* (1993)
(2) Sulphur as electron donor (sulphur oxidation): $2\,S + 3\,O_2 + 2\,H_2O \rightarrow 2\,H_2SO_4$	
Aquifex pyrophilus	Huber *et al.* (1992b)
Sulfolobus spp.	Brock *et al.* (1972), Emmel *et al.* (1986)
Metallosphaera sedula	Huber *et al.* (1989a)
Desulfurolobus ambivalens	Zillig *et al.* (1986), Kletzin (1989), Anemüller *et al.* (1994)
Acidianus spp.	Segerer *et al.* (1986)
(3) Thiosulphate as electron donor: $S_2O_3^{2-} + 2\,H^+ + 2\,O_2 + 3\,H_2O \rightarrow 2\,H_2SO_4 + 2\,H_2O$	
Aquifex pyrophilus	Huber *et al.* (1992b)
(4) Tetrathionate as electron donor: $S_4O_6^{2-} + 3.5\,O_2 + 3\,H_2O \rightarrow 4\,SO_4^{2-} + 6\,H^+$	
Sulfolobus spp.	Wood *et al.* (1987)
(5) Pyrite as electron donor: $FeS_2 + 3.5\,O_2 + H_2O \rightarrow FeSO_4 + H_2SO_4$	
Sulfolobus-like organisms	Norris & Owen (1993)
Sulfolobus metallicus	Huber & Stetter (1991)
Metallosphera sedula	Huber *et al.* (1989a), Clark *et al.* (1993)
Acidianus brierley	Larsson *et al.* (1990)
NO_3^--reduction:	
(1) H_2 as electron donor: $5\,H_2 + 2\,NO_3^- + 2\,H^+ \rightarrow N_2 + 6\,H_2O$	
Pyrobaculum aerophilum	Völkl *et al.* (1993)
Aquifex pyrophilus	Huber *et al.* (1992b)
(2) Sulphur as electron donor: $5\,S + 6\,NO_3^- + 6\,H^+ + 2\,H_2O \rightarrow 5\,H_2SO_4 + 3\,N_2$	
Aquifex pyrophilus	Huber *et al.* (1992b)
(3) Thiosulphate as electron donor: $5\,S_2O_3^{2-} + 18\,H^+ + 8\,NO_3^- + H_2O \rightarrow 10\,H_2SO_4 + 4\,N_2$	
Aquifex pyrophilus	Huber *et al.* (1992b)
Pyrobaculum aerophilum	Völkl *et al.* (1993)

* The references cited include the first description of the energy metabolism and selected publication(s) describing aspects of metabolism discussed in this review. Both the organisms listed and the references given should be considered as representative.

hyperthermophiles have only been studied in a few organisms. In the following sections recent data on the lithotrophic metabolism of some species of the following genera are discussed: the sulphur-reducing *Pyrodictium* and *Desulphurolobus*, the sulphate-reducing *Archaeoglobus*, the CO_2-reducing methanogen, *Methanopyrus*, and the O_2-reducing *Sulfolobus* and *Desulphurolobus*. Available data indicate that the systems in hyperthermophiles are very similar to those of mesophilic lithotrophs.

S Reduction with H_2 to H_2S (Sulphur Respiration). Species of the hyperthermophilic genera *Pyrodictium, Thermoproteus, Pyrobaculum, Desulphurolobus, Thermodiscus, Acidianus* and *Stygiolobus* have been reported to grow lithoautotrophically on H_2 and elemental sulphur as energy source, and CO_2 as carbon source (Table 1). Thus, the organisms gain energy by the mechanism of sulphur respiration forming ATP by electron transport phosphorylation [for a recent review on bacterial sulphur respiration see Schauder & Kröger (1993)]. The mechanism of sulphur respiration has been studied in detail only in the mesophilic eubacterium *Wolinella succinogenes* (see Schauder & Kröger 1993). This organism grows, for example, on formate and sulphur, the actual substrate being polysulphide. A membrane-bound formate dehydrogenase and membrane-bound polysulphide reductase have been isolated and characterized and electron transport from formate to polysulphide has been reconstituted in a liposomal system. Chemiosmotic ATP synthesis coupled to polysulphide reduction by formate has also been demonstrated. A quinone is apparently not involved in electron transport.

For the hyperthermophile *Pyrodictium brockii*, a model for an electron transport chain has been proposed catalysing sulphur reduction by H_2, involving a membrane-bound

NiFeS-containing uptake hydrogenase (Pihl & Maier 1991) similar to that in the mesophiles (Adams 1990), a membrane-bound quinone and cytochrome *c* and membrane-associated sulphur reductase (Pihl *et al.* 1992). *Desulfurolobus ambivalens* can grow anaerobically by sulphur reduction with H_2 or aerobically by sulphur oxidation with O_2 (Zillig *et al.* 1986). Membranes of *Desulfurolobus ambivalens* grown anaerobically with H_2 and sulphur contain hydrogenase and sulphur reductase (measured as H_2S dehydrogenase) but cytochromes are absent (see Kletzin 1994). Membrane-bound menaquinones, probably involved in electron transport, have been identified in the hyperthermophilic sulphur reducers *Thermoproteus tenax* (Thurl *et al.* 1985) and *Pyrobaculum islandicum* (Tindall 1989) [For a distribution of quinones in Archaea see Gambacorta *et al.* (1994)]. So far, it is not known whether sulphur or polysulphides are the substrates for sulphur reduction (or oxidation) in hyperthermophiles (see Schauder & Müller 1993).

SO_4^{2-} ($S_2O_3^{2-}$; SO_3^{2-}) Reduction with H_2 to H_2S (Sulphate Respiration). The only hyperthermophiles known so far to gain energy by dissimilatory sulphate reduction to H_2S belong to the genus *Archaeoglobus* (Stetter 1992) (Tables 1 and 2). These hyperthermophilic sulphate reducers are phylogenetically closely related to methanogens (Woese *et al.* 1991; Figure 1); accordingly, *Archaeoglobus* spp. contain electron carriers (the deazaflavin factor F_{420}) and coenzymes (tetrahydromethanopterin, methanofuran) typical of methanogens (see below).

All *Archaeoglobus* species have been reported to grow lithotrophically at the expense of sulphate reduction, with H_2 as electron donor (Stetter *et al.* 1993), indicating that ATP has to be formed by electron transport phosphorylation in the course of sulphate reduction to H_2S. In contrast to the obligate lithoautotroph *Ar. lithotrophicus* (Stetter *et al.* 1993), *Ar. profundus* has been described as a lithoheterotroph using acetate or other complex compounds as carbon source (Burggraf *et al.* 1990b). *Archaeoglobus fulgidus* is able to grow both lithotrophically and organotrophically, e.g. with lactate as electron donor (Stetter 1988; Möller–Zinkhan *et al.* 1989).

The pathway and energetics of sulphate reduction to H_2S appear to be the same as described for mesophilic sulphate-reducing bacteria, involving endergonic ATP-dependent sulphate activation and exergonic sulphite reduction to H_2S. The latter process is coupled to energy conservation via a chemiosmotic mechanism [for a recent review on the energetics of dissimilatory sulphate reduction see (Thauer 1989)]. The enzymes involved in sulphate reduction have been measured in organotrophically-grown *Ar. fulgidus* but are assumed to be also operative in lithotrophically-grown cells. *Archaeoglobus fulgidus* contains ATP sulphurylase (sulphate adenylyltransferase), pyrophosphatase, adenylylsulphate (APS) reductase and sulphite reductase (Figure 2). The ATP sulphurylase (Dahl *et al.* 1990), APS reductase (Speich & Trüper 1988) and bisulphite reductase (Dahl *et al.* 1993) have been purified. The genes coding for the subunits of sulphite reductase have been cloned and sequenced. They show significant sequence homology to the corresponding enzymes of mesophilic sulphate-reducing bacteria (Dahl *et al.* 1993). Thus, sulphate reduction in *Archaeoglobus* involves adenosine phosphosulphate and sulphite as intermediates, which both serve as terminal electron acceptors. Hydrogenase, the pathway of electrons from H_2 and ATP synthase still need to be studied in *Archaeoglobus*.

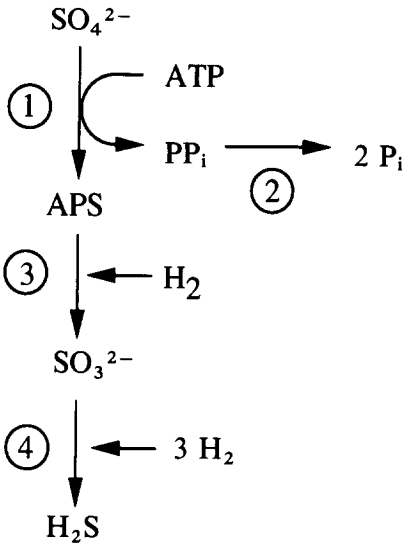

Figure 2. Enzymes involved in sulphate reduction to H_2S in the hyperthermophilic sulphate reducer *Archaeoglobus fulgidus*. ①—ATP sulphurylase; ②—pyrophosphatase (Dahl *et al.* 1990); ③—adenylylsulphate (APS) reductase (Speich & Trüper 1988); ④—sulphite reductase (Dahl *et al.* 1993).

Organotrophically-grown *Ar. fulgidus* contain a membrane-bound lipophilic menaquinone (Tindall *et al.* 1989) probably involved in electron transport. In addition, the presence of cytochromes in *Ar. fulgidus* has been indicated (Kunow *et al.* 1994). Recently, a membrane-bound $F_{420}H_2$:quinone oxidoreductase reductase complex, composed of at least seven subunits, has been purified from *Ar. fulgidus*. The enzyme complex reduces a variety of artificial quinones with reduced F_{420} (Kunow *et al.* 1994), indicating that reduced F_{420} is (the direct electron donor for the membrane-bound quinone found in *Archaeoglobus*. It is proposed that F_{420}:quinone oxidoreductase might be analogous to NADH: quinone oxidoreductase (complex I) of aerobic electron transport chains (Kunow *et al.* 1994).

In summary, the mechanisms of both dissimilatory sulphate reduction and ATP synthesis appear to be similar to those operative in mesophilic bacterial sulphate reducers (see Thauer 1988; Widdel & Hansen 1992).

Reduction of $S_2O_3^{2-}$ with H_2 to H_2S has been reported for *Ar. fulgidus*, *Ar. profundus* and *Pyrodictium occultum*.

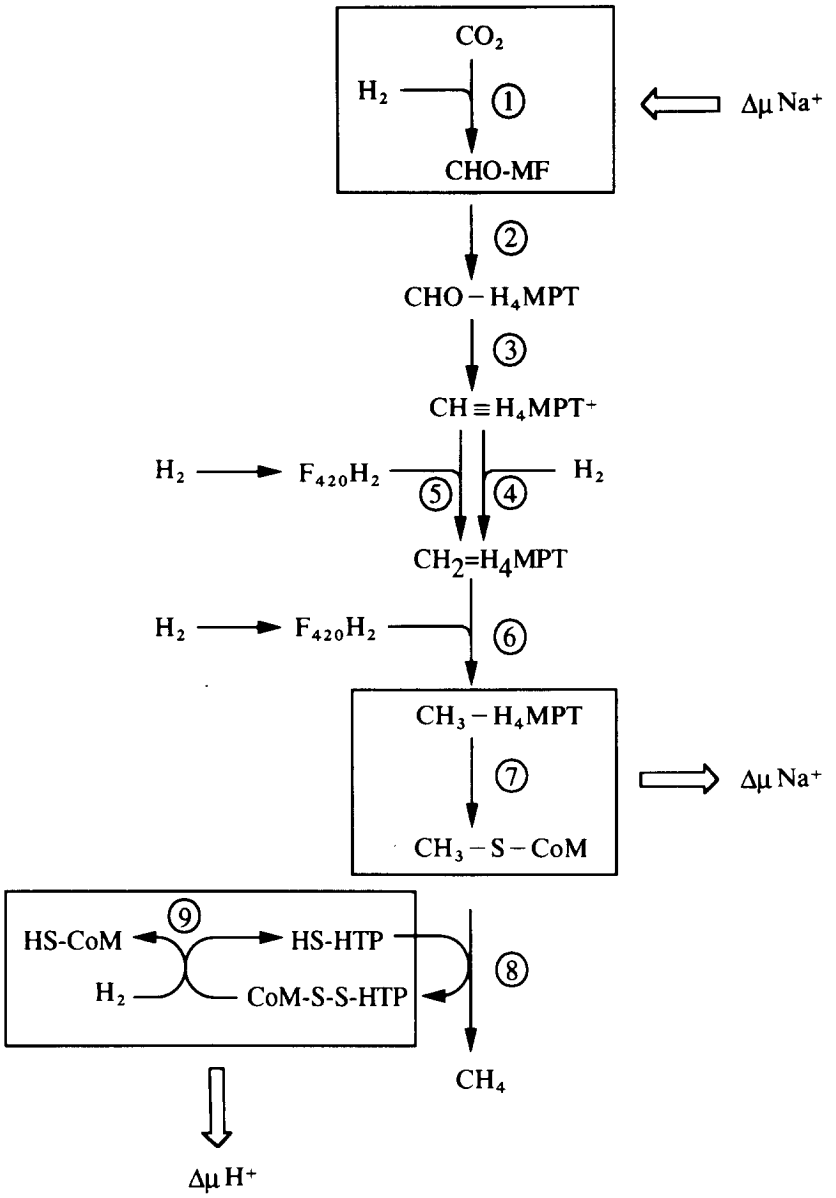

Figure 3. Enzymes involved in CO_2 reduction to CH_4 in the hyperthermophilic methanogen *Methanopyrus kandleri*. MF—Methanofuran; H_4MPT—tetrahydromethanopterin; HS-CoM—coenzyme M; CHO-MF—formyl-MF; $CH{\equiv}H_4MPT^+$—methenyl-H_4MPT; $CH_2{=}H_4MPT$-methylene-H_4MPT; CH_3-H_4MPT—methylH_4MPT; CH_3-S-CoM—methyl – CoM; HS-HTP—mercaptoheptanoylthreonine phosphate; CoM-S-S-HTP—disulphide of HS-CoM and HS-HTP; ①—formylmethanofuran dehydrogenase; ②—formyl-MF:H_4MPT formyltransferase; ③—methenyl-H_4MPT cyclohydrolase; ④—methylene-H_4MPT dehydrogenase (H_2-forming); ⑤—methylene-H_4MPT dehydrogenase (F_{420}dependent); ⑥—methylene-H_4MPT reductase; ⑦—methyl-H_4MPT:HS-CoM methyltransferase; ⑧—Methyl-CoM reductase; ⑨—heterodisulphide reductase; $\Delta\mu Na^+$—electrochemical potential of sodium ions; $\Delta\mu H^+$—electrochemical potential of protons. (Rospert *et al.* 1991; Ma *et al.* 1991a, b; Breitung *et al.* 1991, 1992; Klein *et al.* 1993a.) Boxes indicate sites of energy coupling involved in CO_2 reduction to CH_4 as concluded from studies with mesophilic or thermophilic methanogens (see Müller *et al.* 1993; Schönheit 1993).

Pyrodictium brockii has been said to reduce SO_3^{2-} by H_2 to H_2S; *Pyrodictium* spp. do not reduce sulphate (see Table 1).

