Enzymology and bioenergetics of the glycolytic pathway of *Pyrococcus furiosus*

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hoe ver je gaat
heeft met afstand niets te maken
hoogstens met de tijd

[Bløf – Omarm]

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Introduction

Extremophiles

"In an anthropocentric view, environments hostile to man were designated as extreme" [Stetter, 1999].

Microbial life can be found at nearly all locations on earth, some of which have been defined as "extreme" environments and organisms living in these environments as "extremophiles". These so-called extreme conditions can be related to any environmental parameter such as temperature, pressure, pH, gravity, desiccation, oxygen concentration, salinity, or radiation, each of which requires a distinct strategy for survival of the present organisms. Depending on their optimal growth conditions, they are named thermophiles, psychrophiles, acidophiles, alkalophiles, halophiles, or barophiles. Organisms that thrive in a combination of these conditions, such as *Sulfolobus solfataricus* that lives at pH 3 and 80°C, are called polyextremophiles [Rothschild & Mancinelli, 2001].

Because of the suggested composition of the early atmosphere and environment on earth it is most probable that the first living organisms were extremophiles. Geological evidence shows that life has been present on earth for at least 3.5 - 3.8 billion years. Although there is still controversy on the origin of life and the characteristics of the so-called "last common ancestor", the standard view implies that early life was hot and chemotrophic [Nisbet & Sleep, 2001].

Extremophiles have been extensively studied over the past decade. Their ability to survive and grow under harsh conditions raises the fundamental question which molecular mechanisms are responsible for the adaptation of the micro-organisms. Several specific strategies for adaptation have been identified, many of which are based on protein stability. For example, enzymes from halophiles (organisms growing at salt concentrations above 2.5 M) have more acidic and hydrophobic amino acid residues, and fewer aliphatic residues than their nonhalophilic homologues [Madern *et al*., 2000]. Furthermore, they have clusters of negatively charged residues [Jaenicke & Böhm, 1998]. Enzymes from psychrophiles (growing at temperatures below 15°C) have fewer hydrogen bonds and ion pairs, reduced hydrophobicity, and an increased number of polar or charged groups [Russell, 2000]. On the other hand, enzymes from hyperthermophiles (with optimum temperatures above 80°C) show a decrease in uncharged polar amino acids, an increase in charged residues, an increase in residue

hydrophobicity, and increased residue volume [Fields, 2001]. Another protective strategy of hyperthermophiles and halophiles is the accumulation of organic solutes, so-called compatible solutes, that have a role in the protection of cell components against thermal denaturation [Santos & da Costa, 2002].

The notion that extremophiles are capable of surviving under non-standard conditions has led to the assumption that the properties of their enzymes have been optimised for these conditions. Therefore, extremophilic organisms and their genomes have been screened for the presence of novel enzymes for industrial applications, which has - with help of recent developments in protein engineering and directed evolution techniques - resulted in several novel applications of enzymes in industrial processes [van den Burg, 2003].

Hyperthermophiles

One of the so-called extreme conditions that can occur in a natural environment is an extremely high or low temperature. In a classification based on their optimal growth temperature, organisms growing at temperatures below 15°C are defined as psychrophiles, while mesophiles grow at ambient temperatures with an optimum between 15 and 60°C. In the higher temperature range, two types of thermophiles can be distinguished. Moderately thermophilic organisms have an optimum growth temperature of 60 to 80°C, whereas hyperthermophiles grow optimally at temperatures above 80°C [Rothschild & Mancinelli, 2001]. Recently, the upper temperature limit for life has been extended to 121°C by the isolation of an archaeal iron-reducing strain able to grow at temperatures between 85 and 121°C [Kashefi & Lovley, 2003].

Biotopes for hyperthermophilic organisms are for example water-containing volcanic areas like terrestrial solfataric fields and hot springs, shallow submarine hydrothermal systems and abyssal hot vent systems, the so-called "black smokers". Man-made high-temperature biotopes are for example smouldering coal refuse piles and hot outflows from geothermal power plants [Stetter, 1996], [Stetter, 1999]. Due to the low solubility of oxygen at high temperatures and the presence of reducing gases, most biotopes of hyperthermophiles are anaerobic [Stetter *et al.*, 1990].

Most hyperthermophiles that have been isolated and studied so far belong to the archaeal domain, which is the third domain of life besides

Bacteria and Eukarya. The Archaea consist of two major kingdoms, *i.e.* the Crenarchaeota and the Euryarchaeota, both of which contain many hyperthermophiles [Woese *et al*., 1990]. Also in the bacterial domain some genera of hyperthermophiles can be found, *e.g. Thermotoga* and *Aquifex*. Hyperthermophiles can be found in the deepest and shortest lineages of the universal phylogenetic tree (Figure 1) and they are considered to be closely related to the designated "last common ancestor" of all life on this planet. Therefore, knowledge about the metabolism of hyperthermophiles may give insight into the evolution of metabolic pathways. Furthermore, comparison of metabolic pathways of hyperthermophiles to those of their mesophilic counterparts may provide information about possible mechanisms for adaptation to the extreme environment these organisms live in.

Equally diverse as the environments in which hyperthermophiles have been found are the variations in metabolism present in these organisms. Different types of lithotrophic energy metabolism have been described, like reduction of elemental sulphur, sulphate reduction, methanogenesis, aerobic

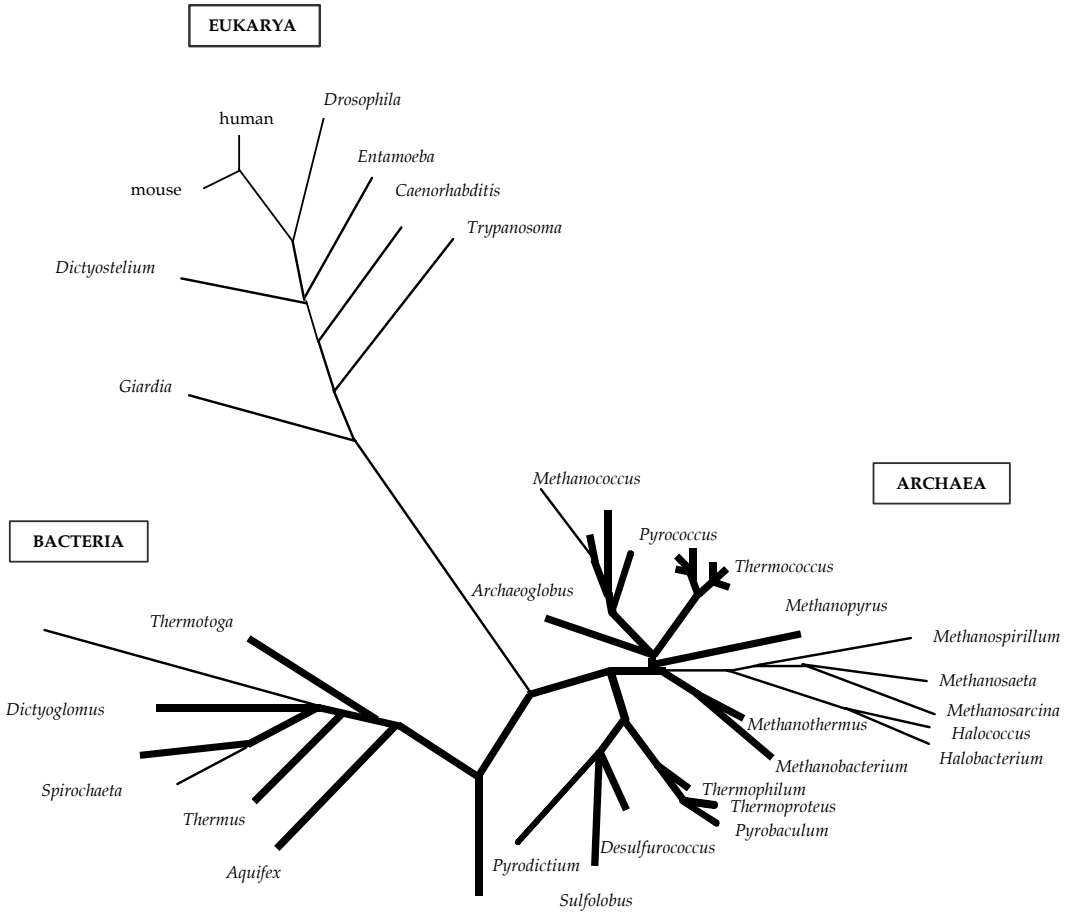

FIGURE 1 Universal tree of life, based on phylogenetic analysis of 16S/18S rRNA sequences (modified from [Woese *et al.*, 1990], [Stetter, 1996]). Thick lines represent (hyper)thermophilic lineages, thin lines represent mesophilic lineages.

respiration and nitrate reduction [Schönheit & Schäfer, 1995]. It has been suggested that iron(III) reduction was an important process on early Earth, since many hyperthermophiles that are closely related to the last common ancestor are able to reduce iron [Vargas *et al*., 1998], [Kashefi & Lovley, 2003]. Furthermore, also organotrophic metabolism can occur in hyperthermophiles, both with (aerobic or anaerobic respiration) and without (fermentation) external electron acceptors [Schönheit & Schäfer, 1995].

Sugar metabolism in hyperthermophiles

A number of hyperthermophiles have the ability to utilise sugars as a source for carbon and energy. Many of them show modifications of the classical sugar degradation pathways, *i.e.* the Entner-Doudoroff pathway and the Embden-Meyerhof pathway. Entner-Doudoroff-like pathways show two types of modifications. In some organisms 2-keto-3-deoxy-gluconate is phosphorylated to 2-keto-3-deoxy-6-phosphogluconate, whereas in other organisms none of the hexose intermediates is phosphorylated [Verhees *et al*., 2003]. In Figure 2 an overview is given of the modifications in Embden-Meyerhof-like pathways that have been found in hyperthermophilic Archaea and Bacteria. These modifications concern the hexo- or glucokinase, that can be ATP- or ADP-dependent, the phosphofructokinase, that can be ATP-, ADP-, or PP_i-dependent, and the step(s) between glyceraldehyde-3-phosphate and 3-phosphoglycerate, that can be catalysed by the conventional enzyme couple glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, by a modified versions of (one of) the enzymes, or by glyceraldehyde-3-phosphate ferredoxin oxidoreductase. While some of the investigated organisms show only one of the possible modifications, in others a combination of modified steps is present. Even two types of the same enzyme have been described, *i.e.* the ATP-dependent and the pyrophosphate-dependent phosphofructokinases in *Thermotoga maritima* [Ding *et al*., 2001] and the NAD-dependent and the NADP-dependent glyceraldehyde-3-phosphate dehydrogenases in *Thermoproteus tenax* [Brunner *et al*., 2001]. The various modifications in the pathways have been investigated in detail by several research groups during the past decade, as reviewed elsewhere [Schönheit & Schäfer, 1995], [Kengen *et al*., 1996], [de Vos *et al*., 1998], [Ronimus & Morgan, 2003], [Verhees *et al*., 2003].

Whereas in many organisms either (a modified form of) the Embden-Meyerhof or the Entner-Doudoroff pathway is present, some organisms use

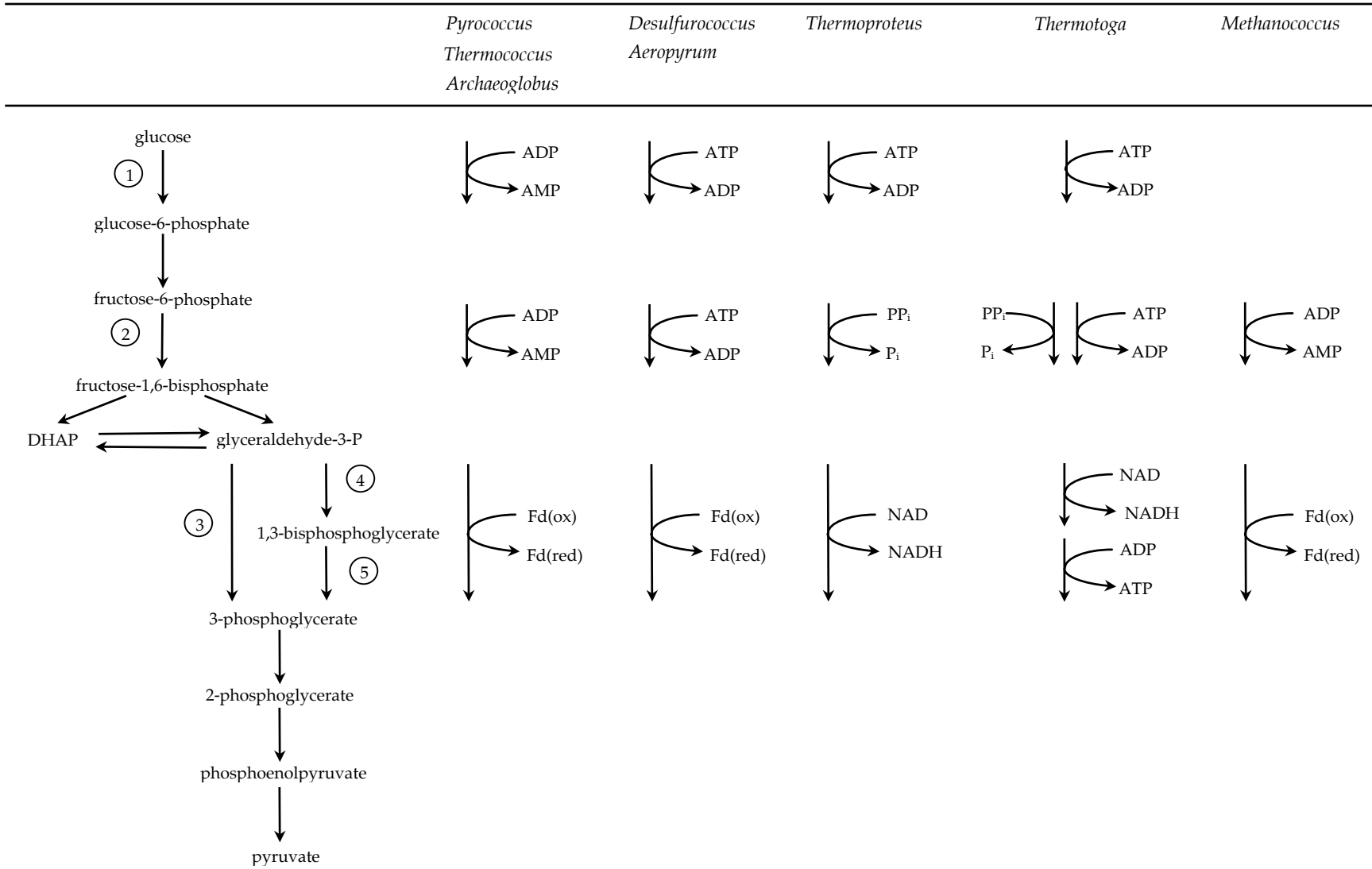

FIGURE 2 Comparison of Embden-Meyerhof-like glycolytic pathways in hyperthermophilic Archaea and Bacteria. The enzymes indicated are: 1. hexo-/glucokinase; 2. phosphofructokinase; 3. glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR); 4. glyceraldehyde-3-phosphate dehydrogenase; and 5. phosphoglycerate kinase. Modified from [Selig *et al.*, 1997] and [Verhees *et al.*, 2003].

both pathways. The hyperthermophilic Archaeon *Thermoproteus tenax* uses both a modified Embden-Meyerhof pathway and a nonphosphorylated Entner-Doudoroff pathway for sugar degradation [Siebers & Hensel, 1993]. Also in the hyperthermophilic bacterium *Thermotoga maritima* two sugar degradation pathways are present. However, in this organism both the classical Embden-Meyerhof and the classical Entner-Doudoroff pathway are present, without any modifications [Selig *et al*., 1997].

Sugar metabolism of *Pyrococcus furiosus* – a historic overview

The hyperthermophilic Archaeon *Pyrococcus furiosus* (Figure 3) was isolated from geothermally heated marine sediments from Vulcano Island in Italy [Fiala & Stetter, 1986] and has since then become one of the most intensively studied hyperthermophilic Archaea. The organism is strictly anaerobic, it has an optimal growth temperature of 100°C and it can use polysaccharides (*e.g.* starch and glycogen), oligosaccharides and disaccharides (*e.g.* cellobiose and maltose) as carbon and energy source. Furthermore, it can grow on pyruvate, peptone and tryptone. The first indication of an unusual sugar metabolism in *P. furiosus* was the discovery of a novel, tungsten-containing aldehyde oxidoreductase. Based on the presence of this enzyme and on the observation that glucose is directly oxidised to gluconate without being phosphorylated first, it was proposed that *P. furiosus* uses a non-phosphorylated variant of the Entner-Doudoroff pathway, named "pyroglycolysis" [Mukund & Adams, 1991]. The apparent absence of activity of the Embden-Meyerhof key enzymes hexokinase and

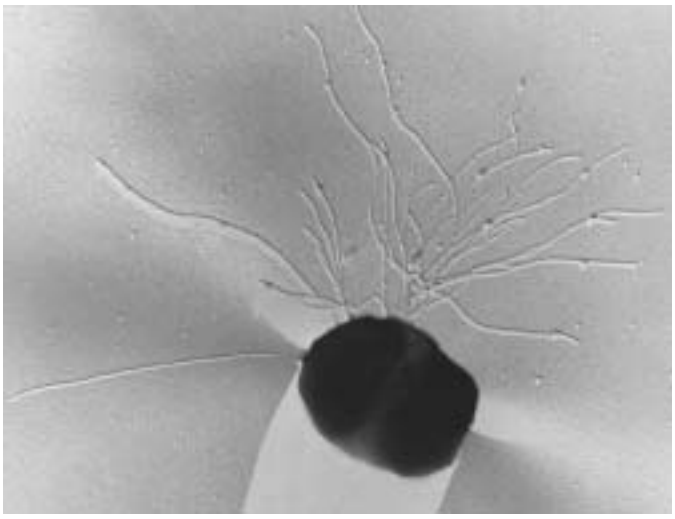

FIGURE 3 Electron microscope picture of *Pyrococcus furiosus*. Picture was taken from www.microbeworld.org.

phosphofructokinase in cell-free extracts of *P. furiosus* [Schäfer & Schönheit, 1992] supported this hypothesis.

However, *in vivo* NMR studies revealed a labelling pattern indicative of the Embden-Meyerhof pathway. When [1-^{13}C]glucose was added to cell suspensions of *P. furiosus*, the label ended up in the C_2 atom of acetate and the C_3 atom of alanine, whereas addition of [3-^{13}C]glucose resulted in the formation of C_1-labelled alanine and labelled HCO_3^- and CO_2 [Kengen *et al.*, 1994]. Furthermore, it was shown that the seemingly absent sugar kinases were in fact present and active in cell-free extracts, but they used ADP instead of ATP as phosphoryl group donor, which was the reason that their activity had not been detected before. This was the first description of ADP-dependent kinases at that time [Kengen *et al.*, 1994]. Thus, the sugar degradation pathway of *P. furiosus* appeared to be a modified version of the Embden-Meyerhof pathway and the so-called pyroglycolysis does not exist. Since then, the glycolytic pathway in *P. furiosus* has been referred to as the modified Embden-Meyerhof pathway (Figure 4).

Shortly after the description of the ADP-dependent kinases, another modification was found in the second half of the pathway. A novel tungsten-containing enzyme, *i.e.* glyceraldehyde-3-phosphate ferredoxin oxido-reductase (GAPOR), catalyses the oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate in one step, coupled to the reduction of ferredoxin [Mukund & Adams, 1995]. In the classical Embden-Meyerhof pathway, glyceraldehyde-3-phosphate is converted to 1,3-bisphosphoglycerate by glyceraldehyde phosphate dehydrogenase. The subsequent conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate, which is catalysed by phosphoglycerate kinase, yields energy in the form of one ATP. Apparently, in *P. furiosus* no energy is gained in this step.

To get more insight into the modified Embden-Meyerhof pathway and the enzymes that catalyse the different steps, purification and characterisation of the enzymes was initiated, starting with the novel ADP-dependent kinases. Meanwhile, sequencing of the total genome of *P. furiosus* was in progress. The results of the sequencing project were published on the internet [www.genome.utah.edu] and thereby became available for BLAST searches and sequence comparison. Using the genome data, genes encoding glycolytic enzymes could be identified and cloned in *Escherichia coli*, which made purification of the enzymes much easier. Because of their high thermostability, purification of overexpressed pyrococcal enzymes can be started with a heat incubation of the cell-free extract at ca. 80°C, thereby denaturating most of the *E. coli* proteins and leaving the