CO_2 reduction with H_2 to CH_4 (Methanogenesis). The metabolic ability to gain energy by methane formation is restricted to Archaea. All methanogens belong to the crenarchaeotal branch of the Archaea and include mesophilic, moderate thermophilic and hyperthermophilic species (see Figure 1). The latter belong to the genera *Methanopyrus*, *Methanococcus* and *Methanothermus* (see Table 1). All these methanogens are obligate lithoautotrophs growing on H_2 and CO_2 as sole carbon and energy sources. Methanol- and acetate-utilizing hyperthermophilic methanogens are not yet known. Most work on the enzymology of the CO_2 reduction pathway to CH_4, as well as most bioenergetic studies, including the identification of ion-translocating

steps coupled to methanogenesis and the mechanism of ATP synthesis, have been performed in the moderate thermophile *Methanobacterium thermoautotrophicum* and the mesophile *Methanosarcina barkeri* [For recent reviews on the enzymology and energetics of methanogenesis see DiMarco *et al.* (1991), Blaut *et al.* (1992), Weiss & Thauer (1993), Thauer *et al.* (1993), Müller *et al.* (1993) and Schönheit (1993)]. In short (see Figure 3): (1) CO_2 reduction to CH_4 starts with an endergonic step, i.e. the reduction of CO_2 with H_2 to formyl-methanofuran ("CO_2 activation"); this endergonic reaction is driven by an electrochemical Na^+ potential ($\Delta\mu Na^+$); (2) The exergonic methyl-group transfer from tetrahydromethanopterin to coenzyme M, catalysed by membrane-bound Na^+ ions translocating methyltransferase, generates a primary electrochemical Na^+ potential ($\Delta\mu Na^+$); $\Delta\mu Na^+$ drives the endergonic activation of CO_2 or can be converted into a H^+ potential ($\Delta\mu H^+$) via Na^+/H^+ antiporter; (3) The exergonic reduction of the heterodisulphide (CoM-S-S-HTP) with H_2 generates a primary $\Delta\mu H^+$ via an electron transport chain, which has yet to be characterized; and (4) Methanogens (*Methanosarcina barkeri*) contain a membrane-bound, functionally analogous H^+translocating F-type ATP synthase with structural similarities to V-type ATPases (see Schäfer & Meyering-Vos 1992) catalysing $\Delta\mu H^+$-driven ATP synthesis.

The most hyperthermophilic methanogen, *Methanopyrus kandleri* (maximum temperature for growth = 110°C) (Huber *et al.* 1989b; Kurr *et al.* 1991) which is distantly related to all other methanogens (Figure 1), contains all the enzymes involved in CO_2 reduction to methane that are found in mesophilic and moderately thermophilic methanogens (see Figure 3). Several enzymes of the CO_2 reduction pathway have been purified and characterized from *Methanopyrus kandleri*, including formyl-methanofuran tetrahydromethanopterin formyltransferase (Breitung *et al.* 1992); methenyl-tetrahydromethanopterin cyclohydrolase (Breitung *et al.* 1991), two different methylene-tetrahydromethanopterin dehydrogenases, either H_2-forming (Ma *et al.* 1991a) or F_{420} dependent (Klein *et al.* 1993a), methylene-tetrahydromethanopterin reductase (Ma *et al.* 1991b) and methyl-CoM reductase (Rospert *et al.* 1991). The *N*-terminal amino acid sequences of these enzymes have been determined and found to show significant homology to the corresponding enzymes of mesophilic (*Methanosarcina barkeri*) and moderately thermophilic (*Methanobacterium thermoautotrophicum*) methanogens. In summary, it is likely that both the pathway of CO_2 reduction to CH_4 and also the mechanism of energy conservation in the hyperthermophilic *Methanopyrus* are similar to the process in other methanogens.

The thermostabilizing factors of enzymes in *Methanopyrus kandleri* have been analysed; for most enzymes salt concentrations in the molar range are required for maximal activity and stability (see Breitung *et al.* 1992; Klein *et al.* 1993a). In accordance, hyperthermophilic methanogens such as *Methanopyrus kandleri* and *Methanothermus fervidus* contain intracellular K^+concentrations > 1 M, the anion being 2,3–diphosphoglycerate (Kanodia & Roberts 1983; Seeley & Fahrney 1983); Hensel & König 1988). The potassium salt of this unusual cyclic phosphate (present at 0.3 M in *Methanothermus fervidus* and 1.1 M in *Methanopyrus kandleri*) has been shown to act as thermostabilizer of L-malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase in *Methanothermus fervidus* (Hensel & König 1988). These two enzymes have been purified and characterized (Fabry *et al.* 1988; Honka *et al.* 1990).

O_2 Reduction with H_2 or S to H_2O (Aerobic Respiration). A few hyperthermophiles of the genera *Aquifex, Sulfolobus, Acidianus, Metallosphaera* and *Pyrobaculum* have been reported to gain energy by the Knallgas reaction with H_2 as electron donor (Table 1). In accordance with the low O_2 concentration present in the natural habitats of hyperthermophiles, all O_2-reducing hyperthermophiles are microaerophilic organisms adapted to low O_2 tensions. For example, growth of the (eu)bacterium *Aquifex pyrophilus*, an obligate lithoautotrophic organism growing on H_2, O_2 (< 1% to 5%) and CO_2 as energy and carbon sources, is inhibited by O_2 concentrations higher than 5%.

The respiratory system in hyperthermophiles with O_2 as electron acceptor and the mechanism of energy conservation have been studied in detail in heterotrophically-grown *Sulfolobus acidocaldarius* (see below); it is assumed that the results are also valid for lithotrophically-grown *Sulfolobus*. In addition, the closely related, obligate lithotrophic, facultative aerobe *Desulfurolobus ambivalens* has been analysed (see Schäfer *et al.* 1994a).

Whole-cell studies with *Sulfolobus acidocaldarius* indicate chemiosmotic energy conservation coupled to oxygen reduction (Lübben & Schäfer 1989; Schäfer *et al.* 1990). The steady-state proton motive force of respiring-cell suspensions was about − 150 mV, consisting (at an external pH of 3.5) of a large proton gradient (2 to 3 pH units, inside alkaline) and a small membrane potential (inside negative); $H^+/2e$ ratios (> 3 to 8) indicated the presence of one or more proton pumps. Inhibitor studies are in accordance with H^+-driven ATP synthesis; *Sulfolobus* contains a membrane-bound ATPase functionally analogous to F-type ATP synthases (of mitochondria and bacteria) but structurally more related to vacuolar ATPases of eukaryotes. Such a chimeric ATPase has also been described in methanogenic Archaea (see above) and gave rise to a proposal evolution of ATPases (Schäfer & Meyering-Vos 1992).

Several enzymes and redox components of the respiratory chain have been isolated and characterized in *Su. acidocaldarius* (see Schäfer *et al.* 1990, 1994a; Lübben *et al.* 1994). A flavin-containing NADH dehydrogenase has been purified (Wakao *et al.* 1987) which appears to be loosely bound to the membranes; its role in energy coupling is not known.

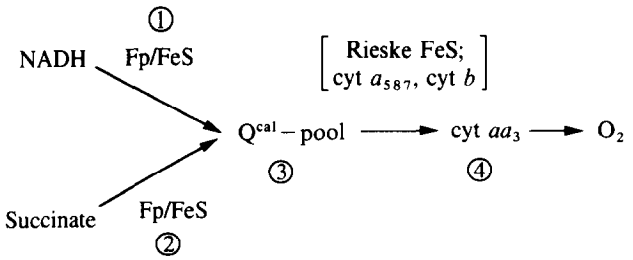

Figure 4. Simplified scheme of the respiratory system in *Sulfolobus acidocaldarius* (Schäfer *et al.* 1990, 1994a modified). Q^{cal}—Caldariella chinone; Fp—flavoprotein; ①—NADH dehydrogenase; ②—succinate dehydrogenase; ③—cyt aa_3 terminal oxidase. The exact roles of the Rieske-Type FeS protein, various cyt *b* and of cyt a_{587} (probably a component of aa_3 oxidase) in electron transport have yet to be defined.

Succinate dehydrogenase has been isolated as an integral membrane protein and shown to be a flavo-iron-sulphur protein similar to the enzyme found in mesophilic bacteria and eukarya (Moll & Schäfer 1991). *Sulfolobus* contains a specific quinone, caldariella quinone (Trincone *et al.* 1986), and a cytochrome aa_3-type terminal oxidase, which functions as a (caldariella) quinone oxidase (Anemüller & Schäfer 1990). This is a novel feature of cytochrome aa_3 oxidase, which normally functions as cytochrome *c* oxidase. The enzyme has been functionally reconstituted in liposomes (see Schäfer *et al.* 1994a). A second terminal oxidase has also been proposed for *Su. acidocaldarius* (Lübben *et al.* 1994). Membranes of *Su. acidocaldarius* also contain *b*-type cytochromes rather than *c*-type cytochromes and an 'Archaeal' Rieske-type, iron-sulphur protein (Anemüller *et al.* 1993), which has been discussed as a possible ancestor of the bc_1 complexes of aerobic bacteria. The role of these redox proteins in electron transport of *Sulfolobus* remains to be defined. It is proposed that the minimal respiratory chain in *Su. acidocaldarius* which is still able to pump protons is composed of membrane-bound, flavin/Fe/S-containing dehydrogenases, caldariella quinone and cytochrome-aa_3-containing terminal oxidase (Figure 4). This simple electron transport chain might represent an archaeal, phylogenetically ancient, respiratory system (Schäfer *et al.* 1994a).

The obligate lithotroph *Desulfurolobus ambivalens* can grow either anaerobically by sulphur reduction with H_2 (see above) or by sulphur oxidation with O_2; the respiratory system of aerobically grown *Desulfa ambivalens* appears to be even more simple than that of *Sulfolobus* in that it contains caldariella quinone (Trincone *et al.* 1989) and a cytochrome-aa_3-type quinone oxidase (Anemüller *et al.* 1994), but is devoid of Rieske-type iron-sulphur proteins and *b*-type cytochromes.

The oxidation of sulphur with O_2 in hyperthermophiles has been described for the genera *Sulfolobus*, *Desulfurolobus*, *Acidianus*, *Metallosphaera* and *Aquifex* (Table 1). The pathway and energy coupling of sulphur oxidation in hyperthermophiles is not understood in detail (see Kletzin 1994). For a review on sulphur oxidation in bacterial *Thiobacillus* spp. see Pronk *et al.* (1990). Aerobically-grown *Desulfurolobus ambivalens* contains a soluble sulphur oxygenase/reductase (SOR) catalysing a combined reaction of sulphur oxidation to sulphite and reduction to sulphide (Kletzin 1989). Details of this complex reaction remain to be studied. SOR is not present in anaerobically-grown cells, indicating that it is induced by O_2. The enzyme has been characterized biochemically and genetically (see Kletzin 1994). A sulphur oxygenase has been characterized in *Acidianus* (*Sulfolobus*) *brierleyi* (Emmel *et al.* 1986) and found to be very similar to the sulphur oxygenase/reductase in *Desulfa ambivalens* in terms of its molecular structure and sulphur-oxidizing activity. Further oxidation of sulphite to sulphate appears to be catalysed by a membrane-bound oxidase system which contains cytochrome aa_3 as the possible terminal oxidase (Kletzin 1994).

Several hyperthermophiles have been shown to use thiosulphate, tetrathionate and sulphides (e.g. as in pyrite) in addition to H_2 and S as electron donors for O_2 reduction; the sulphur compounds are oxidized to H_2SO_4 (Table 1). For a possible biotechnological application of hyperthermophiles in ore leaching see Norris (1992).

NO_3^- Reduction with H_2, S or $S_2O_3^{2-}$ to N_2 (Denitrification). The metabolic ability of hyperthermophiles to utilize nitrate as terminal electron acceptor has been discovered only recently, in the microaerophilic *Aquifex pyrophilus* (Huber *et al.* 1992a) and in *Pyrobaculum aerophilum* (Völkl *et al.* 1993) when grown under strictly anaerobic conditions. This result is of interest since biological nitrate reduction has not been considered to occur in hyperthermophilic habitats because, under pyrite-forming conditions, nitrate was found to be unstable, being reduced abiotically to NH_3 (Blöchl *et al.* 1992). The obligate lithotroph *Aq. pyrophilus* reduces No_3^- to NO_2^- and further to N_2 with either H_2, S or thiosulphate as electron donor (Table 1); N_2 rather than NH_3 was detected as end product, indicating denitrification (Huber *et al.* 1992a). Although the facultative lithotroph *Pyro. aerophilum* utilizes H_2 and thiosulphate during lithotrophic growth, it prefers organotrophic growth with peptides as electron donors for NO_3^- reduction (see below). With organic electron donors the organism also reduces nitrite; N_2 and traces of N_2O and NO were detected as products (Völkl *et al.* 1993). Molecular details on the mechanism of NO_3^- reduction in hyperthermophiles are not yet known. The presence of denitrification in both hyperthermophiles, both of which are phylogenetically ancient organisms, indicates that this type of metabolism developed early in evolution.

Autotrophic CO_2 Fixation

Many lithotrophic hyperthermophiles are autotrophs. The

pathway of CO_2 fixation has been studied in *Thermoproteus neutrophilus, Aquifex pyrophilus, Sulfolobus* spp. and the moderately thermophilic *Methanobacterium thermoautotrophicum*. Two pathways are operative in hyperthermophiles: the reductive citric acid cycle and the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway. The Calvin cycle has not been found so far in autotrophic hyperthermophiles (and other Archaea). For a distribution of the various CO_2-fixation pathways in prokaryotes and a comparison of the energy demand see Fuchs & Stupperich (1985) and Fuchs (1989).

Reductive Citric Acid Cycle. Thermoproteus neutrophilus can grow lithoautotrophically with H_2, elemental sulphur and CO_2 as carbon and energy source (Zillig *et al.* 1981). ^{14}C- and ^{13}C-labelling studies and the determination of enzyme activities in cell extracts indicate that CO_2 fixation proceeds via acetyl-CoA, and involves reverse reactions of the citric acid cycle (Schäfer *et al.* 1986; Fuchs *et al.* 1992; Beh *et al.* 1993; Danson 1993). This requires two enzymes different from those of the conventional citric acid cycle operating in acetyl-CoA oxidation in aerobic bacteria: (1) reductive carboxylation of succinyl-CoA to 2-oxoglutarate ($E^{o\prime}$ = −490 mV) is catalysed by ferredoxin-dependent ($E^{o\prime}$ = −420 mV) 2-oxoglutarate synthase rather than by a pyridine-nucleotide-dependent ($E^{o\prime}$ = −320 mV) irreversible 2-oxoglutarate dehydrogenase complex; and (2) citrate cleavage to oxaloacetate and acetyl-CoA is catalysed by ATP citrate lyase rather than by irreversible citrate synthase. These two enzymes and all other enzymes of the citric acid cycle have been measured in extracts of *Thermop neutrophilus* (Schäfer *et al.* 1986; Beh *et al.* 1993) (Figure 5 A). All enzymes of the reductive citric acid cycle have also been found in the aerobic *Aquifex pyrophilus* (Beh *et al.* 1993) and in the moderately thermophilic Knallgasbacterium *Hydrogenobacter thermophilus* (Shiba *et al.* 1985), which is closely related to *Aquifex* (Kandler 1992). The pathway is possibly also operative in aerobically-growing autotrophic *Sulfolobus* species (Kandler & Stetter 1981) and in *Desulfurolobus ambivalens*. In the latter organism most enzymes of the citric acid cycle, e.g. isocitrate dehydrogenase, 2-oxoglutarate ferredoxin oxidoreductase, succinate dehydrogenase, and malate dehydrogenase (NAD^+, have been detected after autotrophic growth on S and O_2 as energy sources (M. Teixeira & P. Schönheit, unpublished work). The reductive citric acid cycle is also operative in a few anaerobic, autotrophic, mesophilic bacteria, e.g. in the phototroph *Chlorobium limicola* and the sulphate reducer *Desulfobacter hydrogenophilus* (see Fuchs 1989).