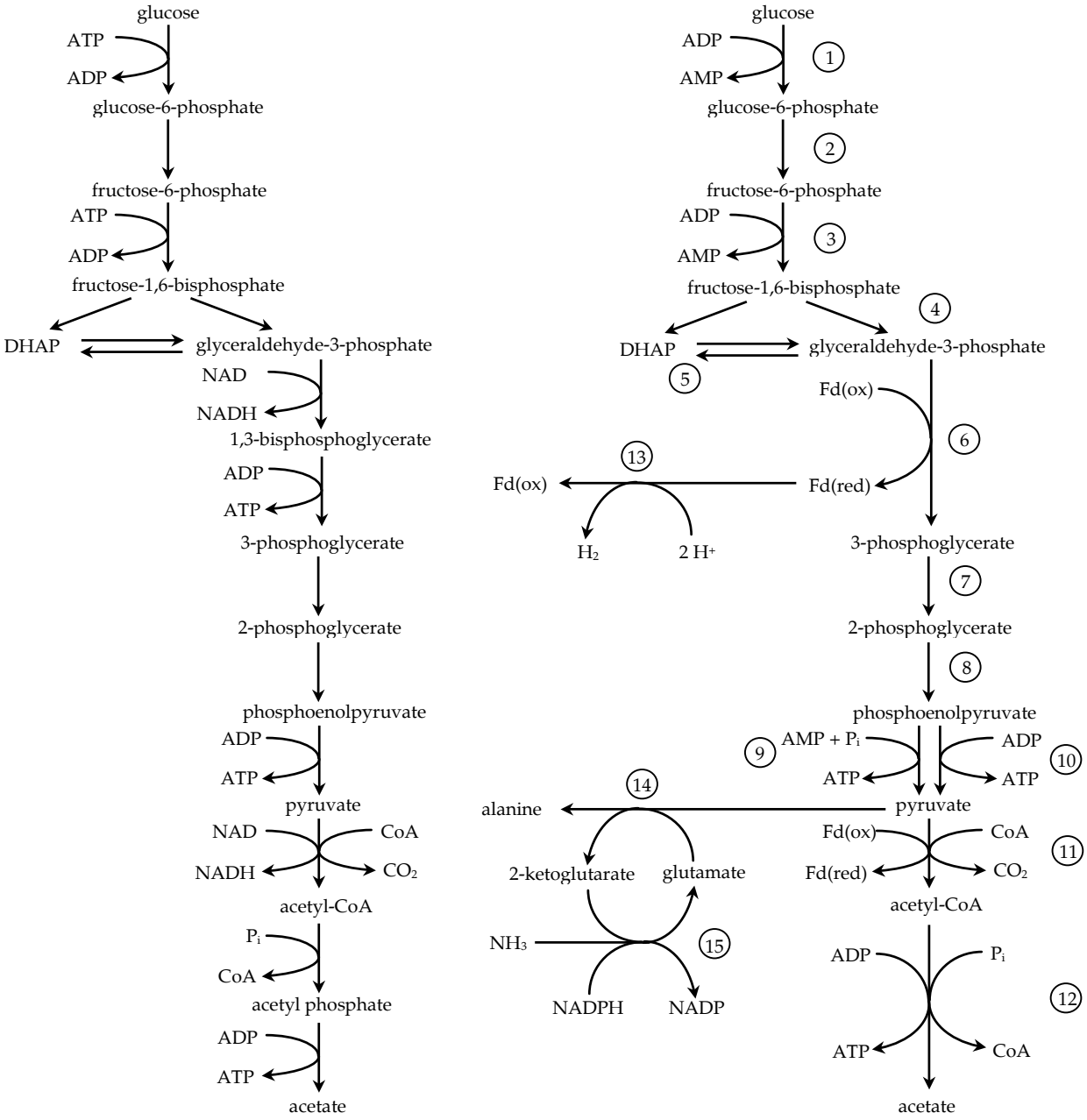

FIGURE 4 Classical Embden-Meyerhof pathway (left) and modified Embden-Meyerhof pathway of *Pyrococcus furiosus* (right). The enzymes involved in the latter pathway are: 1. ADP-dependent glucokinase [Kengen *et al*., 1995]; 2. phosphoglucose isomerase [Verhees *et al*., 2001a]; 3. ADP-dependent phosphofructokinase [Tuininga *et al*., 1999]; 4. fructose-1,6-bisphosphate aldolase [Siebers *et al*., 2001]; 5. triosephosphate isomerase [Kohlhoff *et al*., 1996] (characterised from *P. woesei*); 6. glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) [Mukund & Adams, 1995]; 7. phosphoglycerate mutase [van der Oost *et al*., 2002]; 8. enolase [Peak *et al*., 1994]; 9. PEP synthase [Sakuraba *et al*., 1999], [Hutchins *et al*., 2001], [Tuininga *et al*., 2003]; 10. pyruvate kinase [Tuininga *et al*., 2003]; 11. pyruvate ferredoxin oxidoreductase (POR) [Blamey & Adams, 1993]; 12. acetyl CoA synthetase [Mai & Adams, 1996], [Glasemacher *et al*., 1997]; 13. membrane-bound hydrogenase [Sapra *et al*., 2003]; 14. alanine aminotransferase [Ward *et al*., 2000] and 15. glutamate dehydrogenase [Robb *et al*., 1992].

P. furiosus enzymes unaffected. Typically, a subsequent purification scheme can consist of only one or two columns.

In this thesis, the purification and characterisation of the bioenergetically important enzymes from the modified Embden-Meyerhof pathway, *i.e.* glucokinase, phosphofructokinase, pyruvate kinase, and phosphoenolpyruvate (PEP) synthase, is described. Some other enzymes of this pathway, such as phosphoglucose isomerase, aldolase and pyruvate ferredoxin oxidoreductase (POR), were characterised by other researchers and described elsewhere.

Regulation of glycolysis in *Pyrococcus furiosus*

The regulation of the glycolytic pathway is a very complex process, that relies on the coordination of multiple events, at the level of both DNA (gene expression) and enzymes (for example allosteric regulation). Although many studies have been done on the regulation of glycolysis in mesophilic micro-organisms such as *E. coli* and *Saccharomyces cerevisiae*, not much is known about regulation of glycolysis in Archaea.

Classical control sites of glycolysis are the unidirectional conversions, especially the first irreversible step, which is catalysed by the phospho-fructokinase. This enzyme is usually allosterically controlled by fructose-6-phosphate and fructose-2,6-bisphosphate. The phosphofructokinase from *P. furiosus*, however, is not allosterically regulated and therefore not regarded as the major control point of glycolysis [Tuininga *et al*., 1999]. Also the pyrophosphate-dependent phosphofructokinase in the hyperthermophilic Archaeon *Thermoproteus tenax* is non-allosteric [Siebers *et al*., 1998]. The irreversible step of pyruvate kinase is another possible site of allosteric regulation. The pyruvate kinases of the hyperthermophilic Archaea *Archaeoglobus fulgidus*, *Aeropyrum pernix*, and *Pyrobaculum aerophilum* appeared to be non-allosteric, although the pyruvate kinase in the hyperthermophilic Bacterium *Thermotoga maritima* did show the classical allosteric responses [Johnsen *et al*., 2003]. Again, also the enzyme in *T. tenax* was shown to be non-allosteric [Schramm *et al*., 2000].

To investigate regulation of glycolysis at the level of DNA, transcriptional analyses have been done on a number of glycolytic genes from *P. furiosus*. From Northern blots, it was seen that transcription of the gene encoding the phosphoglucose isomerase was slightly higher in cells grown on maltose than in pyruvate-grown cells [Verhees *et al*., 2001a]. Transcription of the gene encoding the fructose-1,6-bisphosphate aldolase was much higher in

maltose-grown cells than in pyruvate-grown cells [Siebers *et al*., 2001]. Also transcription of the GAPOR-encoding gene was increased by growth on cellobiose, whereas the expression of the gene encoding the gluconeogenic glyceraldehyde-3-phosphate dehydrogenase was independent of the carbon source used for growth of *P. furiosus* [van der Oost *et al*., 1998]. These results all suggest that the expression of the glycolytic genes is induced by growth on sugars.

Recent analysis of the whole-genome DNA microarray of *P. furiosus* confirmed that the genes encoding the glucose-6-phosphate isomerase, phosphofructokinase, and GAPOR are strongly up-regulated in maltose-grown cells compared to peptide-grown cells. Remarkably, the gene encoding the glucokinase is not regulated [Schut *et al*., 2003]. Taken together, the above-mentioned experimental data suggest that the mechanism of regulation for the glycolysis of *P. furiosus* is mainly at the transcriptional level.

Sugar transport in *Pyrococcus furiosus*

Before sugars can be metabolised through the modified Embden-Meyerhof pathway, they need to be taken up from the environment. In mesophilic bacteria, three classes of sugar transporters have been described. By secondary transport, the sugar is transported in symport with protons or sodium ions. Although secondary transporters are abundantly present in Archaea, they are involved in the uptake of anorganic substrates rather than sugars [Koning *et al*., 2002a]. The second class of transporters consists of PEP-dependent phosphotransferase systems (PTS). These systems, in which the sugar is phosphorylated upon transport driven by the energy-rich phosphate bond in PEP, have not been detected in biochemical studies and analysis of genome sequences of Archaea [Koning *et al*., 2002a]. Thus, sugar transport in Archaea seems to be mediated by the third class of sugar transporters, *i.e.* ATP-binding cassette (ABC)-type transporters, that contain a binding protein attached to the cytoplasmic membrane. The sugar is bound to this protein, transferred to the transporter domain in the membrane and subsequently taken up at the expense of ATP [Koning *et al*., 2002a].

In *P. furiosus*, uptake of α- and β-glucosides is done by separate transport systems. Transport of cellobiose is mediated by an ABC transporter with a high affinity (K_m = 175 nM) for cellobiose. Besides cellobiose the sugar binding protein of this transporter binds most other β-glucosides, like cellotriose, cellotetraose, cellopentaose, laminaribiose, and laminaritriose and

was therefore classified as a broad-specificity β-glucoside binding protein [Koning *et al*., 2001]. For transport of α-glucosides, *P. furiosus* has two different ABC-type transport systems. The maltose transporter also transports trehalose, whereas the maltodextrin transport system mediates the uptake of maltotriose and higher malto-oligosaccharides, but not maltose [Koning *et al*., 2002b].

Thus, sugar uptake in *P. furiosus* is an energy-requiring process, although it is not known yet how much ATP is needed for uptake of the sugars. It can be estimated that, in analogy with ABC-transporters in *E. coli*, approximately 1 ATP is needed per molecule of disaccharide transported. The uptake mechanism of pyruvate has not been identified yet, nor is known whether this uptake requires ATP as well. However, the observation that the growth rate of *P. furiosus* on pyruvate is concentration-dependent [S.W.M. Kengen, unpublished results] suggests that the transport of pyruvate is a passive process.

Bioenergetics of *Pyrococcus furiosus*

Some of the modifications in the sugar metabolism of *P. furiosus* have consequences for the bioenergetics of the pathway. The presence of the ADP-dependent kinases in the first steps of the pathway does not give rise to a change in the bioenergetics, because ATP and ADP have the same Gibbs free energy of hydrolysis of -31.8 kJ/mol under standard conditions [Thauer *et al*., 1977]. Whether this amount of energy is exactly the same at high temperature remains to be established, but it can be assumed that the free energy of hydrolysis of both phosphate bonds in ATP is equal at all temperatures. Furthermore, an adenylate kinase is present in *P. furiosus* [Schäfer & Schönheit, 1991] that catalyses the reversible conversion of AMP and ATP into 2 ADP (reaction 1). Together with the reaction catalysed by one of the kinases (reaction 2), the net reaction is the same as that catalysed by ATP-dependent kinases (reaction 3).

$$\text{ATP} + \text{AMP} \leftrightarrow 2\ \text{ADP} \quad (1)$$

$$\text{glucose} + \text{ADP} \rightarrow \text{glucose-6-phosphate} + \text{AMP} \quad (2)$$

$$\text{glucose} + \text{ATP}\ (+\ \text{AMP}) \rightarrow \text{glucose-6-phosphate} + \text{ADP}\ (+\ \text{AMP}) \quad (3)$$

A large difference, however, can be found at the level of glyceraldehyde-3-phosphate. In the classical Embden-Meyerhof pathway one ATP is produced at this level, yielding a total of 2 ATP molecules produced per molecule of glucose converted. Since in *P. furiosus* glyceraldehyde-3-phosphate is oxidised by GAPOR without substrate-level phosphorylation [Mukund & Adams, 1995], the only energy-yielding step in the modified glycolysis is the conversion of acetyl-CoA to acetate. Thus, no net ATP seems to be produced between glucose and pyruvate. As a consequence, Y_{ATP} values for growth on sugars and pyruvate should be in the same range. For growth in batch cultures, however, this was not the case. It appeared that the Y_{ATP} on pyruvate was 9 - 10 g/mol whereas it was 18 - 22 g/mol for disaccharides, which indicates that an additional energy-conserving site may be present in the pathway [Kengen & Stams, 1994b].

A possible site for this extra ATP formation is the conversion of phosphoenolpyruvate (PEP) to pyruvate. In the classical Embden-Meyerhof pathway, the virtually irreversible transfer of a phosphoryl group from PEP to ADP is catalysed by pyruvate kinase. Recently it was suggested that the PEP synthase, that catalyses the reversible conversion of PEP, AMP and phosphate into pyruvate and ATP, might be active in glycolytic direction [Sakuraba *et al*., 1999]. In that case, ATP would be formed from AMP in one step, which would increase the overall ATP yield of the pathway. Whether the above-mentioned hypothesis is correct is still a matter of debate (see Chapter 5).

The extra energy-conserving site might very well be not found in the glycolytic pathway itself. Recently an anaerobic respiratory system has been described for *P. furiosus* in which a membrane-bound hydrogenase reduces protons to hydrogen by using electrons from reduced ferredoxin that is produced in the glycolytic pathway. The production of hydrogen gas is directly coupled to the synthesis of ATP by means of a proton motive force. Thus, the free energy released in the oxidation of glyceraldehyde-3-phosphate is not lost as heat, but conserved in the proton motive force. It was calculated that by means of this energy-conserving system, 1.2 extra mol of ATP can be formed per mol of glucose utilised [Sapra *et al*., 2003], which would be enough to explain the differences in growth yields as observed before [Kengen & Stams, 1994b].

Adenylate energy charge

An important aspect in the bioenergetics of an organism is the adenylate energy charge (AEC), which is a widely used measure of the

available energy stored in the adenine nucleotide pool. After measurement of the intracellular concentrations of ATP, ADP, and AMP the adenylate energy charge can be calculated using equation 4:

$$AEC = \frac{[ATP] + 0.5 * [ADP]}{[ATP] + [ADP] + [AMP]} \quad (4)$$

The energy charge has been proposed as a metabolic regulatory parameter, that is important in the regulation of metabolic pathways such as glycolysis. Generally, the rate of ATP-regenerating enzymes decreases at a higher energy charge, whereas the rate of ATP-utilising enzymes increases. Since the glycolytic pathway is an amphibolic sequence, having a function both in ATP regeneration and in supply of biosynthetic intermediates, the energy charge regulation of the pathway needs to be supplemented by feedback modulation [Atkinson, 1968].

It has been predicted that the energy charge *in vivo* must be stabilised in the range of 0.8 - 0.95 during growth and normal metabolism, and this prediction has been verified in many different organisms and tissues [Chapman & Atkinson, 1977]. Nevertheless, also lower values for the energy charge in growing cells have been determined. In a study comparing different extraction methods, the energy charge of *E. coli* was determined to be 0.59, whereas those of the other tested organisms, *i.e. Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were within the range of 0.80 - 0.97 [Lundin & Thore, 1975]. In cultures of *Salmonella enteritidis* and *S. typhimurium* energy charge values around 0.66 were found [Walker *et al*., 1998]. It was also found that during starvation of microbial cells, the energy charge drops to values around 0.5 in *E. coli* [Chapman *et al*., 1971] and around 0.4 in anaerobically grown *S. cerevisiae* [Ball & Atkinson, 1975]. Furthermore, it has been shown that several bacteria, *i.e. E. coli*, *P. aeruginosa*, and *Streptococcus lactis* are able to survive extreme reduction in energy charge values to as low as 0.05 without loss of viability [Barrette Jr. *et al*., 1988].

After the discovery of the ADP-dependent sugar kinases in *P. furiosus* and other hyperthermophiles, several hypotheses were raised to explain the presence of these novel kinases. One of the suggested explanations was the supposedly higher thermostability of ADP compared to ATP [Kengen *et al*., 1994]. But although ATP indeed is less thermostabile, the half-lives of both ADP and ATP at 90°C are much higher than those of several other intermediates of the Embden-Meyerhof pathway [Dörr *et al*., 2003]. Alternatively, it was suggested that the existence of ADP-dependent kinases

and the remarkably high affinity for ATP of the galactokinase of *P. furiosus* might reflect a low intracellular concentration of ATP [Verhees *et al*., 2002]. This could not be confirmed since the intracellular concentrations of the adenine nucleotides and the adenylate energy charge had not yet been determined in *P. furiosus* or any other hyperthermophilic micro-organism. However, in a comparative study of energy charge values from the moderately thermophilic *Bacillus stearothermophilus* and its mesophilic counterpart *B. subtilis*, it was shown that the energy charge as well as the ATP content were lower in the thermophile than in the mesophile and it was suggested that these values may reflect a decreased activation energy required for metabolic coupling at higher growth temperatures [Webster *et al*., 1988].

Growth yield studies of hyperthermophiles

A means to investigate the bioenergetics of an organism is growth yield studies, in which basically the amount of biomass formed from a certain amount of substrate is calculated. However, in batch cultures the conditions continuously change, *e.g.* the substrate concentration decreases whereas the concentration of possibly inhibitory products increases. Therefore, continuous culture or chemostat culture is a more useful technique for growth yield studies. By definition, in continuous culture the circumstances are kept constant by feeding medium to the culture at a constant rate and by harvesting the culture at the same rate so that the culture volume remains constant. Ideally the mixing should be perfect, *i.e.* a drop of medium entering the vessel should instantly be distributed uniformly throughout the culture. When the dilution rate D (vol/vol/h or h^{-1}) is lower than the maximum growth rate of the organism μ_{max} (h^{-1}), the biomass concentration will initially increase untill the amount of substrate becomes limiting. At that point, the biomass growth rate equals the wash out rate and the specific growth rate of the organism is determined by the medium flow rate ($\mu = D$). This situation is called steady state [Pirt, 1975].

From a chemostat culture, many growth parameters of an organism can be obtained. The maximum growth rate μ_{max} can be determined by increasing the dilution rate D to a value higher than μ_{max} and measuring the biomass concentration x in time. Using equation 5:

$$\ln x = (\mu_{max} - D)t + \ln x_0 \qquad (5)$$

the slope of a logarithmic plot of x against t is (μ_{max} – D) from which μ_{max} (h^{-1}) can be calculated [Pirt, 1975].

From the biomass concentration x and the substrate concentration in the incoming medium s, the growth yield Y can be calculated as Y = x / s (g dry weight per mol substrate). For interpretation of growth yield data it is important to know that the energy that is consumed by the culture is partly used for growth and partly for maintenance purposes, like turnover of cell material, osmotic work and cell motility. Therefore, the maximum growth yield Y_{max} is defined as the growth yield in the case that the maintenance coefficient m is zero. To calculate the maximum growth yield and the maintenance coefficient, continuous cultures should be operated at several dilution rates. After calculation of growth yields for each dilution rate, a plot of 1/Y against 1/μ will be a straight line with slope m and intercept $1/Y_{max}$ (Figure 5A) [Pirt, 1975]. However, inaccuracies in the measurement of biomass concentration and substrate consumption rate are more pronounced at low dilution rates and with a double reciprocal plot heavy emphasis is placed on these data [Tempest & Neijssel, 1984]. Therefore, an alternative plot of the metabolic quotient q (calculated as μ/Y or D/Y) against μ (or D) is more appropiate (Figure 5B). In such a plot, the slope will be $1/Y_{max}$ and the intercept will be m [Pirt, 1975].

When the fermentation pattern of an organism is known, *i.e.* when the amount of ATP from a substrate is known, the yield value can be expressed in g dry weight per mol ATP (Y_{ATP}). Initially, for three fermentative micro-organisms, *i.e. Streptococcus faecalis*, *Pseudomonas lindneri* and *Saccharomyces cerevisiae*, the Y_{ATP} was determined on several substrates. The mean value of the Y_{ATP} was 10.5 (ranging from 8.3 to 12.6) [Bauchop & Elsden, 1960], which has since then dogmatically become the average Y_{ATP} for fermentative micro-

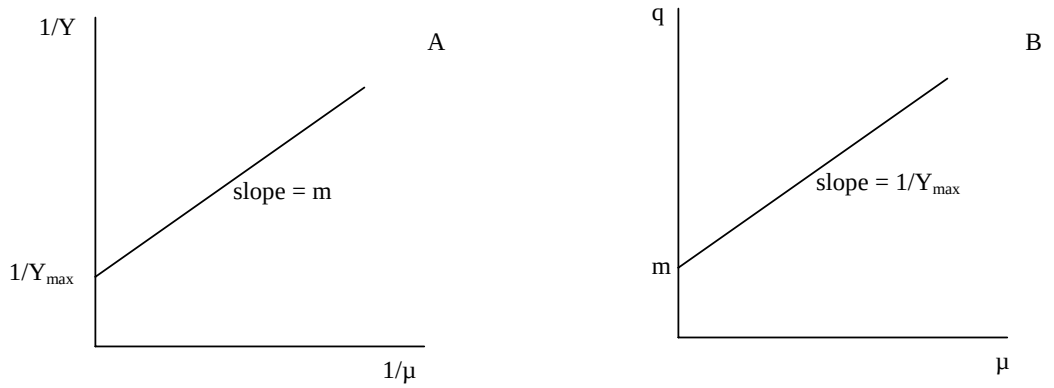

FIGURE 5 Graphical methods for the calculation of the maintenance coefficient (m) and the maximum growth yield (Y_{EG}) from plots of 1/Y against 1/μ (A) and q against μ (B). Taken from [Pirt, 1975].

organisms and has been treated as a biological constant [Russell & Cook, 1995]. However, from many micro-organisms Y_{ATP} values were determined that were more or less deviant from the so-called average value [Stouthamer, 1969], [Tempest & Neijssel, 1984].

Cultivation of hyperthermophilic organisms in chemostat culture requires some technical modifications of the equipment normally used for cultivation of mesophiles. For example, some anaerobic hyperthermophiles produce high levels of reduced sulphur compounds that can damage the fermentor [Rinker *et al.*, 1999]. On the other hand, advantages of cultivation at high temperature are the fact that many reaction components, especially polymeric substrates, are more soluble at elevated temperature and the reduced risk of contamination of the culture with airborne organisms [van den Burg, 2003], [Rinker *et al.*, 1999]. Nevertheless, various hyperthermophilic micro-organisms have been studied in chemostat culture, *e.g. Pyrococcus furiosus* [Brown & Kelly, 1989], [Raven *et al.*, 1992], [Schicho *et al.*, 1993], *P. abyssi* [Godfroy *et al.*, 2000], *Thermococcus litoralis* [Rinker *et al.*, 1999], [Pysz *et al.*, 2001], *Metallosphaera sedula* [Rinker *et al.*, 1999], *Thermotoga maritima* [Rinker *et al.*, 1999], [Pysz *et al.*, 2001], and *Methanocaldococcus jannaschii* [Pysz *et al.*, 2001].