Wächtershäuser (1990, 1992) proposed that the reductive citric acid cycle is one of the first autocatalytic carbon-fixation cycles. In accordance with this postulate, the pathway is present in the phylogenetically ancient hyperthermophilic Knallgas bacteria *Aquifex* and *Hydrogenobacter* rather than in the more distantly related mesophilic Knallgas bacteria, e.g. the genus *Alcaligenes* which belongs to the beta group of the purple bacteria (proteobacteria). These facultative lithoautotrophs assimilate CO_2 via the Calvin cycle (Bowien 1989) and this appears to represent a later evolutionary development (see Fuchs 1989; Kandler 1993).

Reductive Acetyl-CoA/Carbon Monoxide Dehydrogenase Pathway. All hyperthermophilic methanogens (*Methanopyrus, Methanococcus, Methanothermus*) are obligate lithoautotrophs growing on H_2 and CO_2 as sole carbon and energy sources. As shown in detail in the moderate thermophile *Methanobacterium thermoautotrophicum* (optimum temperature for growth = 65°C), CO_2 fixation in methanogens proceeds via the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway (see Fuchs & Stupperich 1986). In this linear pathway, acetyl-CoA is a central intermediate which is formed from two CO_2, molecules (Figure 5B): one CO_2 is reduced to a methyl-tetrahydromethanopterin, via reactions also involved in CO_2 reduction to methane (see above), and the second is reduced to an enzyme-bound carbonyl group ([CO]). Both the reduction of CO_2 to the carbonyl group and the subsequent condensation of methyl-tetrahydromethanopterin, CO, and CoA to acetyl-CoA are catalysed by acetyl-CoA synthase/carbon monoxide dehydrogenase (Fuchs & Stupperich 1986). *Methanopyrus* contains all enzymes of CO_2 reduction via methyl-tetrahydromethanopterin to methane (see Figure 3) as well as carbon monoxide dehydrogenase, thus indicating that the acetyl-CoA/carbon monoxide dehydrogenase pathway is operative in this hyperthermophile. It is probably also operative in autotrophic *Archaeoglobus* species. Organotrophically-grown *Ar. fulgidus* contain all enzymes of the acetyl-CoA/carbon monoxide dehydrogenase pathway used for oxidation of acetyl-CoA (Möller-Zinkhan & Thauer 1990) (see below). It is likely that this reversible pathway catalyses the formation of acetyl-CoA during autotrophic growth. The reductive acetyl-CoA/carbon monoxide pathway is also found in most autotrophic, sulphate-reducing (Schauder *et al.* 1987) and homoacetogenic bacteria (see Fuchs 1986). In contrast to the pathway in hyperthermophilic Archaea, Bacteria reduce CO_2 via free formate as an intermediate and use tetrahydrofolate (instead of tetrahydromethanopterin and methanofuran) as C_1 carrier.

The Calvin cycle has not been found in autotrophic hyperthermophiles and other Archaea. Thus it appears this CO_2 fixation pathway is a relatively late development. Interestingly, ribulose 1,5-bisphosphate carboxylase and phosphoribulokinase, key enzymes of the Calvin cycle, have been detected at low activities in several heterotrophic halophilic Archaea that are unable to grow autotrophically (Altekar & Rajagopalan 1990). The role of Calvin cycle enzymes in these organisms remains to be established.

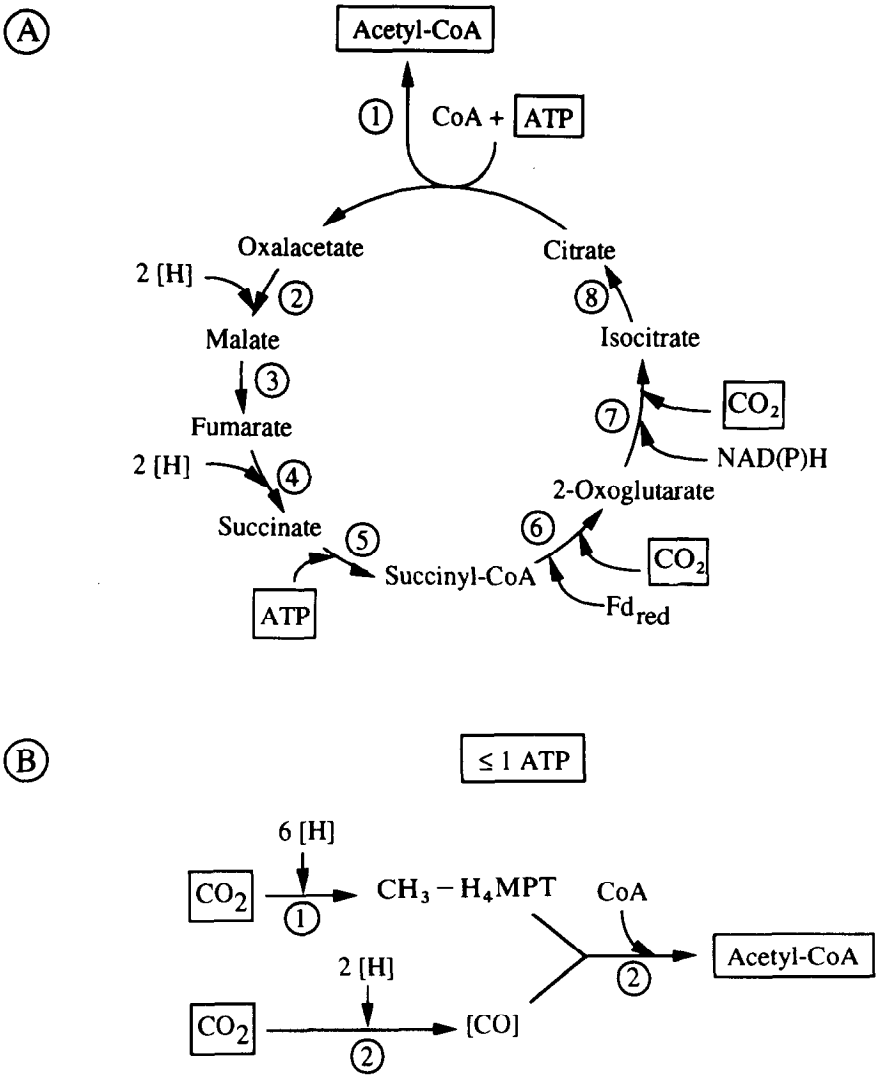

Figure 5. Pathways of autotrophic CO_2 fixation in hyperthermophiles. (A) Acetyl-CoA formation from 2 CO_2 via the reductive citric acid cycle (*Thermoproteus tenax*, *Aquifex pyrophilus*): CoA—acetyl-CoA; Fd_{red}—reduced ferredoxin; ①—ATP citrate lyase; ②—malate-dehydrogenase; ③—fumarase; ④—fumarate reductase; ⑤—succinyl-CoA synthetase; ⑥—2-oxoglutarate:ferredoxin oxidoreductase; ⑦—isocitrate dehydrogenase; ⑧—aconitase) (Schäfer *et al.* 1986; Beh *et al.* 1993). (B) Acetyl-CoA formation from 2 CO_2 via the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway (*Methanobacterium thermoautotrophicum*, *Methanopyrus kandleri*, autotrophic *Archaeoglobus* spp.) CH_3-H_4MPT—methyl-tetrahydromethanopterin; [CO]—enzyme bound carbon monoxide; CoA—coenzyme A; ①—enzymes involved in CO_2 reduction to CH_3-H_4MPT (shown in Figure 2); ②—carbon monoxide dehydrogenase. For the calculation of ATP requirement ($\leq$1 ATP) of acetyl-CoA formation see Fuchs (1986) and Diekert (1990).

Gluconeogenesis from Acetyl-CoA or Pyruvate. Gluconeogenesis (glucose-6-phosphate formation) from acetyl-CoA has been studied in *Methanobacterium thermoautotrophicum* (Fuchs & Stupperich 1986), *Methanococcus jannaschii* (Sprott *et al.* 1993), and *Thermoproteus neutrophilus* (Schäfer *et al.* 1986; Strauss *et al.* 1992), *Aquifex pyrophilus* (Beh *et al.* 1993), and *Hydrogenobacter thermophilus* (Shiba *et al.* 1985), and from pyruvate in the obligate organoheterotroph *Pyrococcus furiosus* (Schäfer & Schönheit 1993). As deduced from enzyme activities in cell extracts and from ^{14}C- or ^{13}C-labelling studies, gluconeogenesis in all these hyperthermophiles has been shown to proceed via the reversal of the Embden–Meyerhof pathway. Reductive carboxylation of acetyl-CoA to pyruvate is catalysed by pyruvate:ferredoxin oxidoreductase (pyruvate synthase). For *Methanobacterium: thermoautotrophicum*, reduced factor F_{420} rather than ferredoxin has been proposed as electron donor for reductive carboxylation of acetyl-CoA (Zeikus *et al.* 1977). However, the mesophilic methanogen *Methanosarcina barkeri* contains pyruvate:ferredoxin oxidoreductase rather than 'pyruvate F_{420} oxidoreductase' (Bock *et al.* 1994) when grown on H_2/CO_2; the cofactor specificity of pyruvate oxidoreductases in other methanogens has to be tested. Sugar phosphate (glucose-6-phosphate) formation from pyruvate involves

Table 2. Modes of respiration with organic [H]-donors in hyperthermophilic Archaea.

Organism	[H]-donor*	[H]-acceptor	References†
Thermoproteales			
Thermoproteus tenax	Glycogen, starch, amylose, amylopectin, glucose, ethanol, methanol, formamide, formate, malate, fumarate, peptides	S	Zillig *et al.* (1981), Siebers & Hensel (1993) Selig & Schönheit (1994)
Thermofilum pendens	Peptides	S	Zillig *et al.* (1983a), Stetter *et al.* (1986)
Thermodiscus maritimus	Peptides	S	Stetter *et al.* (1990)
Pyrobaculum islandicum	Peptides	S, $S_2O_3^{2-}$, SO_3^{2-} Cystine, glutathione	Huber *et al.* (1987), Tindall (1989) Selig & Schönheit (1994)
Pyrobaculum organotrophum	Peptides	S, cystine, glutathione	Huber *et al.* (1987), Tindall *et al.* (1991)
Pyrobaculum aerophilum	Peptides, acetate, propionate	O_2, NO_3^-, NO_2^-	Völkl *et al.* (1993)
Archaeoglobales			
Archaeoglobus fulgidus	Glucose, starch, lactate, formate, formamide, peptides	SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$	Stetter *et al.* (1987), Stetter (1988) Möller-Zinkhan & Thauer (1990), Dahl *et al.* (1993) Schwörer *et al.* (1993)
Sulfolobales			
Sulfolobus acidocaldarius	Glucose, galactose, sucrose, lactose, ribose, glutamate, glutamine, alanine, aspartic acid, peptides	O_2	Brock *et al.* (1972), Schäfer *et al.* (1994a)
Sulfolobus solfataricus	Glucose, xylose, sucrose, lactose, maltose, rhamnose, peptides	O_2	De Rosa *et al.* (1975, 1984)
Acidianus brierleyi	Peptides	O_2	Segerer *et al.* (1986)
Metallosphaera sedula	Peptides	O_2	Huber *et al.* (1989a)

* 'Peptides' indicates complex compounds, e.g. yeast extract, peptone, tryptone, casamino acids, trypticase and caseine.
† The references cited include the first description of the energy metabolism and selected publication(s) describing aspects of metabolism discussed in this review. Both the organisms listed and the references given should be considered as representative.

phosphoenolpyruvate synthetase in all hyperthermophiles tested so far, the reversible enzymes of the Embden–Meyerhof pathway catalysing fructose-1,6-bisphosphate formation from phosphoenolpyruvate, fructose-1,6-bisphosphatase and hexose-phosphate isomerase (Figure 6). The complete gluconeogenetic pathway from pyruvate in other hyperthermophiles (*Sulfolobus, Thermoplasma*) and in halophilic Archaea remains to be elucidated (see Danson 1993).

The operation of the reversed Embden–Meyerhof pathway in gluconeogenesis in hyperthermophiles and all other organisms studied so far, including those utilizing a different pathway for sugar catabolism (e.g. the Entner–Doudoroff pathway), can be explained by the fact that the Embden–Meyerhof pathway has the highest degree of reversibility of all glycolytic pathways (for a discussion see Schäfer & Schönheit 1993).

Organotrophic Metabolism

Many hyperthermophiles are able to grow organotrophically, mostly on complex media containing peptides (proteins, casamino acids, yeast extract, peptone, amino acid mixtures) and sugars (see below). In addition, pyruvate and lactate are good substrates for some hyperthermophiles. Other organic substrates reported for hyperthermophiles are given in Tables 2 and 3.

In principle, two different modes of organotrophic catabolism have been reported for hyperthermophiles (Figure 7):

(1) Growth of the organisms is dependent on the presence of external electron acceptors, e.g. sulphur, sulphate, thiosulphate, oxygen or nitrate. Under these conditions, organic compounds are oxidized to CO_2 and energy is conserved via anaerobic or aerobic respiration. These types of respiratory metabolism have been reported for organisms which belong to the *Thermoproteales, Archaeoglobales* and *Sulfolobales* (Table 2).

(2) Sugars or peptides serve as fermentable substrates. Various fermentation products, such as acetate and other volatile fatty acids, lactate or butanol, were formed in addition to CO_2 and H_2. This fermentative metabolism is found in species of the orders *Thermococcales, Desulphurococcales, Pyrodictiales, Thermotogales* and *Thermoproteales* (Table 3). Almost all of these

Table 3. Modes of fermentation in hyperthermophilic Archaea and Bacteria.

Organism	Substrates*	Products	References†
Thermococcales			
Pyrococcus furiosus	Maltose, cellobiose, pyruvate, peptides	Acetate, alanine, CO_2, H_2	Fiala & Stetter (1986), Schäfer & Schönheit (1991, 1992), Kengen & Stams (1994a)
Pyrococcus woesei	Starch, pyruvate, peptides	Acetate, CO_2, H_2	Zillig *et al.* (1987), Zwickl *et al.* (1990) Schäfer *et al.* (1993)
Pyrococcus abyssi	Pyruvate, peptides	Acetate, CO_2 isovalerate, isobutyrate, propionate	Erauso *et al.* (1993)
Thermococcus stetteri	Starch, peptides	Acetate, CO_2, H_2	Miroshnichenko *et al.* (1989), Pusheva *et al.* (1992)
Thermococcus celer	Pyruvate, peptides	Acetate, CO_2, H_2	Zillig *et al.* (1983b), Schäfer *et al.* (1993)
Thermococcus litoralis	Peptides, pyruvate	N.D.	Belkin & Jannasch (1985), Neuner *et al.* (1990)
Desulfurococcales			
Desulfurococcus amylolyticus	Starch, pectine, glycogen, alanine, phenylalanine, serine, tyrosine, ornithine, peptides	Acetate, CO_2, H_2	Bonch-Osmolovskaya *et al.* (1988) Schäfer *et al.* (1993)
Desulfurococcus mucosus	Peptides	CO_2	Zillig *et al.* (1982)
Desulfurococcus mobilis	Peptides	CO_2	Zillig *et al.* (1982)
Desulfurococcus strain S/SE	Peptides	CO_2	Jannasch *et al.* (1988b)
Staphylothermus marinus	Peptides + S	Acetate, isovalerate, CO_2, H_2S	Fiala *et al.* (1986), Stetter *et al.* (1986)
Pyrodictiales			
Pyrodictium abyssi	Starch, glycogen, lactose, raffinose, peptides	CO_2, isovalerate, butanol, isobutyrate, acetate	Pley *et al.* (1991)
Hyperthermus butylicus	Peptides	Acetate, propionate, butanol, phenylacetate, CO_2	Zillig *et al.* (1990, 1991), Schäfer *et al.* (1993)
Thermotogales			
Thermotoga maritima	Ribose, xylose, sucrose, glucose, maltose, lactose, galactose, starch, glycogen, pyruvate, peptides	Acetate, lactate, CO_2, H_2	Huber *et al.* (1986), Wrba *et al.* (1990), Schäfer *et al.* (1993), Schröder *et al.* (1994)
Thermotoga neapolitana strain NS-E	see *T. maritima*	Acetate, lactate, CO_2, H_2	Jannasch *et al.* (1988a), Childers *et al.* (1992)
Thermotoga strain FjSS3.B1	Xylose, glucose, fructose, maltose, starch, amylopectin, carboxymethyl-cellulose	Acetate, lactate, CO_2, H_2	Huser *et al.* (1986), Janssen & Morgan (1992)
Thermotoga thermarum	Glucose, maltose, starch, peptides	N.D.	Windberger *et al.* (1989)
Fervidobacterium islandicum	Cellulose, ribose, glucose, maltose, raffinose, starch, pyruvate, peptides	Lactate, acetate, ethanol, CO_2, H_2	Huber *et al.* (1990)
Thermosipho africanus	Peptides	N.D.	Huber *et al.* (1989c)
Thermoproteales			
Thermoproteus uzoniensis	Peptides	Acetate, isobutyrate, isovalerate	Bonch-Osmolovskaya *et al.* (1990)
Other			
Caldococcus litoralis	Peptides	N.D.	Svetlichnyi *et al.* (1987)

* 'Peptides' indicates complex compounds, e.g. yeast extract, peptone, tryptone, casamino acids, trypticase and caseine.
† The references cited include the first description of the energy metabolism and selected publication(s) describing aspects of metabolism discussed in this review. Both the organisms listed and the references given should be considered as representative.
N.D., not determined.

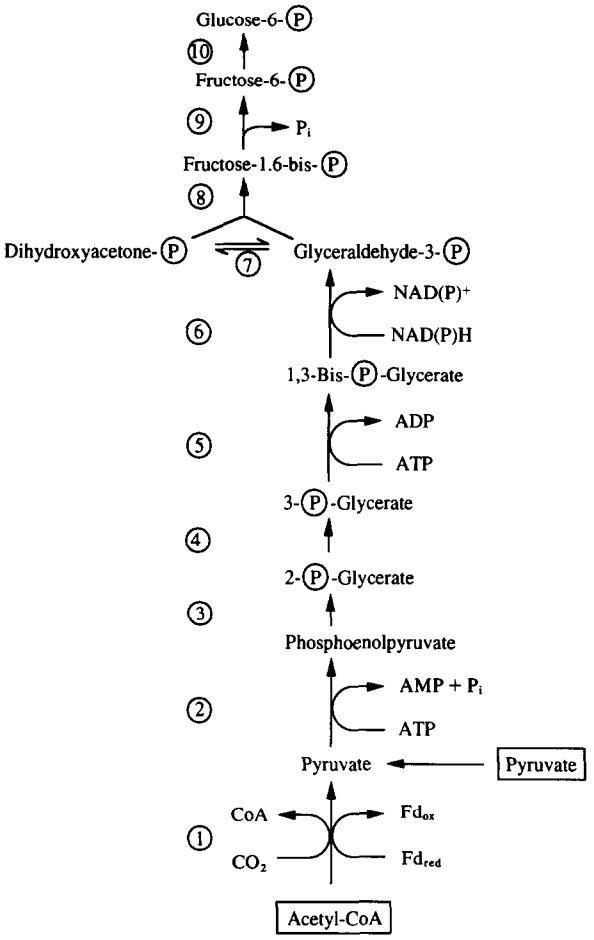

Figure 6. Gluconeogenesis (glucose-6-phosphate formation) in hyperthermophiles from acetyl-CoA or pyruvate as deduced from labelling studies and/or enzyme activities in cell extracts. Included are the moderate thermophiles *Methanobacterium thermoautotrophicum* (see Fuchs & Stupperich 1986), *Methanococcus jannaschii* (Sprott *et al.* 1993), *Aquifex pyrophilus* (Beh *et al.* 1993) and gluconeogenesis from pyruvate (*Pyrococcus furiosus*; Schäfer & Schönheit 1993). For pathways of acetyl-CoA formation in autotrophic hyperthermophiles see Figure 4. Fd—Ferredoxin; ①—pyruvate:ferredoxin oxidoreductase; ②—phosphoenolpyruvate synthetase; ③—enolase; ④—phosphoglycerate mutase; ⑤—phosphoglycerate kinase; ⑥—glyceraldehyde-3-phosphate dehydrogenase; ⑦—triose phosphate isomerase; ⑧—phosphofructose-1,6-bisphosphate aldolase; ⑨—fructose-1,6-bisphosphatase; ⑩—hexose phosphate isomerase.

organisms are facultative sulphur reducers. There is no evidence that sulphur reduction to H_2S is coupled with ATP synthesis via sulphur respiration (see below for *Pyrococcus* and *Thermotoga*). Complete fermentation balances and, in the case of peptide-containing complex substrates, quantitative product formation have only been described for a few organisms (see below). For most organisms, substrates and products have not been quantified so that the mode of energy metabolism cannot be defined.

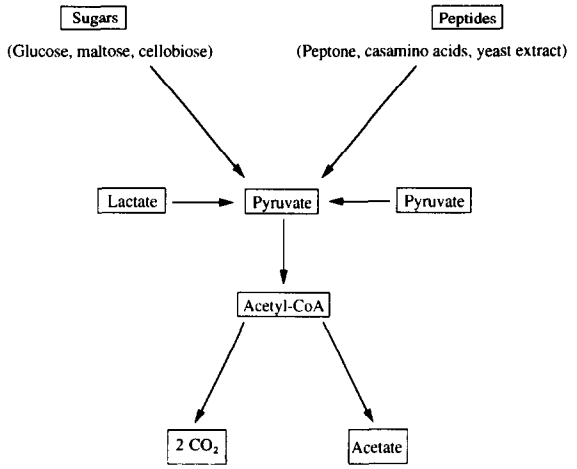

Figure 7. Pathways and reactions of organotrophic catabolism in the hyperthermophiles discussed in this review.

The following part of the review summarizes the metabolic pathways of hyperthermophiles that involve sugar, peptide, lactate or pyruvate oxidation to CO_2 or fermentation of these organic substrates to acetate and other products. In particular, the following topics will be discussed (Figure 7): (1) pathways of sugar degradation to pyruvate; (2) mechanism(s) of pyruvate conversion to acetyl-CoA; (3) mechanisms of acetyl-CoA oxidation to two CO_2 molecules with either sulphur, sulphate or oxygen as terminal electron acceptor; and (4) mechanisms of acetyl-CoA conversion to acetate. In addition, some aspects of peptide catabolism are described.

Sugar Catabolism

Various sugars have been reported to be substrates for hyperthermophiles. They include polymeric sugars (starch, amylose, glycogen, dextrin, xylan), disaccharides (maltose, cellobiose, sucrose), hexoses (glucose, galactose, fructose) and pentoses (ribose, xylose). Polymeric sugars are attacked by extracellular hydrolases, e.g. amylases, pullulanases and xylanases. Several of these enzymes have been purified and characterized, e.g. α-amylase and pullulanase from *Pyroc. furiosus* and *Pyroc. woesei*. The pullulanase gene of *Pyroc. woesei* has been cloned and expressed in *Escherichia coli*. For literature on the sugar-degrading exoenzymes of hyperthermophiles see Leuschner & Antranikian (1995).

The pathways of sugar catabolism in hyperthermophiles have been studied in detail in five different genera that are distantly related phylogenetically: the aerobic Archaea *Sulfolobus* and *Thermoplasma* (moderate thermophile), the anaerobic Archaea *Pyrococcus* and *Thermoproteus* and the anaerobic (eu)bacterium *Thermotoga*. In addition, the sulphate-reducing *Archaeoglobus* has been reported to grow on sugars; so far the pathway of lactate oxidation has been studied in this organism.

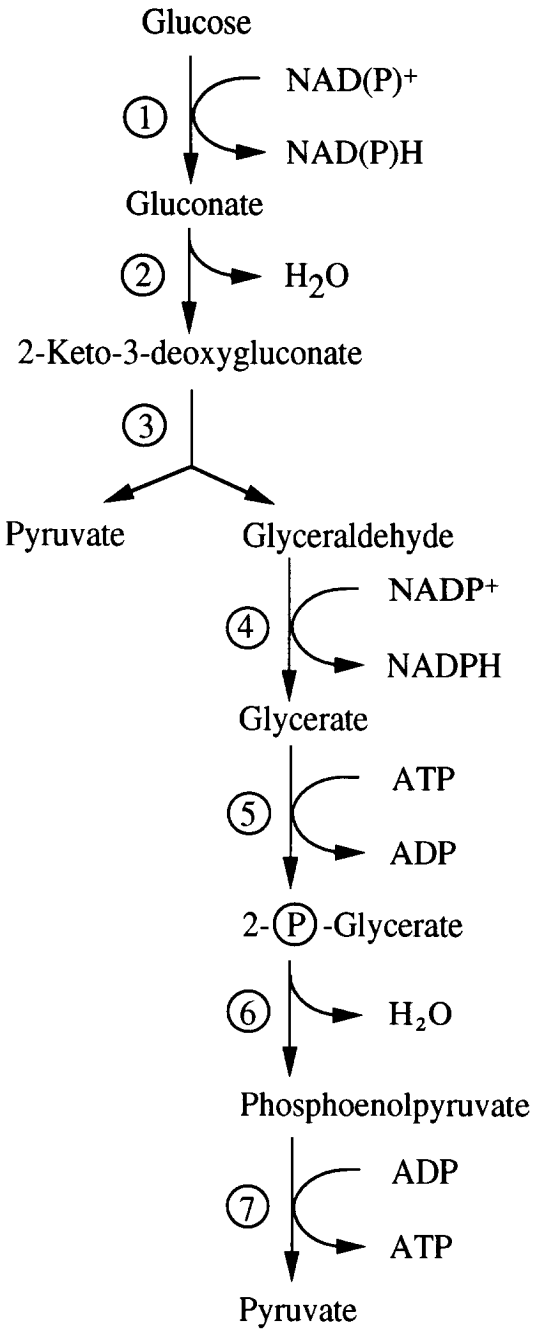

Figure 8. Proposed pathway of glucose conversion to pyruvate, in the aerobic hyperthermophilic Archaea *Sulfolobus* (S. *solfataricus*; *S. acidocaldarius*) and the moderate thermophile *Thermoplasma acidophilum*, via the non-phosphorylated Entner–Doudoroff pathway. ①—Glucose dehydrogenase [NAD(P)$^+$]; ②—gluconate dehydratase; ③—2-keto-3-deoxy-gluconate aldolase; ④—glyceraldehyde dehydrogenase (NAD$^+$); ⑤—glycerate kinase; ⑥—enolase; ⑦—pyruvate kinase. After De Rosa *et al.* (1984), Bartels (1989) and Budgen & Danson (1986). For a review of this topic see Danson (1988).

Sugar Conversion to Pyruvate. Sulfolobus species are microaerophilic, facultative organotrophs growing on glucose and oxygen (Table 2; see Segerer & Stetter 1992). The pathway of glucose conversion to pyruvate has been studied in detail in *Su. solfataricus* and *Su. acidocaldarius*. Based on enzyme activities found in cell extracts, on the detection of intermediates after pulse labelling of cell extracts with ^{14}C-glucose, and on radio-respirometry experiments. (De Rosa *et al.* 1984; Giardina *et al.* 1986; Wood *et al.* 1987; Danson 1988; Bartels 1989), it was concluded that glucose is degraded to pyruvate via a modified Entner–Doudoroff pathway, involving glucose oxidation to glycerate via non-phosphorylated intermediates. This non-phosphorylated Entner–Doudoroff pathway and the enzymes involved are shown in Figure 8. Glucose is oxidized to gluconate via glucose dehydrogenase [NAD(P)$^+$-dependent], then the action of gluconate dehydratase gives 2-keto-3-deoxy-gluconate (KDG). KDG is cleaved to pyruvate and glyceraldehyde via KDG aldolase and the glyceraldehyde is further oxidized to glycerate via specific glyceraldehyde dehydrogenase (NADP$^+$). A specific kinase phosphorylates glycerate to 2-phosphoglycerate which is then converted to pyruvate via enolase and pyruvate kinase. According to this pathway, glucose conversion to pyruvate is not coupled with net ATP synthesis, since the free energy change associated with the oxidation of the two aldehydes, glucose and glyceraldehyde, to the corresponding acids, is apparently not conserved in the form of ATP or another energized state. ATP can be generated, however, during oxidation of reduced pyridine nucleotides by O_2 in the respiratory chain (see above).

The non-phosphorylated Entner–Doudoroff pathway has also been proposed for the aerobic moderate thermoacidophile *Thermoplasma acidophilum* (Budgen & Danson 1986; Danson 1988, 1989). It is interesting to note that, in aerobic and extremely halophilic Archaea, glucose degradation involves a partially phosphorylated Entner–Doudoroff pathway in which glucose is converted to KDG followed by phosphorylation — via specific kinase — to 2-keto-3-deoxy-6-phospho-gluconate (KDPG), which is further converted along reactions of the classical Entner-Doudoroff pathway (Tomlinson *et al.* 1974; Danson 1989, 1993).

Pyrococcus species are strictly anaerobic and obligately organotrophic hyperthermophiles growing on various sugars, including starch, maltose and cellobiose and on pyruvate as energy and carbon source (Table 3). Growth of *Pyroc. furiosus* (Fiala & Stetter 1986) on maltose and cellobiose and on pyruvate has been studied in detail with respect to fermentation balances, molar growth yields and enzyme activities involved in both catabolism and gluconeogenesis (Schäfer & Schönheit 1991, 1992, 1993; Kengen *et al.* 1993; Kengen & Stams 1994a, b). Glucose does not serve as a growth substrate but is converted by cell suspensions of *Pyroc. furiosus* at low rates. Recently, labelling studies on cell suspensions with specifically labelled ^{13}C-glucose have been reported (Kengen *et al.* 1994; Schäfer *et al.* 1994b).