Several chemostat culture studies have been done with *P. furiosus*. The specific production rate of both H_2S and CO_2 appeared to increase linearly with an increased dilution rate from 0.1 to 0.6 h^{-1}. Above this range, the increase in gas production rate became even higher [Brown & Kelly, 1989]. Steady-state cell densities were found to increase with higher inert gas flow rates, reaching a maximum with 0.5 vol/vol/min of nitrogen [Raven *et al.*, 1992]. When growth of *P. furiosus* in the presence of sulphur was compared to that without sulphur, using maltose as the carbon source, the maximal yield coefficient appeared to be higher with than without sulphur, which indicates that sulphur reduction is an energy-conserving reaction. Furthermore, the maintenance coefficients were found to be not significantly different from those determined for mesophilic organisms, indicating that growth at elevated temperature does not necessarily require more maintenance energy [Schicho *et al.*, 1993]. Although in batch cultures of *P. furiosus* growth yields on sugars were compared to those on pyruvate [Kengen & Stams, 1994b], these studies had not been repeated in continuous culture.

Outline of the thesis

The research described in this thesis was based on the first description of the ADP-dependent kinases in *P. furiosus*, and later formed part of the ALW-NWO-project "Metabolic pathways for glycosides in the hyperthermophile *Pyrococcus furiosus*" that aimed to study novel metabolic processes in *P. furiosus* by unravelling the catabolism of glycosides, focusing on the analysis of the enzymology, kinetics, bioenergetics, and genetics of key proteins involved in uptake, metabolism, and excretion of glycosides. The research was done by three Ph.D. students at the Rijksuniversiteit Groningen and Wageningen University. This thesis deals with the enzymological and bioenergetical aspects of the sugar metabolism.

In **Chapter 2** the purification and characterisation of the ADP-dependent glucokinase, that catalyses the first step of the glycolysis, is described. It concerns the first characterisation of an ADP-dependent kinase.

Using the N-terminal amino acid sequence of the glucokinase, the gene encoding this enzyme and also the gene encoding the ADP-dependent phosphofructokinase was identified in the genome sequence of *P. furiosus*. The latter gene was expressed in *E. coli* and the protein was purified and characterised, as described in **Chapter 3**.

Following the identification of the phosphofructokinase gene in *P. furiosus*, high similarity to a gene in the hyperthermophilic methanogen *Methanocaldococcus jannaschii* was found. In **Chapter 4**, the expression of this gene in *E. coli* and the subsequent purification and characterisation of the ADP-dependent phosphofructokinase of *M. jannaschii* is described, together with the results of a screening of a number of methanogenic micro-organisms for genes and activity of ADP-dependent phosphofructokinase.

In **Chapter 5** the two enzymes that are involved in the interconversion of phosphoenolpyruvate and pyruvate in the last step of the glycolysis, namely pyruvate kinase and PEP synthase were studied. This was done to investigate the previously done suggestion that the PEP synthase of *P. furiosus* could be active in glycolytic direction, thereby increasing the energy yield of the pathway.

Chapter 6 deals with the bioenergetic studies that were done with *P. furiosus* in continuous culture on cellobiose and pyruvate and the determination of the adenylate energy charge in steady-state cells grown on cellobiose.

In **Chapter 7** the results of the thesis are summarised, followed by a layman's version of the summary in Dutch (**Chapter 8**).

2

Purification and characterisation of a novel ADP-dependent glucokinase from the hyperthermophilic Archaeon *Pyrococcus furiosus*

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SUMMARY

Pyrococcus furiosus uses a modified Embden-Meyerhof pathway during growth on poly- or disaccharides. Instead of the usual ATP-dependent glucokinase, this pathway involves a novel ADP-dependent (AMP-forming) glucokinase. The level of this enzyme and some other glycolytic enzymes appeared to be closely regulated by the substrate. Growth on cellobiose resulted in a high specific activity of 0.96 U/mg, whereas on pyruvate a 10-fold lower activity was found. The ADP-dependent glucokinase was purified 1350-fold to homogeneity. The oxygen-stable enzyme had a molecular mass of 93 kDa and was composed of two identical subunits (47 kDa). The glucokinase was highly specific for ADP, which could not be replaced by ATP, phosphoenolpyruvate, GDP, PP_i, or polyphosphate. D-Glucose could be replaced only by 2-deoxy-D-glucose, albeit with a low efficiency. The K_m values for D-glucose and ADP were 0.73 and 0.033 mM, respectively. An optimum temperature of 105°C and a half-life of 220 min at 100°C are in agreement with the requirements of this hyperthermophilic organism. The properties of the glucokinase are compared to those of less thermoactive gluco-/hexokinases.

INTRODUCTION

During the past decade, an increasing number of micro-organisms have been described that have their optimum growth temperature above 80°C [Stetter *et al*., 1990], [Blöchl *et al*., 1995]. Except for two bacterial genera, all of the more than 50 hyperthermophilic species isolated thus far are classified as Archaea (formerly Archaebacteria), the third domain of life [Woese *et al*., 1990].

Because of its favorable culturing conditions, *Pyrococcus furiosus* is the best-studied anaerobic hyperthermophile to date. Next to some polypeptides and polysaccharides, *P. furiosus* is able to use maltose and cellobiose as simple substrates [Fiala & Stetter, 1986], [Schäfer & Schönheit, 1992], [Kengen *et al*., 1993]. These disaccharides are transported into the cell, hydrolysed to glucose, and fermented to mainly acetate, alanine, H_2, and CO_2 [Kengen & Stams, 1994b]. Initially, *P. furiosus* was believed to use a novel non-phosphorylated type of Entner-Doudoroff pathway, called pyroglycolysis [Schäfer & Schönheit, 1992], [Mukund & Adams, 1991]. However, recent ^{13}C *in vivo* NMR data were not consistent with a major role for the pyroglycolysis [Kengen *et al*., 1994], [Schäfer *et al*., 1994]. The ^{13}C labelling pattern suggested that an Embden-Meyerhof-like pathway was most likely to be involved [Kengen *et al*., 1994]. Conventional glucokinase and phosphofructokinase could, however, not be detected in cell-free extracts [Schäfer & Schönheit, 1992]. Remarkably, two novel sugar kinases were recently discovered that required ADP instead of ATP [Kengen *et al*., 1994]. In contrast to the key enzymes of the pyroglycolysis, the specific activities of both kinases were sufficiently high to envisage a major catabolic role. Instead of a classical NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, *P. furiosus* was recently shown to harbour a novel tungsten-containing glyceraldehyde-3-phosphate ferredoxin oxidoreductase [Mukund & Adams, 1995]. The presence of an enzyme that converts glyceraldehyde-3-phosphate instead of glyceraldehyde also substantiates the operation of a modified Embden-Meyerhof pathway instead of the pyroglycolysis. The discovery of the novel type of kinases and the tungsten proteins in *P. furiosus* supports the idea that life at elevated temperatures may involve different metabolic steps as a result of an altered biochemistry or a decreased stability of biomolecules.

In this paper, we describe the purification and characterisation of the novel ADP-dependent glucokinase. The results show that ADP-dependent conversion of glucose is not only found in crude *P. furiosus* extracts but is

catalysed by a single enzyme that shows a characteristic specificity. The properties of the *Pyrococcus* enzyme are compared to those of glucokinases from less thermophilic sources.

MATERIALS AND METHODS

Materials

ADP (monopotassium salt, less than 0.2% ATP), ATP (disodium salt), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxido-reductase, EC 1.1.1.49; yeast), phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9; yeast), and phosphomannose isomerase (D-mannose-6-phosphate ketol-isomerase, EC 5.3.1.8; yeast) were obtained from Boehringer GmbH (Mannheim, Germany). Fructose-6-phosphate, D-galactose, 2-deoxy-D-glucose, sodium phosphate glass (type 35), and adenosine-5-diphosphate-agaroses were from Sigma Chemie (Bornem, Belgium). D-Glucose, D-fructose, and D-mannose were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Phenyl Sepharose CL-4B, Mono Q HR 5/5, and Phenyl Superose HR 5/5 were purchased from Pharmacia LKB Biotechnology (Woerden, The Netherlands). Hydroxyapatite Bio-Gel HT and the Prep-Gel system were from Bio-Rad (Veenendaal, The Netherlands). Gasses were supplied by Hoek-Loos (Schiedam, The Netherlands). *P. furiosus* (DSM 3638) was obtained from the German Collection of Micro-organisms (Braunschweig, Germany).

Growth of organism

P. furiosus was routinely grown at 90°C on an artificial seawater medium, supplemented with tungsten (10 µM Na_2WO_4), yeast extract (1 g/litre), and vitamins, as described before [Kengen *et al.*, 1993]. Routine culturing was performed in stoppered 120-ml serum bottles, containing 50 ml of medium and pressurised with 150 kPa N_2/CO_2 (80:20). Starch (5 g/litre), maltose (10 mM), cellobiose (5 mM), pyruvate (40 mM), or peptone (5 g/litre) were used as substrates. For the preparation of cell extracts, cultures were subcultured at least five times (1% inoculum) on the substrate of interest prior to extraction.

Mass culturing (200 litres) was performed on the same medium except that Na_2S was omitted, the fermentor was sparged with N_2, and potato starch was used as substrate (5 g/litre).

Preparation of cell-free extracts

To determine the effect of the substrate on enzyme levels, cell extracts were prepared aerobically from the 50-ml cultures. The contents of each bottle were centrifuged for 20 min at 22,800 × g. The supernatant was discarded and the cell pellet was resuspended in 1 ml of distilled water. The cell suspension was sonicated three times for 30 s. Cell debris was removed by centrifugation, and the supernatant was used as cell-free extract. For use in enzyme purifications, cells and cell extracts were handled aerobically. Cells were suspended in 50 mM Tris/HCl buffer (pH 7.8) (0.5 g of cells/ml of buffer) containing DNase (10 µg/ml), and the suspension was passed twice through a French press at 138 MPa. Cell debris were removed by centrifugation for 1 h at 100,000 × g. The cell extract, containing 35 - 45 mg protein/ml, was stored at -20°C until use.

Protein was determined with Coomassie Brilliant Blue G250 as described by Bradford [Bradford, 1976], using bovine serum albumin as a standard. Occasionally, a modified more sensitive Coomassie Brilliant Blue G250 method was used as described before [Löffler & Kunze, 1989].

Determination of enzyme activity

The enzyme assays were performed aerobically in stoppered 1-ml quartz cuvettes as described before [Kengen *et al.*, 1994]. Specific enzyme activities were calculated from initial rates and expressed in U/mg protein. 1 U (unit) was defined as that amount of enzyme required to convert 1 µmol of glucose per min.

ADP-dependent glucokinase was determined by measuring the formation of NADPH in a coupled assay with yeast glucose-6-phosphate dehydrogenase. The assay was performed at 50°C. At this temperature, the yeast enzyme remained active, and the *Pyrococcus* enzyme was sufficiently active to measure its activity. The assay mixture contained 100 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 0.5 mM NADP, 15 mM D-glucose, 2 mM ADP, 0.35 U of D-glucose-6-phosphate dehydrogenase, and 5 - 50 µl of enzyme preparation. The absorbance of NADPH was followed at 334 nm (ε = 6.18

$mM^{-1}cm^{-1}$). Care was taken that the activity of the auxiliary enzyme was always in excess of the glucokinase activity.

Phosphoglucose isomerase (EC 5.3.1.9) and ADP-dependent phosphofructokinase were determined at 50°C using auxiliary enzymes as described before [Kengen *et al.*, 1994].