Growing cultures of *Pyroc. furiosus* ferment maltose, cellobiose or pyruvate to acetate, alanine, CO_2 and H_2. H_2

has been shown to inhibit growth of *Pyroc. furiosus* (Fiala & Stetter 1986; Schäfer & Schönheit 1991) but inhibition could be prevented by keeping the hydrogen partial pressure (pH_2) low. This is accomplished: (1) by the addition of sulphur, which *Pyroc. furiosus* reduces by H_2 to H_2S; (2) by growing the organism in an open fermenter system gassed with N_2; or (3) by growing the organism in co-culture with a H_2-consuming hyperthermophilic methanogen (see Fiala & Stetter 1986; Malik *et al.* 1989; Bonch-Osmolovskaya & Stetter 1991; Schäfer & Schönheit 1991, 1992; Raven *et al.* 1992; Rüdiger *et al.* 1992; Kengen & Stams 1994a, b).

The H_2 pressure determined the ratio of alanine/acetate during sugar fermentation, which ranged from 0.07 at low pH_2 to 0.8 at high pH_2 (Kengen & Stams 1994b). Thus, mainly acetate and low amounts of alanine were formed at low pH_2, e.g. in an open fermentor gassed with N_2, *Pyroc. furiosus* ferments maltose almost completely (about 90% C and [H] recovery) to acetate, CO_2 and H_2 (Schäfer & Schönheit 1992). Conversely, at high pH_2 alanine formation was the major electron sink reaction and less acetate was formed (Kengen & Stams 1994b). Molar growth yields on maltose and cellobiose of 40 to 60 g cell dry wt/mol have been determined, indicating ATP yields between 4 and 6 mol ATP/mol disaccharide assuming Y_{ATP} (= g cells/mol ATP) to be about 10 g cell dry mass/mol (Decker *et al.* 1970; Stouthamer 1979). Sulphur reduction to H_2S by *Pyroc. furiosus* is apparently not coupled with energy conservation via sulphur respiration. The higher molar growth yields during growth on maltose or cellobiose observed in the presence of sulphur (see Schicho *et al.* 1993; Kengen & Stams 1994b) might be explained by a shift of fermentation products from alanine to acetate and therefore higher ATP yields coupled to increased acetate formation. It has recently been shown that the soluble hydrogenase (Bryant & Adams 1989) of *Pyroc. furiosus* has sulphur reductase activity (Ma *et al.* 1993), arguing against sulphur reduction being coupled to energy conservation.

The pathway of maltose or cellobiose degradation to pyruvate in *Pyroc. furiosus* is still a matter of debate. After transport of the disaccharides into the cells by a mechanism not yet known, maltose and cellobiose are most likely split into two glucose molecules by cytoplasmatic α-glucosidase (Costantino *et al.* 1990) and β-glucosidase (Kengen *et al.* 1993), respectively. Both extremely heat stable enzymes have been purified and characterized. Thus, free glucose appears to be the substrate for further degradation. The glycolytic pathway involved in glucose conversion to pyruvate was studied by measuring enzyme activities in cell extracts and by labelling experiments with ^{13}C-glucose. Three different pathways have been proposed. One proposed route is a modified non-phosphorylated Entner–Doudoroff pathway. All enzymes, except gluconate dehydratase, of this pathway have been detected in cell extracts (Schäfer & Schönheit 1992). *Pyrococcus furiosus* contained glucose:ferredoxin oxidoreductase and glyceraldehyde:ferredoxin oxidoreductase rather than the pyridine-nucleotide-dependent dehydrogenases present in the aerobic hyperthermophiles, *Sulfolobus* and *Thermoplasma*. The ferredoxin-dependent dehydrogenases together with ferredoxin-dependent hydrogenase enables *Pyroc. furiosus* to release reducing equivalents as molecular H_2, an advantage for a fermenting organism. Glyceraldehyde:ferredoxin oxidoreductase, catalysing the oxidation of various aldehydes *in vitro*, is a tungsten-iron-sulphur protein (Mukund & Adams 1991) in which tungsten is bound to a pterin moiety (Johnson *et al.* 1993). Furthermore, KDG aldolase and glycerate kinase have also been detected (Schäfer & Schönheit 1992).

When grown on pyruvate, *Pyroc. furiosus* contains all reversible enzymes of the Embden–Meyerhof pathway (see Figure 6), catalysing gluconeogenesis from pyruvate (Schäfer & Schönheit 1993). These enzymes were also present in maltose-grown cells (Figure 9) but kinetic and regulatory properties of several enzymes suggest a gluconeogenetic rather than a catabolic role for the Embden–Meyerhof pathway. For instance, glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-reducing) was 10-fold more active in pyruvate-grown cells than in maltose-grown cells (Schäfer & Schönheit 1993). This enzyme has been purified from *Pyroc. woesei* and the gene has been cloned, sequenced and expressed in *E. coli* (Zwickl *et al.* 1990). For a discussion of thermostability of glyceraldehyde-3-phosphate dehydrogenases in hyperthermophiles see Hensel & Jakob (1994).

Recent ^{13}C-labelling experiments, however, seem to favour an Embden–Meyerhof pathway also as a route for glucose degradation. Cell suspensions converted [1 − ^{13}C]glucose and [3 − ^{13}C]glucose to acetate, alanine, CO_2 and H_2; alanine and acetate were distinctly labelled (Schäfer *et al.* 1994b). With [1 − ^{13}C]glucose, the methyl groups of both alanine and acetate was labelled; with [3 − ^{13}C]glucose only the carboxyl group of alanine was labelled whereas acetate was unlabelled. These labelling patterns were not consistent with a non-phosphorylated Entner–Doudoroff pathway but support an Embden–Meyerhof glycolytic pathway. Similar ^{13}C-labelling experiments have been described by Kengen *et al.* (1994).

Two modifications of the Embden–Meyerhof pathway have been proposed on the basis of enzyme studies and both would fit the ^{13}C-labelling data. Cell extracts *Pyroc. furiosus* contain glucose isomerase, ketohexokinase (ATP: fructose-1-phosphotransferase) and fructose-1-phosphate aldolase. Activities of these enzymes can explain glucose conversion to dihydroxyacetone phosphate and glyceraldehyde, the products of fructose-1-phosphate cleavage. Further conversion of both trioses to pyruvate involves enzymes of both the Embden–Meyerhof pathway and the non-phosphorylated Entner–Doudoroff pathway which have previously been reported (Schäfer & Schönheit 1992, 1993). Hexokinase or 6-phosphofructokinase, either ATP-

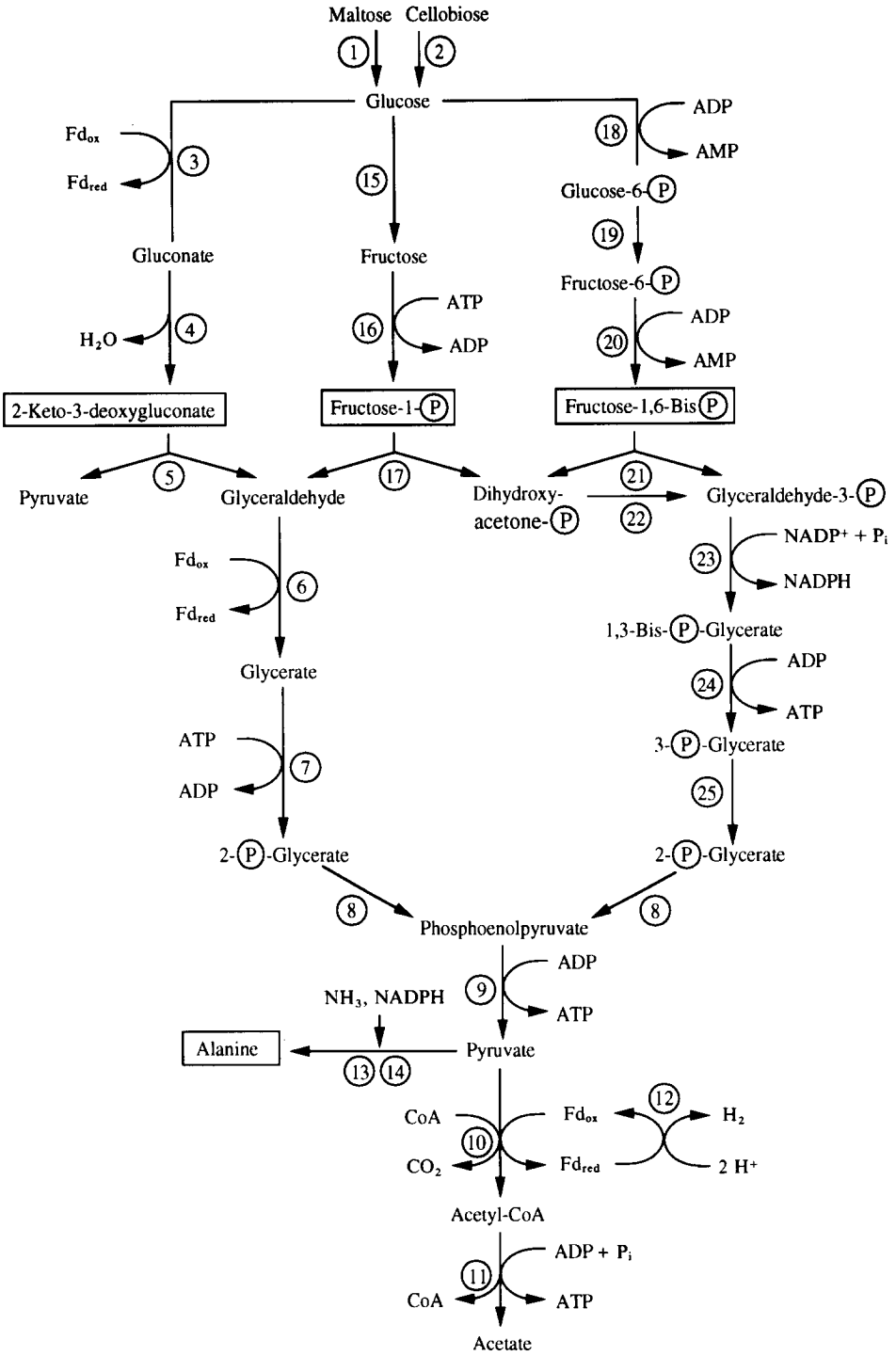

Figure 9. Possible pathways involved in maltose or cellobiose fermentation to acetate, alanine, H_2 and CO_2 in *Pyrococcus furiosus*. Fd—Ferredoxin; ①—α-glucosidase (Costantino *et al.* 1990); ②—β-glucosidase (Kengen *et al.* 1993); ③—glucose ferredoxin oxidoreductase (Mukund & Adams 1991; Schäfer & Schönheit 1992); ④—gluconate dehydratase (not yet detected); ⑤—2-keto-3-deoxygluconate aldolase (Schäfer & Schönheit 1992); ⑥—glyceraldehyde:ferredoxin oxidoreductase (Mukund & Adams 1991; Schäfer & Schönheit 1992); ⑦—glycerate kinase; ⑧—enolase; ⑨—pyruvate kinase; ⑩—pyruvate ferredoxin oxidoreductase; ⑪—acetyl-CoA synthetase (ADP-forming) (Schäfer & Schönheit 1991, 1992); ⑫—hydrogenase (Bryant & Adams 1989); ⑬—glutamate dehydrogenase; ⑭—alanine aminotransferase (Kengen & Stams 1994a); ⑮—glucose isomerase; ⑯—ketohexokinase; ⑰—fructose-1-phosphate aldolase (Schäfer *et al.* 1994b); ⑱—ADP-dependent hexokinase (Kengen *et al.* 1994); ⑲—glucose-6-phosphate isomerase (Schäfer & Schönheit 1992); ⑳—ADP-dependent fructose-6-phosphate kinase (Kengen *et al.* 1994); ㉑—fructose-1,6-bisphosphate aldolase; ㉒—triosephosphate isomerase; ㉓—glyceraldehyde 3-phosphate dehydrogenase; ㉔—phosphoglycerate kinase; ㉕—phosphoglycerate mutase (Schäfer & Schönheit 1992, 1993).

or pyrophosphate-dependent, could not be detected in *Pyroc. furiosus* (Schäfer *et al.* 1994b; Kengen *et al.* 1994).

Kengen *et al.* (1994) recently reported the presence of a two novel kinases that substitute for the missing enzymes of the Embden–Meyerhof pathway. Hexokinase and 6-phosphofructokinase depend on ADP as phosphoryl donor, forming AMP as product. ADP-dependent kinases have not been reported so far in prokaryotes. Kengen *et al.* (1994) propose that glucose is degraded by *Pyroc. furiosus* via a modified Embden–Meyerhof pathway involving these novel ADP-dependent kinases and the hexose phosphate isomerase and fructose-1,6-bisphosphate aldolase described previously (Schäfer & Schönheit 1992, 1993). The different possible pathways of glucose catabolism to acetate and alanine, as concluded from enzyme data, are summarized in Figure 9. Both the Embden–Meyerhof type glycolysis and Entner–Doudoroff pathway may be operative at the same time to different extents, regulated by various physiological conditions as has been proposed for the hyperthermophile *Thermoproteus tenax* (see below).

More work is necessary to completely understand sugar catabolism in *Pyroc. furiosus*, including experiments to detect and quantify intermediates of sugar degradation analysed by *in vivo* and *in vitro* ^{13}C-NMR spectroscopy, ^{14}C-pulse labelling experiments in cell extracts and radiorespirometry.

Thermoproteus tenax is an obligate sulphur-dependent, facultative organotroph growing on various sugars (glucose, starch, amylose) (Zillig *et al.* 1981; Huber & Stetter 1992b). Evidence has been provided that glucose is completely oxidized by this organism to CO_2 (Zillig *et al.* 1981; Selig & Schönheit 1994). During exponential growth, CO_2 and H_2S but no other fermentation products were detected. Two mol H_2S were formed per mol CO_2 indicating complete oxidation of glucose with sulphur as electron acceptor ($C_6H_{12}O_6 + 12\ S \rightarrow 6\ CO_2 + 12\ H_2S$). The pathway of glucose catabolism to pyruvate was studied in *Thermoproteus tenax* by ^{14}C-glucose pulse labelling experiments using dialysed cell extracts (Siebers & Hensel 1993). In the presence of ATP and pyrophosphate (PPi), intermediates of the Embden–Meyerhof pathway were detected; whereas, in the absence of ATP and PPi but in the presence of NAD^+, typical intermediates of the non-phosphorylated Entner–Doudoroff pathway were found. All enzymes of the Embden–Meyerhof pathway could be detected in the extracts. The organism contained ATP-dependent hexokinase and a 6-phosphofructokinase which was dependent on pyrophosphate rather than on ATP (Siebers & Hensel 1993). Two distinct glyceraldehyde-3-phosphate dehydrogenases were present and both have been purified. One is specific for $NADP^+$, the other for NAD^+; both enzymes are also present in autotrophically-grown *T hermoproteus tenax* (see above). The exact role of these enzymes in sugar catabolism or gluconeogenesis remains to be defined (Hensel *et al.* 1987). Cell extracts also contain activities of glucose dehydrogenase [$NAD(P)^+$-dependent], 2-keto-3-deoxygluconate aldolase and glyceraldehyde dehydrogenase (viologen-dye-dependent), i.e. enzymes typical of the non-phosphorylated Entner–Doudoroff pathway (Siebers & Hensel 1993; Selig & Schönheit 1994). Thus, the ^{14}C-labelling data and enzyme studies indicate that both glycolytic pathways, a modified Embden–Meyerhof pathway and the non-phosphorylated Entner–Doudoroff pathway, might be operative concomitantly in glucose catabolism in *T. hermaproteus tenax*. The degree of contribution of either pathway might be regulated by various physiological conditions, e.g. by the phosphorylation potential. This hypothesis has to be tested.