Substrate specificity

The substrate specificity was tested using purified glucokinase. The use of 2-deoxy-D-glucose and D-galactose as possible substrates for the glucokinase was tested using the standard enzyme assay because the auxiliary enzyme from yeast is also able to use galactose-6-phosphate. For the determination of D-fructose as a possible substrate, phosphoglucose isomerase (1.4 U) was added to the standard assay mixture. D-Mannose was tested by adding phosphomannose isomerase (0.6 U) and phosphoglucose isomerase (1.4 U) as auxiliary enzymes. Proper functioning of the assay was tested using yeast glucokinase instead of *P. furiosus* glucokinase. All sugars were tested at a concentration of 15 mM. As possible phosphoryl group donor, ATP, GDP, PP_i, phosphoenolpyruvate (each 2 mM), and polyphosphate (sodium phosphate glass, type 35; 0.2 g/litre) were used instead of ADP. The divalent cation requirement was tested by adding 10 mM of $MgCl_2$, $MnCl_2$, $CaCl_2$, $ZnCl_2$, or $CoCl_2$ to the standard assay mixture containing 2 mM disodium EDTA.

Purification of the glucokinase

All purification steps were performed without protection against oxygen. To prevent microbial contamination, all buffers contained 0.02% sodium azide. The standard buffer used was 100 mM Tris/HCl, pH 7.8 (buffer A). Cell-free extract (50 ml) was first brought to 58% ammonium sulphate saturation (2 h, 0°C). After centrifugation, the pellet fraction was discarded, and the supernatant was loaded on a Phenyl Sepharose 6 Fast Flow (high sub) column (3.2 × 4 cm), equilibrated in buffer A containing 2.5 M ammonium sulphate. The column was developed using two successive linear gradients from 2.5 to 0.75 M $(NH_4)_2SO_4$ (120 ml) and from 0.75 to 0 M $(NH_4)_2SO_4$ (360 ml). The glucokinase eluted at 0.5 M $(NH_4)_2SO_4$. Active fractions were pooled and desalted by ultrafiltration (Amicon YM-5) using buffer A, supplemented with 5 mM CHAPS. The desalted glucokinase pool was applied to a Mono Q HR 5/5 column equilibrated in buffer A containing 1 mM CHAPS. The

glucokinase eluted during a 60-ml linear gradient (0 - 0.5 M NaCl) at 0.18 M NaCl. Active fractions were pooled, and CHAPS was added up to 5 mM. The enzyme pool was loaded on a Hydroxyapatite column (2 × 20 cm) equilibrated in 1 mM potassium phosphate buffer (pH 6.8) containing 1 mM CHAPS. The column was developed using two successive linear gradients from 0 to 0.25 M potassium phosphate (140 ml) and from 0.25 to 0.5 M potassium phosphate (50 ml). Glucokinase-containing fractions eluted at 0.35 M potassium phosphate. The buffer of the active pool was exchanged for 50 mM potassium phosphate buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$ by Amicon YM-5 ultrafiltration. The concentrated pool was loaded on a Phenyl Superose HR 5/5 column equilibrated in the same buffer. Glucokinase eluted from the column at 1.2 M $(NH_4)_2SO_4$ during a linear 30-ml gradient from 1.7 to 0 M $(NH_4)_2SO_4$. Active fractions were combined, and the buffer was exchanged for 50 mM Pipes/HCl (pH 6.2) by ultrafiltration. The enzyme pool was applied to a Mono Q HR 5/5 column, equilibrated in 50 mM Pipes/HCl (pH 6.2). The glucokinase eluted during a linear 40-ml gradient (0 - 1 M NaCl) at 0.2 M NaCl. The enzyme pool was desalted and concentrated with Macrosep (30K) concentrators (Filtron). Complete purification was accomplished by continuous elution electrophoresis on a Prep Gel apparatus (Bio-Rad). A 1-ml sample from the concentrated enzyme pool was loaded on the gel (8% acrylamide), and electrophoresis of proteins was performed according to the instructions of the manufacturer. Protein was eluted from the gel in Tris (25 mM), glycine (192 mM) buffer, pH 8.3. Only those fractions that gave one single band on a native gel were combined.

Purity of the enzyme was checked by native and denaturing SDS-PAGE as described before [Kengen *et al.*, 1993]. For determination of the subunit composition by SDS-PAGE, protein samples were diluted in sample buffer, containing 2% SDS (w/v) and 5% 2-mercaptoethanol, and subsequently heated at 100°C. In some cases, 2-mercaptoethanol was omitted from the sample buffer, and the sample was not heated. Silver staining was performed using the reagent kit from E. Merck (Darmstadt, Germany).

Activity staining was performed on native PAGE gels by coupling the glucokinase activity to the reduction of nitro blue tetrazolium. Therefore, the gel was incubated at 37°C in the dark for 30 min in a staining mix with the following composition: 100 mM Tris/HCl, pH 7.8, 0.001% phenazin methosulphate, 0.035% nitro blue tetrazolium, 15 mM $MgCl_2$, 0.5 mM NADP, 3 mM ADP, 15 mM D-glucose, 185 mM NaCl, and D-glucose-6-phosphate dehydrogenase (1.75 U).

Molecular mass determination

The molecular mass of the native glucokinase was determined by performing PAGE at various acrylamide percentages (5, 6, 7, 8, 9, and 10%), as described before [Hedrick & Smith, 1968]. The following molecular mass standards were used: lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin monomer and dimer (66 and 132 kDa), and urease hexamer (545 kDa).

pH optimum

The pH optimum was determined at 50°C in 200 mM Tris/maleate buffer over the pH range 5.5 - 9.0. Care was taken that the auxiliary enzyme was not limiting at the various pH values.

Temperature effect and thermostability

The effect of temperature on the activity was determined by incubating an appropriate amount of purified enzyme in 1-ml crimp-sealed vials containing 200 mM Tris/maleate buffer (pH 8.5), 20 mM $MgCl_2$, and 20 mM D-glucose. The vials were submerged in an oil bath at temperatures varying from 30 to 110°C, and the enzyme reaction was started by injecting 10 µl of 100 mM ADP. After 15 - 30 min, the reaction was stopped by putting the vials on ice, and the amount of glucose-6-phosphate formed was determined spectrophotometrically by measuring the reduction of NADP (334 nm) in an assay with glucose-6-phosphate dehydrogenase.

Thermostability of the glucokinase was determined by incubating purified glucokinase in 200 mM Tris/maleate buffer (pH 8.5) at 100°C in crimp-sealed vials, submerged in an oil bath. At certain time intervals, 50-µl aliquots were withdrawn and analysed for activity in the standard assay.

Kinetic analysis

Kinetic parameters were determined at 50°C by varying the concentration of glucose or ADP in the presence of a saturating concentration of ADP (4 mM) or glucose (15 mM), respectively. The 980-fold purified enzyme was used for these determinations.

N-terminal amino acid sequence analysis

The N-terminal sequence of the purified glucokinase was determined according to the Edman degradation method and was performed on two independent enzyme preparations by the sequencing facilities of Eurosequence (Groningen, The Netherlands) and SON (Leiden, The Netherlands). Because of the presence of Tris and glycine in the final preparations, the samples were subjected to PAGE and electroblotted on a polyvinylidene difluoride membrane prior to analysis.

RESULTS

Glucokinase levels on different carbon sources

To discern the inducible or constitutive nature of the ADP-dependent glucokinase, cells of *P. furiosus* were grown on various carbon sources (Figure 1). The level of the ADP-dependent glucokinase was found to vary from almost zero (0.003 U/mg) during growth on peptone to 0.96 U/mg during growth on cellobiose. The non-glycolytic substrate pyruvate showed a relatively low glucokinase activity (0.074 U/mg). Starch and maltose gave intermediate values of 0.43 and 0.49 U/mg, respectively. The activity of the subsequent enzymes in the glycolysis, *viz.* phosphoglucose isomerase and ADP-dependent phosphofructokinase, were also determined. The level of both enzymes appeared to vary in a similar way as the glucokinase, *i.e.* the

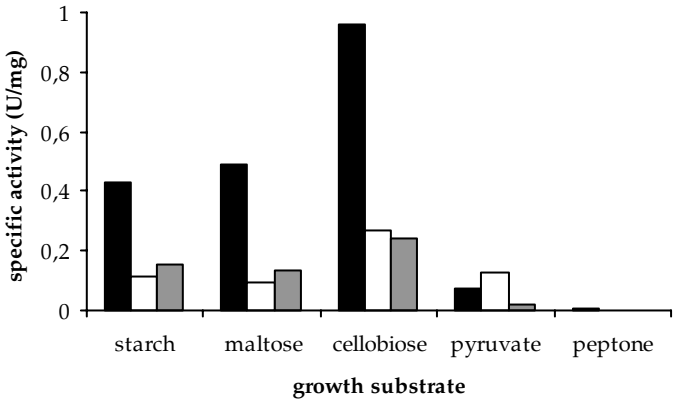

FIGURE 1 Levels of glycolytic enzymes in cell-free extracts of *P. furiosus* grown on various substrates. The specific activities were determined as given under "Materials and Methods". For each substrate, the bars indicate the specific activity of the ADP-dependent glucokinase (black), the phosphoglucose isomerase (white), and the ADP-dependent phosphofructokinase (grey).

highest activity on cellobiose and lower activities on starch, maltose, and pyruvate.

Purification of the glucokinase

The glucokinase was purified aerobically because no enzyme activity was lost upon storage of cell-free extracts at 4°C under air. After fractionation of the broken cell suspension at 100,000 × g, most of the total amount of activity (92%) was recovered in the supernatant, indicating that the enzyme is located in the cytoplasm. During initial purification attempts, PAGE showed that the enzyme copurified with several other proteins, suggesting that the enzyme adhered to these proteins. Therefore, the zwitterionic detergent CHAPS was added to the buffers, which did not negatively affect the activity.

The use of affinity chromatographic techniques, like ADP-agarose (either ribose-linked or N6-linked) or various dye-ligand-agaroses (Dyematrex screening kit, Amicon), was unsuccessful because the enzyme did not bind to any of the ligands, even in the presence of 10 mM $MgCl_2$. Therefore, a series of seven sequential purification steps were required to obtain a homogeneous preparation as judged by a silver-stained PAGE gel (Table 1). The colorless enzyme was 1346-fold purified with 2.1% recovery and showed a specific activity of 307 U/mg at 50°C. The identity of the band was confirmed by activity staining.

Physical properties

The molecular mass of the native enzyme as determined by PAGE at various acrylamide concentrations was 93 kDa (not shown). SDS-PAGE of the

TABLE 1 Purification of the ADP-dependent glucokinase from *P. furiosus*. Specific activities were determined at 50°C.

purification step	volume	total activity	protein	specific activity	purification factor	recovery
	ml	*U*	*mg/ml*	*U/mg*	*-fold*	*%*
cell-free extract	34.5	211	29.4	0.228	1	100
ammonium sulphate prec.	33.5	173	7.54	0.638	3	82
Phenyl Sepharose	34.5	144	0.72	5.79	25	68
Mono Q (pH 7.8)	20.6	107	0.205	25.4	111	51
Hydroxyapatite	18.2	92	0.049	103	452	44
Phenyl Superose	18.6	51	0.023	120	526	24
Mono Q (pH 6.2)	8.9	50	0.025	224	982	24
gel electrophoresis	1.9	4.4	0.0076	307	1346	2.1

980-fold purified protein gave a single band of 47 kDa, irrespective the time of heating in sample buffer or the presence of 2-mercaptoethanol (Figure 2). Apparently, the 93-kDa native enzyme easily disintegrates into two identical 47-kDa subunits. This result is in accordance with the immediate and complete inhibition of glucokinase activity that was found upon addition of 5 mM SDS (data not shown).