The order *Thermotogales* belongs to the deepest branches within the bacterial domain and thus represents an ancient phenotype of the Bacteria. *Thermotoga* spp. obligate are organoheterotrophs growing on various sugars (mono-and di-saccharides, starch and xylan) as carbon and energy sources (Table 3; Huber & Stetter 1992a). Various hydrolytic exoenzymes, amylases and xylanases, have been purified from several *Thermotoga* spp. (Leuschner & Antranikian 1995).

The pathway of glucose fermentation has been studied in *Thermotoga maritima* (Huber *et al.* 1986) by measuring fermentation balances, molar growth yields and enzyme activities in cell extracts (Schröder *et al.* 1994). Furthermore, ^{13}C-NMR studies were performed in cell suspensions. The data indicate the operation of a "classical" Embden–Meyerhof pathway in glucose fermentation; growing cultures of *T hermotoga maritima* fermented glucose almost completely to acetate, CO_2 and H_2 (glucose + $2H_2O \rightarrow 2$ acetate$^-$ + $2\ H^+ + 2\ CO_2 + 4\ H_2$; $\Delta G^{o\prime} = -212$ kJ/mol). The $\Delta G'$ value of the fermentation under the experimental conditions was about -300 kJ/mol (Schröder *et al.* 1994), which is the free energy change sufficient to allow the formation of four mol of ATP under cellular conditions [for a thermodynamic explanation see Thauer *et al.* (1977) and Tewes & Thauer (1980)]. A similar fermentation balance has been found only in one moderate thermophilic (eu)bacterium, *Acetomicrobium flavidum* (Soutschek *et al.* 1984). All other glucose-fermenting anaerobes tested form less than two mol acetate and four mol H_2 per mol glucose. Instead, various amounts of reduced products such as lactate, ethanol or butyrate are formed (see Tewes & Thauer 1980). L-Lactate, a major product of glucose fermentation by cell suspensions of *Thermotoga maritima* (Huber *et al.* 1986), was not formed in significant concentrations in growing cultures although the organism contained lactate dehydrogenase (Hecht *et al.* 1989). The molar growth yield, $Y_{glucose}$, was about 45 g cell dry mass/mol glucose, indicating an ATP yield of about four mol assuming Y_{ATP} to be about 10 g cell dry mass/mol. Since two mol ATP are formed during acetate formation (see below), two mol ATP have to be formed during glucose conversion to pyruvate, indicative of the operation

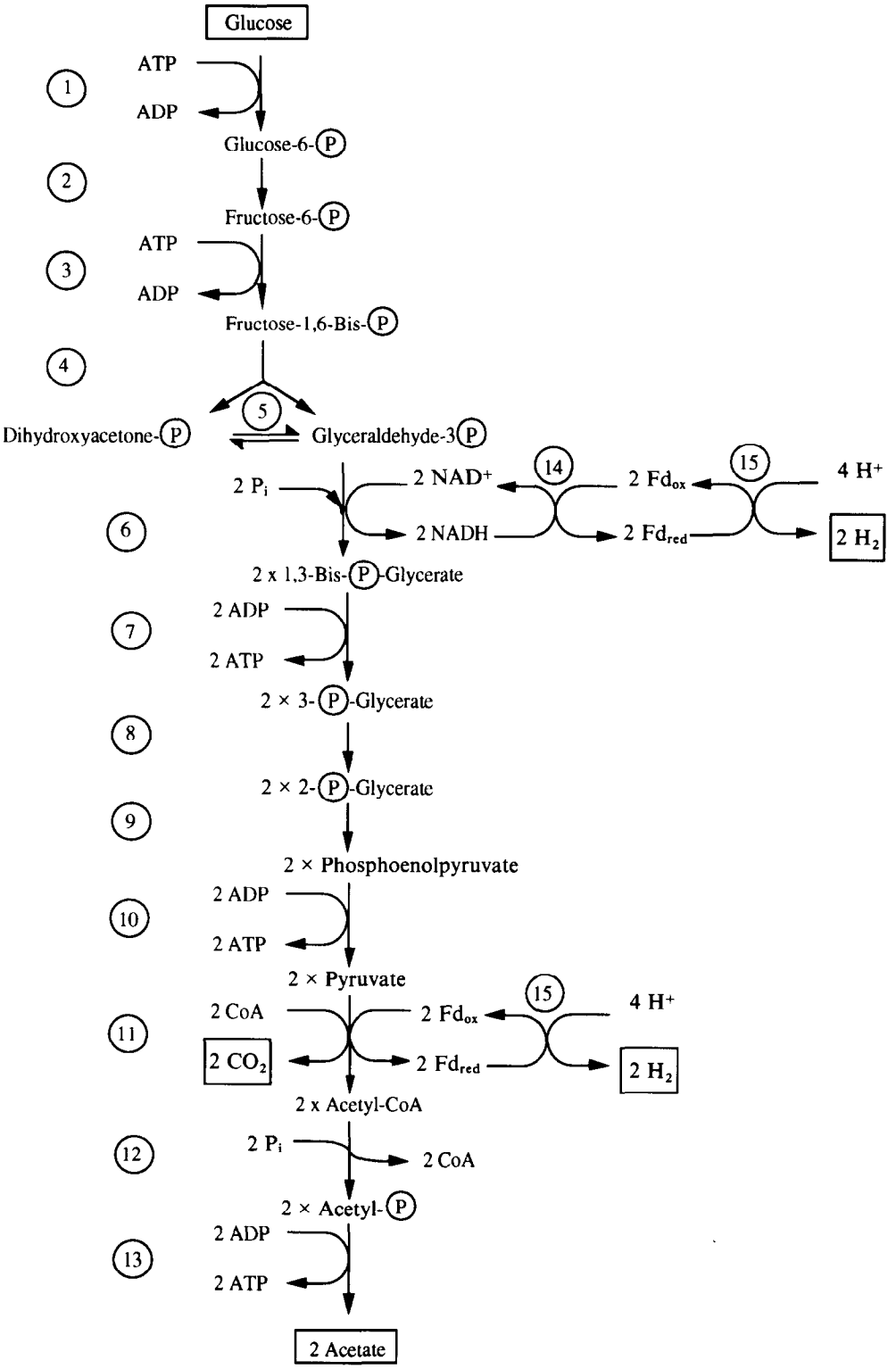

Figure 10. Pathway of glucose fermentation to acetate, CO_2 and H_2 (glucose + 2 $H_2O \rightarrow$ 2 acetate$^-$ + 2 H^+ + 4 H_2) in the hyperthermophilic (eu)bacterium *Thermotoga maritima* via the 'classical' Embden–Meyerhof pathway (Schröder *et al.* 1994). ①—ATP-dependent hexokinase; ②—glucose-6-phosphate isomerase; ③—ATP-dependent 6-phosphofructokinase; ④—fructose-1,6-bisphosphate aldolase; ⑤—triose-phosphate isomerase; ⑥—glyceraldehyde-3-phosphate dehydrogenase; ⑦—phosphoglycerate kinase; ⑧—phosphoglycerate mutase; ⑨—enolase; ⑩—pyruvate kinase; ⑪—pyruvate; ferredoxin oxidoreductase; ⑫—phosphate acetyltransferase; ⑬—acetate kinase; ⑭—NADH:ferredoxin oxidoreductase; ⑮—hydrogenase.

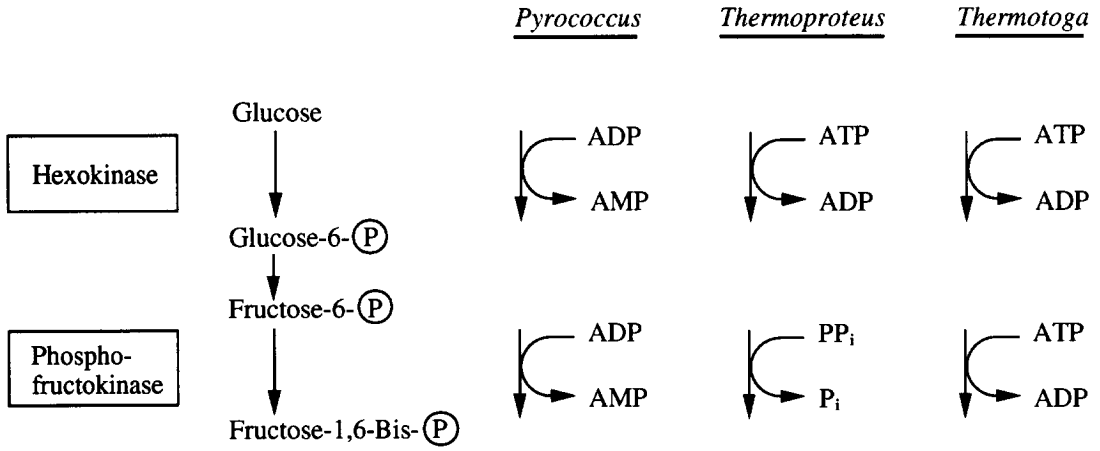

Figure 11. Phosphoryl-donors for hexokinase and 6-phosphofructokinase involved in sugar catabolism of hyperthermophiles.

of the Embden–Meyerhof pathway. Cell extracts contained all enzymes of the "classical" Embden–Meyerhof pathway, including ATP-dependent hexokinase and ATP-dependent 6-phosphofructokinase (Figure 10).

^{13}C-Labelling patterns of the fermentation products acetate and lactate obtained after fermentation of [1-^{13}C]glucose and [3-^{13}C]glucose by cell suspensions of *Thermotoga maritima* are compatible with the operation of the Embden–Meyerhof pathway to about 85%. About 15% of the labelling pattern can be explained by the operation of an Entner–Doudoroff pathway (H. Santos and P. Schönheit, unpublished work). Cell extracts contained glucose-6-phosphate dehydrogenase activity.

The complete pathway of glucose fermentation to acetate, CO_2 and H_2 is shown in Figure 10. Conversion of the intermediate pyruvate to acetate, CO_2 and H_2 is catalysed by pyruvate:ferredoxin oxidoreductase, hydrogenase, phosphate acetyltransferase and acetate kinase (see below). Formation of H_2 from NADH, the product of glyceraldehyde-3-phosphate dehydrogenase, is catalysed by NADH: ferredoxin oxidoreductase and hydrogenase. These enzymes, and also glyceraldehyde-3-phosphate dehydrogenase and 6-phosphofructokinase, have also been reported for *Thermotoga* strain FjSS3.B1 (Janssen & Morgan 1992). Glyceraldehyde-3-phosphate dehydrogenase (Wrba *et al.* 1990; Tomschy *et al.* 1993) and hydrogenase (Jusczak *et al.* 1991) from *Thermotoga maritima* have been purified and characterized.

Sulphur stimulates growth of *Thermotoga maritima* on glucose at H_2 concentrations higher than 2% to 3%, sulphur being reduced to H_2S (Huber *et al.* 1986; Schröder *et al.* 1994) [for other *Thermotoga* strains see also Belkin *et al.* (1986), Janssen & Morgan (1992) and Huber & Stetter (1992a)]. This effect has been explained by an electron-sink reaction preventing H_2 to accumulate (Huber & Stetter 1992a). Sulphur reduction is apparently not coupled with energy conservation since, as shown for *Thermotoga maritima*, the molar growth yield and the stoichiometry of acetate/CO_2 formation from glucose were almost identical in the presence or absence of sulphur (see also Janssen & Morgan 1992; Schröder *et al.* 1994).

In summary, the present state of investigation of the glycolytic pathways in hyperthermophiles indicates: (1) In the aerobic Archaea *Sulfolobus* (*Thermoplasma*), the non-phosphorylated Entner–Doudoroff pathway appears to be the main catabolic pathway. (2) In the anaerobic Archaea, the sulphur-reducing *Thermoproteus* and the fermenting *Pyrococcus*, modifications of both a non-phosphorylated Entner–Doudoroff pathway and an Embden–Meyerhof pathway might be operative. (3) The nucleotide specificities of the hexokinase and phosphofructokinase differ in hyperthermophiles: *Thermoproteus tenax* contains ATP-dependent hexokinase and pyrophosphate-dependent 6-phosphofructokinase whereas both kinases in *Pyrococcus furiosus* are dependent on ADP. The ADP dependency of the kinases might represent an phylogenetically ancestral mechanism. (4) The "classical" Embden–Meyerhof pathway involving both ATP-dependent 6-phosphofructokinase and ATP-dependent 6-phosphofructokinase has not been found in hyperthermophilic (or other) Archaea but is present in the hyperthermophilic eubacterium *Thermotoga* (and all other glucose-fermenting anaerobic bacteria) (Figure 11);

Thus, ATP-dependent 6-phosphofructokinase probably evolved after diversification of the Archaea and Bacteria.

Pyruvate Conversion to Acetyl-CoA in Hyperthermophiles. Pyruvate, formed as an intermediate in sugar, peptide or lactate degradation, or supplied as a growth substrate (Figure 7), is oxidized to acetyl-CoA and CO_2. All hyperthermophiles and all other Archaea tested contain pyruvate:ferredoxin oxidoreductase catalysing pyruvate oxidation with ferredoxin as electron acceptor:

$$\text{Pyruvate} + \text{ferredoxin}_{ox} + \text{CoA} \rightarrow \text{acetyl-CoA} + CO_2 + \text{ferredoxin}_{red}$$

The enzyme has been found in the aerobes *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum* (Kerscher *et al.*

1982), in the sulphur-reducing *Thermoproteus tenax* and *Pyrobaculum islandicum* (Selig & Schönheit 1994), in the fermenting *Pyrococcus furiosus, Thermococcus celer, Desulfurococcus amylolyticus, Hyperthermus butylicus* and *Thermotoga maritima* (Schäfer *et al.* 1993) and probably in the sulphate-reducing *Archaeoglobus fulgidus* (Möller–Zinkhan *et al.* 1989). Pyruvate:ferredoxin oxidoreductase is also present in mesophilic Archaea, in the aerobic extreme halophiles, including *Halobacterium halobium* (see Kerscher & Oesterhelt 1982) and *Halobacterium saccharovorum* (Schäfer *et al.* 1993), and the anaerobic methanogen *Methanosarcina barkeri* (Bock *et al.* 1994). So far, neither the pyruvate dehydrogenase multienzyme complex typical of aerobic Bacteria and Eukarya nor the pyruvate formate lyase present in facultative Bacteria have been found in Archaea. Thus, pyruvate:ferredoxin oxidoreductase appears to represent the only mechanism of acetyl-CoA generation from pyruvate in the Archaeal domain. For a mechanistic comparison of the pyruvate:ferredoxin oxidoreductase and pyruvate dehydrogenase complex of aerobic Bacteria and Eukarya see Danson (1988, 1993). Interestingly, several Archaea have been shown to contain dihydrolipoamide dehydrogenase, a constituent of pyruvate dehydrogenase complex; the function of this enzyme is not known (see Danson 1993).