Catalytic properties

The ADP-dependent glucokinase exhibited a high activity (>65% of maximum) between pH 6 and 9, with an optimum at pH 7.5. As all other kinases, the enzyme required divalent cations for activity (Table 2). $MgCl_2$ was most effective, followed by $MnCl_2$, which resulted in 77% of the activity found with $MgCl_2$. No activity was found in the absence of divalent cations in the presence of EDTA. With respect to the phosphoryl group donor, the glucokinase was highly specific for ADP. ATP, GDP, phosphoenolpyruvate, PP_i, or polyphosphate were unable to replace ADP (Table 2). The glucokinase was also rather specific for the type of sugar. D-Fructose, D-mannose, and D-galactose could not be phosphorylated, and only 2-deoxy-D-glucose was able to replace glucose to a limited (9.2%) extent (Table 2).

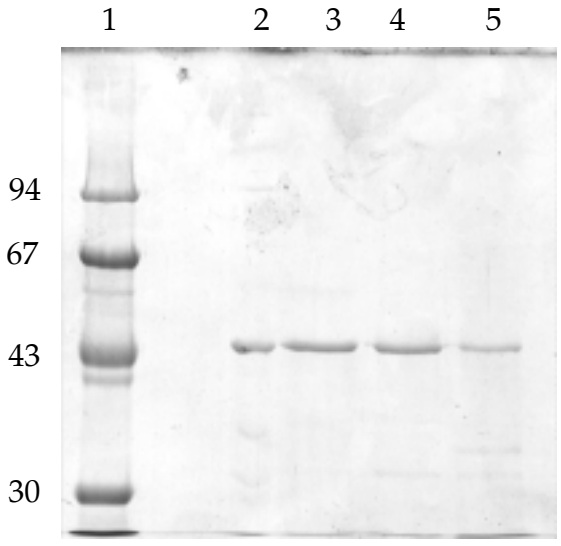

FIGURE 2 SDS-polyacrylamide gel electrophoresis of the glucokinase from *P. furiosus*. Lane 1 shows a set of marker proteins with their molecular mass indicated. Lanes 2–5 contained the 980-fold purified protein (0.45 µg protein/lane). Lanes 2 and 3 contained glucokinase diluted in sample buffer without and with 2-mercaptoethanol, respectively, and which were not boiled. Lanes 4 and 5 contained glucokinase that was boiled in sample buffer for 2 and 45 min, respectively. Proteins were stained with Coomassie Brilliant Blue R250.

TABLE 2 Substrate specificity and cation dependence of the glucokinase from *P. furiosus*. Enzyme assays were performed at 50°C as described under "Materials and Methods". 100% activity corresponds to a specific activity of 285 U/mg.

sugar	relative activity %	phosphoryl group donor	relative activity %	divalent cation	relative activity %
D-glucose	100	ADP	100	Mg^{2+}	100
2-deoxy-D-glucose	9.2	ATP	ND[a]	Mn^{2+}	77
D-fructose	ND	GDP	ND	Ca^{2+}	17
D-mannose	ND	phosphoenolpyruvate	ND	Zn^{2+}	5
D-galactose	ND	pyrophosphate	ND	Co^{2+}	1
		polyphosphate	ND		

[a] ND, not detectable, *i.e.* the activity was less than 0.3% of the activity under optimal conditions.

Kinetic parameters

Michaelis-Menten constants were determined according to Lineweaver-Burk. A K_m value of 0.73 ± 0.06 and 0.033 ± 0.003 mM was found for glucose and ADP, respectively. Apparent V_{max} values were 249 ± 18 and 194 ± 15 U/mg for glucose and ADP, respectively.

Thermostability and temperature optimum

The thermostability of the purified glucokinase was determined at 100°C and 110°C. At 110°C, all activity was lost after 30 min of incubation. Addition of MgADP, glucose, or both did not affect the stability. Therefore, no attempts were made to determine the half-life of the enzyme at this temperature. At 100°C, however, the glucokinase was remarkably stable. Inactivation followed first-order kinetics with a half-life value of 220 min (not shown).

The temperature dependence of the activity is shown in Figure 3A. The optimum temperature was found at 105°C (15-min incubation period). Because of a rapid denaturation above this temperature, this optimum value may increase or decrease depending on the time of incubation (shorter or longer incubation time, respectively). An Arrhenius plot of the data (Figure 3B) showed a breakpoint at 60°C, resulting in activation energy values of 54.3 kJ/mol between 30 and 60°C and 37.4 kJ/mol between 60 and 105°C.

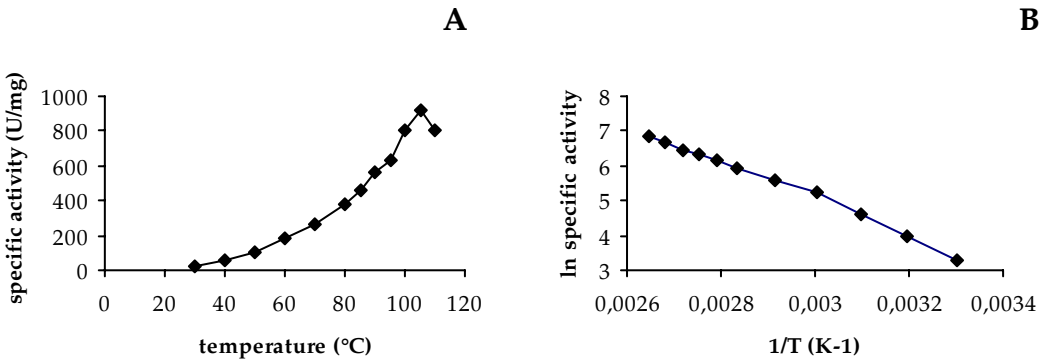

FIGURE 3 A. Dependence of glucokinase activity on temperature. Activity was determined by measuring the amount of glucose-6-phosphate formed after incubation for an appropriate period of time at the desired temperature. B. Arrhenius plot of the data from 30 to 105°C.

N-terminal amino acid sequence analysis

Two independent attempts to determine the N-terminal sequence did not give an unambiguous and ungapped sequence, indicating that the N terminus may be blocked. Those amino acids that were identified as identical by both analyses gave the following sequence (first 10 residues, X = ambiguous residue): NH_2, MTXEXLYKN(I/A). This sequence did not show similarity with any sequence given in the SWISSPROT data base.

DISCUSSION

P. furiosus has recently been shown to utilise a modified Embden-Meyerhof pathway, which involves a glucokinase and a phosphofructokinase that are both ADP-dependent [Kengen *et al.*, 1994]. Here, the ADP-dependent glucokinase was purified and characterised. Cell-free extracts of *P. furiosus* contained high levels of this enzyme, especially when the organism was grown on cellobiose. This high level of the glucokinase and also of the phosphoglucose isomerase and the phosphofructokinase in cellobiose-grown cells as compared to maltose-grown cells clearly shows that at least the first steps of the glycolysis are closely regulated in this hyperthermophilic Archaeon. This also follows from the low activity of these enzymes on pyruvate- and peptone-grown cells.

The cytoplasmic and oxygen-stable glucokinase was purified more than 1000-fold to homogeneity. Thus, the glucokinase constitutes less than 0.1% of the total cellular protein. This seems a rather low value for such a key enzyme. However, using the experimentally determined relationship between

activity and temperature, the specific activity at 100°C amounts to 2,233 U/mg (k_{cat} = 3,500 s^{-1}), which is the highest reported (Table 3). Moreover, it has been calculated before that the specific activity in cell-free extracts is more than satisfactory to sustain the glucose flux [Kengen *et al.*, 1994].

The ADP-dependent glucokinase had a native molecular mass of approximately 93 kDa and consisted of two identical subunits of approximately 47 kDa. This α_2 composition is observed also for bacterial glucokinases and eukaryotic hexokinases, but it differs from the eukaryotic glucokinases (baker's yeast, rat liver), which show a monomeric structure (Table 3). Furthermore, the *P. furiosus* glucokinase differs from most bacterial enzymes by its native molecular mass, which is about twice the usual size of about 50 kDa. In this respect, it resembles more the hexokinases from Eukarya. All the glucokinases described in detail (with sequence information) can be grouped into three evolutionary disconnected clusters, *i.e.* the mammalian glucokinases, the yeast glucokinases, and the bacterial glucokinases [Bork *et al.*, 1993]. However, based on the partial N-terminal sequence obtained for the *P. furiosus* enzyme, the latter does not group within any of these. Also, no similarity was found within other kinase families [Bork

TABLE 3 Comparison of the glucokinase from *P. furiosus* with other gluco- or hexokinases. Representatives of the Archaea, Bacteria and Eukarya are given.

domain	species	type	temp. optimum °C	native mass *kDa*	subunit mass *kDa*	specific activity[a] *U/mg*	reference[b]
Archaea	*Pyrococcus furiosus*	gluco	100	93	47	2233	this paper
Bacteria	*Bacillus stearothermophilus*	gluco	50	67	34.5	678	1
	Escherichia coli	gluco	37	49	24.5	20	2
	Streptococcus mutans	gluco	37	41	24	198	3
	Myxococcus corralloides	gluco	30	47	ND[c]	ND	4
	Propionibacterium shermanii	gluco	30	63	30	51	5
Eukarya	*Saccharomyces cerevisiae*	gluco	25	55	55	ND	6
		hexo[d]	25	104	52	450	7
	Aspergillus niger	hexo	30	100	50	32	8
	rat (liver)	gluco	37	50	50	9	9, 10
		hexo[e]	37	100	50	ND	10

[a] The values given represent specific activities of the purified enzymes, determined at the temperature optimum of the organism, or converted to the temperature optimum assuming a Q_{10} of 2.
[b] References: 1. [Hengartner & Zuber, 1973], 2. [Fukuda *et al.*, 1984], 3. [Porter *et al.*, 1982], 4. [González *et al.*, 1990], 5. [Phillips *et al.*, 1993], 6. [Albig & Entian, 1988], 7. [Hoggett & Kellett, 1992], 8. [Steinböck *et al.*, 1994], 9. [Parry & Walker, 1966], and 10. [Kogure *et al.*, 1993].
[c] ND, not determined.
[d] *S. cerevisiae* contains two hexokinases, PI and PII, with similar subunit composition.
[e] Mammalian tissues contain three isozymes of hexokinase (type I, II, and III) next to the glucokinase (type IV).

et al., 1993]. Nevertheless, many of these kinases, if not all, exhibit a striking structural feature; each subunit contains two lobes separated by a cleft [Anderson *et al.*, 1979]. Upon binding of the substrate the two lobes come together. Whether the *Pyrococcus* enzyme also shows this substrate-induced cleft closing remains to be elucidated.