Pyruvate:ferredoxin oxidoreductases have been purified from the hyperthermophiles *Pyroc. furiosus* and *Thermotoga maritima*. The enzymes of both organisms have molecular masses of about 115 000, composed of four dissimilar subunits, and contain thiamine pyrophosphate and two ferredoxin-like [4Fe/4S] clusters. The *Pyroc. furiosus* enzyme appears to contain copper which is not found in the enzyme of *Thermotoga maritima*; accordingly, different catalytical mechanisms have been proposed for the two hyperthermophiles (Blamey & Adams 1993, 1994; Smith *et al.* 1994). Pyruvate:ferredoxin oxidoreductases from mesophilic bacteria differ from those of hyperthermophiles in that they have about twice the molecular mass and are composed of two identical subunits (see Blamey & Adams 1994). Another archaeal pyruvate:ferredoxin oxidoreductase studied in detail is the enzyme of the aerobe *Halobacterium halobium*; this enzyme has a similar molecular mass to that of mesophilic bacteria but is a tetramer of two different types of subunits (Kerscher & Oesterhelt 1981). The encoding genes have been sequenced and the catalytical mechanism has been elucidated (see Kerscher & Oesterhelt 1982; Plaga *et al.* 1992). For a discussion of the evolution of pyruvate:ferredoxin oxidoreductases see Kerscher & Oesterhelt (1982), Danson (1988, 1993) and Blamely & Adams (1994).

Pyruvate:ferredoxin oxidoreductase is also present in obligate lithoautotrophic hyperthermophiles, in which the reversible enzyme functions as pyruvate synthase, catalysing the reductive carboxylation of acetyl-CoA, with reduced ferredoxin as electron donor (see above).

Ferredoxins, operating as electron carriers of 2-oxoacid oxidoreductases (pyruvate oxidoreductase, 2-oxoglutarate oxidoreductase) and of hydrogenases have been purified and characterized from various hyperthermophiles. Ferredoxins from *Sulfolobus, Thermoplasma* and *Desulfurococcus* contain two [4Fe/4S] clusters (Kerscher *et al.* 1982). In *Pyrococcus* (Aono *et al.* 1989) and *Thermotoga* (see Adams 1993) extremely heat-stable ferredoxins carrying one [4Fe/4S] cluster have been described; *Halobacterium* contains a plant-type [2Fe/2S] ferredoxin (Kerscher *et al.* 1976).

Acetyl-CoA, the product of pyruvate oxidation, is either oxidized to CO_2 with external electron acceptors or fermented to acetate and other products. The pathways of acetyl-CoA oxidation to CO_2 and of acetate formation from acetyl-CoA in hyperthermophiles are summarized below.

Acetyl-CoA Oxidation to CO_2. Various hyperthermophiles have been reported to completely oxidize organic compounds to CO_2 in the presence of external electron acceptors, implicating the oxidation of acetyl-CoA (see Table 2).

Two mechanisms are known catalysing acetyl-CoA oxidation to two CO_2 molecules, the citric acid cycle and the acetyl-CoA/carbon monoxide dehydrogenase pathway (see Thauer 1988; Thauer *et al.* 1989). The citric acid cycle is operative in all aerobic Bacteria and Eukarya and also in several anaerobic sulphur- and few sulphate-reducing Bacteria. In contrast to the citric acid cycle, the acetyl-CoA/carbon monoxide dehydrogenase pathway is a linear pathway catalysing direct carbon–carbon bond cleavage of acetyl-CoA to an enzyme-bound methyl-group and enzyme-bound carbon monoxide. Both intermediates are further oxidized to CO_2. The key enzyme of this pathway is carbon monoxide dehydrogenase, catalysing both acetyl-CoA cleavage and the oxidation of CO to CO_2. The pathway is found in most bacterial sulphate reducers.

The mechanism of acetyl-CoA oxidation to CO_2 in hyperthermophiles has been studied in the aerobic *Sulfolobus* and *Thermoplasma* (moderate thermophile) (see Danson 1988, 1993), in the anaerobic sulphate-reducing *Archaeoglobus* (Thauer *et al.* 1989), and in sulphur-reducing *Thermoproteus* and *Pyrobaculum* (Selig & Schönheit 1994).

In the aerobic *Sulfolobus* and *Thermoplasma* most (*Sulfolobus*) or all (*Thermoplasma*; H. Görisch, unpublished work) enzymes of the citric acid cycle have been demonstrated (see Danson 1988), indicating that acetyl-CoA is oxidized to CO_2 via the citric acid cyle. In contrast to the citric acid cycle of aerobic Bacteria, hyperthermophiles (and all other aerobic Archaea, including mesophilic extreme halophiles) contain 2-oxoglutarate:ferredoxin oxidoreductase rather than pyridine-nucleotide-dependent 2-oxoglutarate dehydrogenase complex. Furthermore, the pyridine-nucleotide-dependent dehydrogenases, isocitrate dehydrogenase and malate dehydrogenase, show a dual cofactor specificity, using both NAD^+ and $NADP^+$ as electron acceptors, a typical property of various archaeal dehydrogenases (see Danson 1988). Several enzymes of the citric acid cycle of

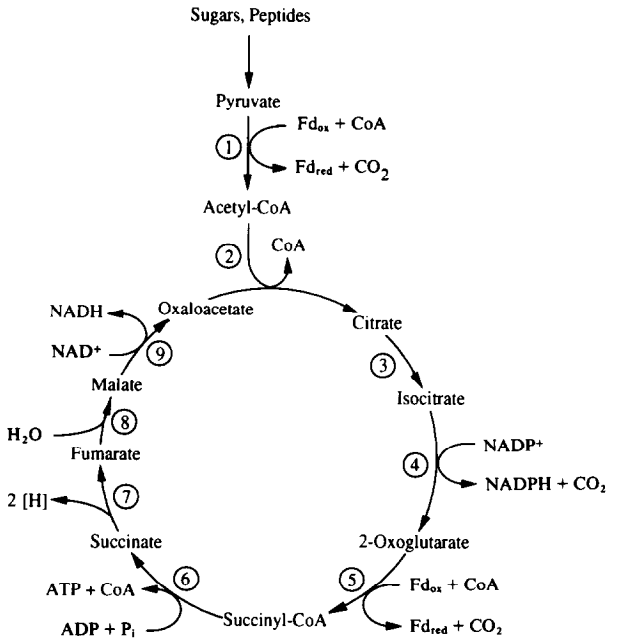

Figure 12. Acetyl-CoA oxidation to two mol CO_2 via the citric acid cycle in the anaerobic hyperthermophilic sulphur reducer *Thermoproteus tenax* and *Pyrobaculum islandicum* (Selig & Schönheit 1994). ①—pyruvate:ferredoxin oxidoreductase; ②—citrate synthase; ③—aconitase; ④—isocitrate dehydrogenase; ⑤—2-oxoglutarate:ferredoxin oxidoreductase; ⑥—succinyl-CoA synthetase; ⑦—succinate dehydrogenase; ⑧—fumarase; ⑨—malate dehydrogenase.

Sulfolobus acidocaldarius and *Thermoplasma acidophilum* have been purified, including citrate synthase, succinate thiokinase, fumarate hydratase, and malate dehydrogenase (Grossebüter & Görisch 1985; Grossebüter *et al.* 1986; Danson *et al.* 1985; Danson 1988; Puchegger *et al.* 1990). Malate dehydrogenases from both organisms have been crystallized (Stezowski *et al.* 1989; Hartl *et al.* 1987); the genes coding for citrate synthase of *Thermoplasma acidophilum* (Sutherland *et al.* 1990) and *Su. solfataricus* (Lill *et al.* 1992) have been cloned and sequenced. The various data on citric acid cycle enzymes of hyperthermophiles allow comparative studies with the corresponding enzymes from phylogenetically distantly-related mesophilic bacteria or Eukarya (see Danson 1988, 1993; Muir *et al.* 1994).

The anaerobic hyperthermophilic Archaea *Thermoproteus tenax* (Zillig *et al.* 1981) and *Pyrobaculum islandicum* (Huber *et al.* 1987) grow on sugars (only *Thermoproteus tenax*) or peptides (both species, with sulphur as electron acceptor (see Table 2). *Pyrob. islandicum* can also use thiosulphate as electron acceptor. Recent evidence indicates that organic compounds in both organisms are completely oxidized to CO_2, with either sulphur or thiosulphate as electron acceptor; oxidation of acetyl-CoA proceeds via the citric acid cycle (Selig & Schönheit 1994). Cultures of both organisms exponentially growing on glucose or peptides and sulphur or thiosulphate formed only CO_2 and H_2S and no other fermentation products (see Zillig *et al.* 1981; Huber *et al.* 1987). The stoichiometry of CO_2/H_2S formation was 1:2 with sulphur as electron acceptor and 1:1 with thiosulphate as electron acceptor, a result consistent with complete oxidation of glucose and peptides, which both have an average carbon redox state equal to formaldehyde (>HCHO<), with sulphur (>HCHO< + 2 S + $H_2O \rightarrow$ CO_2 + 2 H_2S) or thiosulphate (>HCHO< + 0.5 $S_2O_3^{2-}$ + $H^+ \rightarrow$ CO_2 + H_2S + 0.5 H_2O). Cell extracts of both organisms contained all enzymes of the citric acid cycle (Figure 12) in catabolic activities. Carbon monoxide dehydrogenase activity could not be detected.

The citric acid cycle has also been found in all sulphur-reducing bacteria tested so far, suggesting that the cycle is a general mechanism for acetyl-CoA oxidation with sulphur as electron acceptor (see Thauer *et al.* 1989; Selig & Schönheit 1994).

The presence of a complete citric acid cycle in *Pyrob. islandicum* and *Thermoproteas tenax*, representing deep branches within the Archaea (see Figure 1), supports the proposal by Wächtershäuser (1990) that the citric acid cycle constitutes one of the first metabolic pathways. The cycle is assumed to operate in the reductive direction for acetyl-CoA formation in lithoautotrophic metabolism (see above) and might then, secondarily, be used for acetyl-CoA oxidation during organotrophic metabolism. This dual function of the citric acid cycle has now been demonstrated in *Thermoproteus* spp. (Schäfer *et al.* 1986; Selig & Schönheit 1994).

During acetyl-CoA oxidation via the citric acid cycle (Figure 12), reduced pyridine nucleotides (NADH or NADPH) ($E^{\circ\prime}$ = −320 mV), reduced ferredoxin ($E^{\circ\prime}$ = −420 mV for Clostridial ferredoxin) and, in the succinate dehydrogenase reaction, probably a reduced menaquinone ($E^{\circ\prime}$ = −75 mV) are generated. Lipophilic menaquinones have been demonstrated in *Thermoproteus tenax* (Thurl *et al.* 1985) and *Pyrob. islandicum* (Tindall *et al.* 1991); these probably serve as physiological electron acceptors of succinate dehydrogenase. During growth on sulphur the reduced electron carriers have to be reoxidized by sulphur reduction to H_2S ($E^{\circ\prime}$ [S/H_2S] = −270 mV). The mechanism and energetics of sulphur reduction in both hyperthermophiles are not known. In analogy to the well studied mesophilic sulphur-reducing (eu)bacterium, *Desulfuromonas acetoxidans*, it can be assumed that, in the hyperthermophiles, sulphur reduction by ferredoxin could be the site of energy conservation and that the endergonic reoxidation of reduced menaquinone by sulphur involves reversed electron flow (see Paulsen *et al.* 1986; Thauer 1988).

Hyperthermophiles able to oxidize organic compounds to CO_2 with sulphate as electron acceptor belong to the genus *Archaeoglobus*. *Archaeoglobus fulgidus* (Stetter *et al.* 1987; Stetter 1988) grows on sugars, peptides, or lactate and sulphate (Table 2). The pathway of lactate oxidation

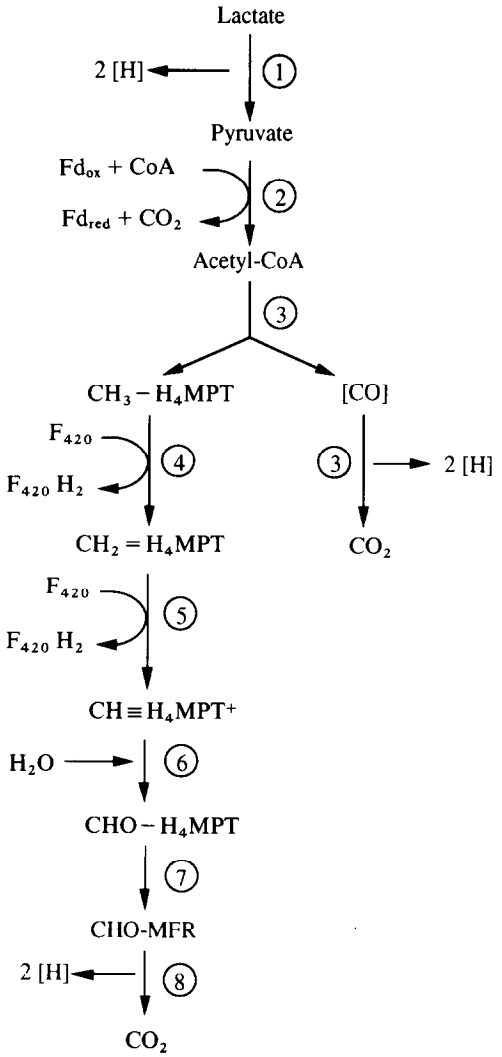

Figure 13. Proposed pathway of lactate oxidation to CO_2 in the hyperthermophilic sulphate reducer *Archaeoglobus fulgidus*: acetyl-CoA is oxidized to two mol CO_2 via a modified acetyl-CoA/carbon monoxide dehydrogenase pathway (Thauer *et al.* 1989; Möller-Zinkhan & Thauer 1990). CoA—Coenzyme A; H_4MPT—tetrahydromethanopterin; MF—methanofuran; CH_3-H_4MPT—methyl-H_4MPT; CH_2=H_4MPT—methylene-H_4MPT; $CH=H_4MPT^+$—methenyl-H_4MPT; CHO-H_4MPT—formyl-H_4MPT; [CO]—enzyme-bound carbon monoxide; $F_{420}H_2$—reduced coenzyme F_{420}; ①—lactate dehydrogenase; ②—pyruvate:ferredoxin oxidoreductase; ③—carbon monoxide dehydrogenase; ④—methylene-H_4MPT reductase (Schmitz *et al.* 1991); ⑤—methylene-H_4MPT dehydrogenase (Schwörer *et al.* 1993); ⑥—methenyl-H_4MPT cyclohydrolase (Klein *et al.* 1993b); ⑦—formyl-H_4MPT:MF formyltransferase (Schwörer *et al.* 1993); ⑧—formyl-MF dehydrogenase.

to CO_2 has been elucidated by Thauer and coworkers (Möller-Zinhan *et al.* 1989; Thauer *et al.* 1989; Möller-Zinhan & Thauer 1990) (Figure 13). Lactate is oxidized to acetyl-CoA and CO_2 by membrane-bound lactate dehydrogenase and pyruvate:ferredoxin oxidoreductase. Oxidation of acetyl-CoA to 2 CO_2 proceeds via a modified acetyl-CoA/carbon monoxide dehydrogenase (CO-DH) pathway rather than via the citric acid cycle. Surprisingly, C_1 transformation involves the coenzymes tetrahydromethanopterin and methanofuran, the electron carrier factor F_{420}, a deazaflavin, and enzymes typical of methanogenic Archaea. All enzymes involved in acetyl-CoA oxidation to CO_2 according to the modified acetyl-CoA/carbon monoxide pathway were detected in cell extracts (Figure 13). Carbon monoxide dehydrogenase, catalysing both acetyl-CoA cleavage to methyl-tetrahydromethanopterin and bound carbon monoxide, [CO], as well as the oxidation of [CO] to CO_2, is present in high activities (Möller-Zinkhan & Thauer 1990). Various enzymes of the pathway have been purified from *Ar. fulgidus* (Figure 13); the *N*-terminal aminoacid sequences and other molecular properties show a high degree of similarity with those of the respective enzymes of methanogens (Schmitz *et al.* 1991; Schwörer *et al.* 1993). An F_{420}-dependent $NADP^+$ reductase, linking catabolism to anabolism, has been purified (Kunow *et al.* 1993).

Several organotrophic bacterial mesophilic sulphate reducers, e.g. *Desulfotomaculum*, also oxidize acetyl-CoA via the oxidative acetyl-CoA/carbon monoxide dehydrogenase pathway. The pathway in Bacteria (see Fuchs 1986; Wood *et al.* 1986; Diekert 1990) differs from that of *Archaeoglobus* in that it involves tetrahydrofolate instead of tetrahydromethanopterin as C_1 carrier and free formate rather than formylmethanofuran as an intermediate.

During lactate oxidation by *Archaeoglobus*, reduced ferredoxin and reduced F_{420} are generated and these reduce sulphate via adenosine phosphosulphate (APS) and sulphite to H_2S. The *Ar. fulgidus* enzymes involved in sulphate reduction to H_2S have been discussed above. The redox potential differences of ferredoxin (oxidized/reduced) ($E^{o\prime}$ = −420 mV), F_{420} (oxidized/reduced) ($E^{o\prime}$ = −360 mV) and the electron acceptor couples APS/SO_3^{2-} ($E^{o\prime}$ = −60 mV) and SO_3^{2-}/H_2S ($E^{o\prime}$ = −105 mV) are high enough to allow ATP formation by the mechanism of electron transport phosphorylation.

Acetyl-CoA Conversion to Acetate. Several hyperthermophiles have been shown to ferment organic compounds (peptides, sugars, pyruvate etc) to acetate as major fermentation product (see Table 3). The enzymes involved in acetate formation from acetyl-CoA were studied in the hyperthermophilic Archaea *Pyrococcus furiosus, Pyroc. woesei, Thermococcus celer, Desulfurococcus amylolyticus* and *Hyperthermus butylicus* and in the hyperthermophilic (eu)bacterium *Thermotoga maritima*. All hyperthermophilic acetate-forming Archaea tested contain an acetyl-CoA synthetase (ADP-forming); phosphate acetyltransferase and acetate kinase were not found (Schäfer & Schönheit 1991; Schäfer *et al.* 1993). Acetyl-CoA synthetase, a novel enzyme in prokaryotes, couples acetate formation from acetyl-CoA with the phosphorylation of ADP via the mechanism of substrate level phosphorylation:

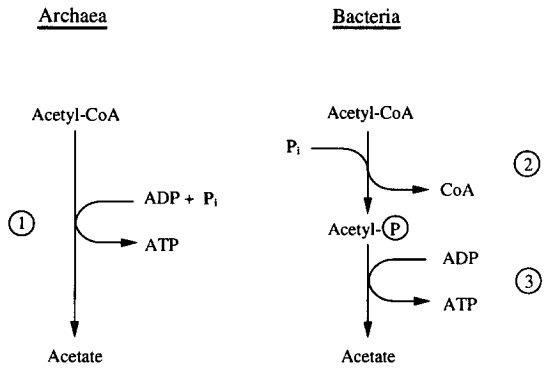

Figure 14. Mechanisms of acetate formation and of ATP synthesis from acetyl-CoA, ADP and P_i in (hyperthermophilic) Archaea and Bacteria. ①—Acetyl-CoA synthetase (ADP-forming); ②—phosphate acetyltransferase; ③—acetate kinase.

$$\text{Acetyl-CoA} + \text{ADP} + \text{P} \rightarrow \text{acetate} + \text{ATP} + \text{CoA}$$

The enzyme constitutes the main energy conserving site of pyruvate or sugar fermentation in *Pyroc. furiosus* (Schäfer & Schönheit 1991). In contrast to hyperthermophilic Archaea, the hyperthermophilic acetate-forming *Thermotoga maritima*, and all other acetate-forming Bacteria tested so far, contain the 'classical' enzymes, phosphate acetyltransferase and acetate kinase, but no acetyl-CoA synthetase (ADP-forming). Thus, acetyl-CoA synthetase represents an archaeal enzyme rather than an enzyme typical of hyperthermophiles (Schäfer *et al.* 1993). In accordance, acetyl-CoA synthetase (ADP-forming) (but no phosphate acetyltransferase or acetate kinase) was also found in the mesophilic aerobic archaeon *Halobacterium saccharovorum* (Schäfer *et al.* 1993), which forms significant amounts of acetate during growth on glucose (Tomlinson *et al.* 1974). The enzyme has also been reported for *Thermoplasma acidophilum* (see Danson 1988).

An enzyme, which appears to be typical of hyperthermophiles, is reverse gyrase, a novel DNA topoisomerase which might have a function in thermostabilization of DNA; however, the exact role of the enzyme is not known. Reverse gyrase has been detected only in (hyper)thermophiles, both Archaea and Bacteria, and not in mesophiles (Kikuchi *et al.* 1986; Bouthier De La Tour *et al.* 1990, 1991).

In summary, in acetate-forming prokaryotes two different mechanisms exist for the formation of acetate and ATP from acetyl-CoA, ADP and P_i, depending on the phylogenetic domain to which they belong (Figure 14). Archaea utilize one enzyme, acetyl-CoA synthetase (ADP-forming), and Bacteria two enzymes, phosphate acetyltransferase and acetate kinase. Thus, acetyl-CoA synthetase (ADP-forming) probably represents the phylogenetically 'older' mechanism of ATP synthesis via substrate level phosphorylation compared with the acetate kinase reaction. One may speculate that phosphate acetyltransferase and acetate kinase might have originated from acetyl-CoA synthetase (ADP-forming), e.g. by gene splitting.

In contrast, hyperthermophilic and other Archaea use different mechanisms for the activation of acetate to acetyl-CoA, both in anabolism and catabolism, which do not include acetyl-CoA synthetase (ADP-forming). Acetate activation involves either acetyl-CoA synthetase (AMP-forming), as in *Thermoproteus neutrophilus*, or acetate kinase/phosphate acetyltransferase, as in several methanogens (see Schäfer *et al.* 1993).

It should be mentioned that acetyl-CoA synthetase (ADP-forming) was first detected in anaerobic Eukarya, *Entamoeba histolytica* (Reeves *et al.* 1977) and *Giardia lamblia* (Lindmark 1980). In these protozoa the enzyme is apparently involved in acetate formation and ATP synthesis as part of the anaerobic metabolism (see Müller 1988; Adam 1991).

Peptide Catabolism

Most organotrophic hyperthermophiles can grow on complex media containing peptides as carbon and energy source. Table 2 lists the hyperthermophiles which have been reported to utilize peptides as electron donor for the reduction of external electron acceptors (sulphur, sulphate, thiosulphate, oxygen or nitrate); Table 3 includes organisms that can ferment peptides. Almost all of these organisms are facultative sulphur reducers. In the presence of sulphur, H_2S rather than the inhibitory H_2 is produced. Sulphur reduction is apparently not coupled with energy production. There have only been a few studies on the quantitative determination of fermentation products and on catabolic pathways involved in peptide metabolism.

Several reports on extracellular proteinases and enzymes involved in amino-acid metabolism have appeared. Various proteases have been isolated and characterized from different hyperthermophiles (*Pyrococcus, Thermococcus, Desulfurococcus, Sulfolobus, Staphylothermus, Fervidobacterium*). They are mostly serine-type proteases (see Leuschner & Antranikian 1995). Glutamate dehydrogenases from various hyperthermophiles (*Pyroc. furiosus, Pyroc. woesei, Su. solfataricus* and *Thermotoga maritima*) have been studied in detail (Consalvi *et al.* 1991a, b; Schinkinger *et al.* 1991; Maras *et al.* 1992; Robb *et al.* 1992; Sanangelantoni *et al.* 1992; Eggen *et al.* 1993). Kinetic analysis of the enzyme from *Pyroc. furiosus* (Consalvi *et al.* 1991b) indicates that, *in vivo*, the enzyme catalyses glutamate conversion to oxoglutarate, a compound of the citric acid cycle. The extremely thermostable enzyme amounted up to 20% of the cytoplasmic protein in *Pyrococcus*. In contrast to the enzyme of mesophilic bacteria, the enzyme of hyperthermophiles (and other Archaea) can utilize either NAD^+ or $NADP^+$ as cofactors, with a preference for $NADP^+$. The glutamate dehydrogenase gene was cloned, expressed in *E. coli* and sequenced. Comparison of the primary sequence of various enzymes

from Bacteria, Archaea, and Eukarya indicate significant homology (Eggen *et al.* 1993). Glutamate dehydrogenase from *Pyroc. woesei* has been crystallized (see Leuschner & Antranikian 1995). Glutamine synthetases from *Pyroc. woesei* and *Thermotoga maritima* have been cloned and sequenced (Tiboni *et al.* 1993). A comparative study of the pathways of arginine synthesis has been reported in various hyperthermophiles (Van De Casteele *et al.* 1990). A tungsten-containing formaldehyde:ferredoxin oxidoreductase has been purified from the obligately peptide-fermenting *Thermococcus litoralis*; the enzyme is present in high concentrations and is thought to be involved in peptide catabolism (Mukund & Adams 1993).

Peptide Oxidation to CO_2. Several hyperthermophiles of the orders *Thermoproteales, Archaeoglobales* and *Sulfolobales* have been reported to grow on complex media containing peptides and various electron acceptors such as sulphur, thiosulphate, sulphate, oxygen and nitrate and nitrite (see Table 2). These organisms are assumed to completely oxidize peptides to CO_2, gaining energy by anaerobic or aerobic respiration. Since the compounds used from complex peptides are not known, substrate consumption and energetic details are unknown in most cases.

For the hyperthermophilic Archaea, *Thermoproteus tenax* (Zillig *et al.* 1981) and *Pyrobaculum islandicum* (Huber *et al.* 1987), it has been indicated that peptides are completely oxidized, with sulphur or thiosulphate as electron acceptor, and that the oxidation of acetyl-CoA proceeds via the citric acid cycle (Selig & Schönheit 1994) (Figure 12).

Sulfolobus spp. and the recently-isolated, microaerophilic *Pyrobaculum aerophilum* can grow on peptides and molecular oxygen (Table 2). *Pyrob. aerophilum* is also able to grow anaerobically at the expense of dissimilatory nitrate or nitrite reduction (see above). Details of the metabolic pathways and enzymes involved are not known. Thus, *Pyrobaculum* spp. are the most versatile organisms within the hyperthermophiles, able to use elemental sulphur, thiosulphate, sulphite, oxygen, nitrate or nitrite as terminal electron acceptors, indicating that these forms of respiratory metabolism were already operative in this phylogenetically ancient organism early in evolution.

Peptide Fermentation to Acetate. Almost all species of the hyperthermophilic *Thermococcales, Desulphurococcales, Thermotogales, Pyrodictiales* and *Thermoproteales* have been reported to ferment peptides to acetate (see Schäfer *et al.* 1993) and other products (see Table 3). Except for the mechanisms of acetate formation from acetyl-CoA (Schäfer *et al.* 1993; see above) the metabolic pathways involved in peptide fermentation are not known.

Concluding Remarks

Studies on the lithotrophic energy metabolism of hyperthermophiles have revealed that most of the types of energy metabolism known from mesophilic lithotrophic bacteria are operative in hyperthermophiles. Due to the compounds present in hyperthermophilic habitats, anaerobic H_2-dependent reduction of sulphur compounds and of CO_2 (methanogenesis) are the predominant energy-yielding reactions. Contrary to earlier belief, hyperthermophiles can gain energy by H_2-dependent O_2 reduction (Knallgas reaction) and by denitrification.

The presence of various modes of lithotrophic metabolism in all prokaryotes indicates that the metabolic pathways involved had developed before diversification of the phylogenetic domains. Therefore, a comparative molecular analysis of lithotrophic energy metabolism in hyperthermophiles and mesophiles might give hints on the evolution of lithotrophy.

The study of organotrophic metabolism of hyperthermophiles, in particular of sugar catabolism, has revealed several novel pathways and enzymes. However, it appears that in most cases the distribution of particular pathways and enzymes is determined by the phylogenetic position rather than by the thermophilic nature of the organism.

The glycolytic pathways found in hyperthermophilic Archaea include modifications of the Embden–Meyerhof pathway and of the Entner–Doudoroff pathway, whereas hyperthermophilic Bacteria such as *Thermotoga* contain a conventional Embden–Meyerhof pathway. The modifications observed include ADP-dependent hexokinase and ADP-dependent 6-phosphofructokinases in *Pyrococcus* and ATP-dependent hexokinase and pyrophosphate-dependent 6-phosphofructokinase in *Thermoproteus*. ATP-dependent 6-phosphofructokinase is not found in Archaea but is present in the eubacterium *Thermotoga*, indicating the enzyme has evolved after diversification of the Archaea and Bacteria. The rationale behind the different phosphoryl donor specificities of kinases in the various hyperthermophiles is not known.

Pyruvate:ferredoxin oxidoreductase appears to be a phylogenetically ancient enzyme; pyruvate dehydrogenase complex or pyruvate formate lyase are absent in Archaea and probably developed after separation of the domains.

The citric acid cycle is operative in the phylogenetically ancient sulphur-dependent hyperthermophilic Archaea, both in acetyl-CoA oxidation and autotrophic CO_2 fixation, supporting the proposal of Wächtershäuser that the citric acid cycle was one of the first metabolic cycles to evolve.

A modified acetyl-CoA/carbon monoxide dehydrogenase pathway, involving coenzymes (tetrahydromethanopterin, methanofuran, F_{420}) and enzymes of methanogenesis, is operative in (hyperthermophilic) Archaea, methanogens and sulphate reducers. The corresponding pathway of Bacte-

ria, including lithotrophic homoacetogens and most lithotrophic or organotrophic sulphate reducers, uses tetrahydrofolate folates as C_1 carriers. Tetrahydrofolate-dependent C_1 transformation has not yet been found in the Archaeal domain.

Acetyl-CoA synthetase (ADP-forming) is a novel prokaryotic enzyme, involved in acetate formation and energy conservation from acetyl-CoA in all acetate-forming hyperthermophilic Archaea. The corresponding mechanism in acetate-forming Bacteria involves two enzymes, phosphate transacetylase and acetate kinase.

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