A comparison with respect to the substrate specificity is difficult since for many enzymes these data are incomplete. Nevertheless, the glucokinase from *P. furiosus* showed a high specificity for the type of sugar as well as the phosphoryl group donor. From previous NMR experiments, it can be concluded also that the glucokinase is able to use both the α- and β-anomer of D-glucose [Kengen *et al.*, 1994]. Thus, the enzyme is a true glucokinase and highly specific for ADP. The specificity for ADP is also reflected in the high affinity found for this compound, *i.e.* the K_m value of 0.033 mM is the among the lowest reported [Hengartner & Zuber, 1973], [Fukuda *et al.*, 1984], [Porter *et al.*, 1982], [González *et al.*, 1990], [Phillips *et al.*, 1993], [Albig & Entian, 1988], [Hoggett & Kellett, 1992], [Steinböck *et al.*, 1994], [Parry & Walker, 1966], [Kogure *et al.*, 1993]. The reason for the ADP dependence may lie in the ability to activate sugars at conditions of low energy charge, *e.g.* after a period of starvation. The ATP level is then probably very low, and the relatively high ADP-level may still enable the phosphorylation of sugars.

The dependence of the activity on temperature showed an optimum at 105°C, which is in accordance with the requirements of the organism. The breakpoint in the Arrhenius plot at 60°C has been observed before for other thermophilic enzymes and may reflect a conformational change of the protein [Pisani *et al.*, 1990], [Bryant & Adams, 1989].

The purified glucokinase showed a high thermostability (half-life of 220 min) at the physiological growth optimum of 100°C. As has been found for most other enzymes from (hyper)thermophiles [Leuschner & Antranikian, 1995], this thermostability is apparently not determined by extrinsic factors, like high salt concentrations or specific compatible solutes.

It is evident that *P. furiosus* harbours an exceptional ADP-specific glucokinase, perfectly fit for catalysing the phosphorylation of glucose at elevated temperatures. Up to now, the ADP dependence is unique for *P. furiosu*s. Other sugar-converting thermophiles examined thus far use either the classical Embden-Meyerhof pathway [Siebers & Hensel, 1993], [Schröder *et al.*, 1994] or a non-phosphorylated version of the Entner-Doudoroff pathway [De Rosa *et al.*, 1984], [Budgen & Danson, 1986]. Further research is focussing on the distribution of this type of enzyme among other (hyper)thermophiles and on the determination of their primary structure,

which both will shed light on the evolutionary position of the ADP-dependent glucokinase. Moreover, the function of the ADP-dependent enzymes in relation to the bioenergetics of the organism is under present investigation.

3

Molecular and biochemical characterisation of the ADP-dependent phosphofructokinase from the hyperthermophilic Archaeon *Pyrococcus furiosus*

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SUMMARY

Pyrococcus furiosus uses a modified Embden-Meyerhof pathway involving two ADP-dependent kinases. Using the N-terminal amino acid sequence of the previously purified ADP-dependent glucokinase, the corresponding gene as well as a related open reading frame were detected in the genome of *P. furiosus*. Both genes were successfully cloned and expressed in *Escherichia coli*, yielding highly thermoactive ADP-dependent glucokinase and phosphofructokinase. The deduced amino acid sequences of both kinases were 21.1% identical but did not reveal significant homology with those of other known sugar kinases. The ADP-dependent phosphofructokinase was purified and characterised. The oxygen-stable protein had a native molecular mass of approximately 180 kDa and was composed of four identical 52-kDa subunits. It had a specific activity of 88 U/mg at 50°C and a pH optimum of 6.5. As phosphoryl group donor, ADP could be replaced by GDP, ATP, and GTP to a limited extent. The K_m values for fructose-6-phosphate and ADP were 2.3 and 0.11 mM, respectively. The phosphofructokinase did not catalyse the reverse reaction, nor was it regulated by any of the known allosteric modulators of ATP-dependent phosphofructokinases. ATP and AMP were identified as competitive inhibitors of the phosphofructokinase, raising the K_m for ADP to 0.34 and 0.41 mM, respectively.

INTRODUCTION

During growth on poly- or disaccharides, the hyperthermophilic Archaeon *Pyrococcus furiosus* uses a novel type of glycolytic pathway that is deviant from the classical Embden-Meyerhof pathway in several respects [Kengen *et al*., 1996], [de Vos *et al*., 1998]. First, instead of the classical ATP-dependent hexokinase, the pathway involves a novel ADP-dependent glucokinase [Kengen *et al*., 1994], [Kengen *et al*., 1995]. Second, a novel ADP-dependent phosphofructokinase replaces the more common ATP-dependent phosphofructokinase [Kengen *et al*., 1994]. Third, the pathway is modified in the degradation of glyceraldehyde-3-phosphate, which involves glyceraldehyde-3-phosphate ferredoxin oxidoreductase instead of the conventional couple glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase [Mukund & Adams, 1995], [van der Oost *et al*., 1998]. Modifications of the classical Embden-Meyerhof pathway at one or more of these three steps have also been observed in members of the hyperthermophilic archaeal genera *Thermococcus*, *Desulfurococcus*, and *Thermoproteus* [de Vos *et al*., 1998], [Selig *et al*., 1997]. The presence of these modifications in *P. furiosus* and other hyperthermophilic micro-organisms suggests that these are adaptations to elevated temperatures as a result of an altered biochemistry or a decreased stability of biomolecules.

Although ATP is regarded as the universal energy carrier and the most common phosphoryl group donor for kinases, several gluco- and phosphofructokinases with a different cosubstrate specificity have been described. Besides ADP-dependent gluco- and phosphofructokinases that have been demonstrated in *Pyrococcus* and *Thermococcus* spp. [Kengen *et al*., 1994], [Kengen *et al*., 1995], [Selig *et al*., 1997], polyphosphate-dependent glucokinases have been found in several other micro-organisms. In addition, the glucokinase of *Propionibacterium* can use both ATP and polyphosphate as phosphoryl group donor [Phillips *et al*., 1993]. Furthermore, PP_i-dependent phosphofructokinases have been described in several Eukarya and Bacteria and the hyperthermophilic Archaeon *Thermoproteus tenax* [Siebers *et al*., 1998].

Phylogenetic analyses of phosphofructokinases grouped these enzymes into three clusters. In a multiple alignment of representatives of each cluster, functionally important residues were identified that were highly conserved between all phosphofructokinases [Siebers *et al*., 1998]. ADP-dependent phosphofructokinases were not included in this study, because primary sequences of these enzymes were not yet available.

In this paper, we describe the cloning, expression, purification, and characterisation of the ADP-dependent phosphofructokinase from *P. furiosus*. It is the first molecular and biochemical characterisation of an ADP-dependent phosphofructokinase to date.

MATERIALS AND METHODS

Materials

Acetyl phosphate (potassium-lithium salt, crystallised), ADP (disodium salt), AMP (disodium salt, crystallised), aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13; rabbit muscle), ATP (disodium salt), fructose-1,6-bisphosphate (trisodium salt, crystallised), GDP (dilithium salt), glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49; yeast), glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8; rabbit muscle), NADH (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt), phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9; yeast), and triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1; rabbit muscle) were obtained from Roche Molecular Biochemicals. D-Fructose-1-phosphate (barium salt), D-fructose-2,6-bisphosphate (sodium salt), D-fructose-6-phosphate (disodium salt), β-NADP (sodium salt), sea salts, sodium phosphate glass type 35, tetrapotassium pyrophosphate, tripolyphosphate pentasodium, and trisodium trimetaphosphate were from Sigma. All other chemicals were of analytical grade. *Pfu* DNA polymerase was obtained from Life Technologies Inc. Mono Q HR 5/5, Phenyl Superose HR 5/5, Q Sepharose fast flow, and Superdex 200 prep grade were obtained from Amersham Pharmacia Biotech, Hydroxyapatite Biogel HT was from Bio-Rad. *P. furiosus* (DSM 3638) was obtained from the German Collection of Micro-organisms (Braunschweig, Germany). *Escherichia coli* XL-1 Blue and *E. coli* BL21(DE3) were obtained from Stratagene (La Jolla, CA, USA). The expression vector pET9d was obtained from Novagen Inc. (Madison, WI, USA).

Organisms and growth conditions

P. furiosus was mass-cultured (200 litres) in an artificial seawater medium supplemented with Na_2WO_4 (10 µM), yeast extract (1 g/litre), and vitamins, as described before [Kengen *et al.*, 1993] but with lower concentrations of Na_2S (0.25 g/litre) and NaCl (20 g/litre). The fermentor (Bioengineering AG, Wald, Switzerland) was sparged with N_2, and potato starch was used as substrate (8 g/litre).

E. coli XL1 Blue was used as a host for the construction of pET9d derivatives. *E. coli* BL21(DE3) was used as an expression host. Both strains were grown in Luria Bertani medium with kanamycin (50 µg/ml) in a rotary shaker at 37°C.

Preparation of cell-free extract from *P. furiosus*

P. furiosus cells from a 200-litre culture were harvested by continuous centrifugation (Sharples, Rueil, France) and stored at -20°C until used. Cell-free extract was prepared by suspending cells in 2 volumes (w/v) of 50 mM Tris/HCl buffer, pH 7.8, and treatment in a French press at 100 MPa. Cell debris was removed by centrifugation for 1 h at 100,000 × g at 10°C. The supernatant was used for purification of the phosphofructokinase.

Purification of the phosphofructokinase from *P. furiosus* cell-free extract

The phosphofructokinase was partially purified from cell-free extract of *P. furiosus*. All purification steps were done without protection against oxygen. To prevent microbial contamination, all buffers contained 0.02% sodium azide. Phosphofructokinase activity was recovered from cell-free extract following precipitation between 40 and 60% ammonium sulphate saturation. The subsequent purification included chromatography on Phenyl Superose HR 5/5, Q Sepharose fast flow, Hydroxyapatite Bio-Gel HT, Mono Q HR 5/5, and Superdex 200 prep grade gel filtration. Alternatively, cell-free extract was applied to a dye affinity chromatography system as described before [Hondmann & Visser, 1990].

Cloning of the phosphofructokinase gene

The previously obtained N-terminal amino acid sequence of the ADP-dependent glucokinase from *P. furiosus*, partially published as MTXEXLYKN(I/A), where X = ambiguous residue [Kengen *et al.*, 1995], was used for BLAST search of the *P. furiosus* data base [Utah Genome Center, http://www.genome.utah.edu]. After exchanging the ambiguous residues with several possible amino acids, a putative glucokinase gene was identified. Using the sequence of this gene, another open reading frame was identified by nucleotide sequence similarity in the *P. furiosus* data base. The following primer set was designed to amplify this open reading frame by polymerase chain reaction: BG447 (5'-GCGCGTCATGATAGATGAAGTCAGAGAG-CTCG, sense) and BG448 (5'-GCGCGGGATCCTTACTGATGCCTTCTTAGG-AGGGA, antisense), with *Bsp*HI and *Bam*HI restriction sites underlined.

The 100-µl polymerase chain reaction mixture contained 100 ng of *P. furiosus* DNA, isolated as described before [Sambrook *et al.*, 1989], 100 ng each of primer BG447 and BG448, 0.2 mM dNTPs, *Pfu* polymerase buffer, and 5 units (U) of *Pfu* DNA polymerase and was subjected to 35 cycles of amplification (1 min at 94°C, 45 sec at 60°C, and 3 min 30 sec at 72°C) on a DNA Thermal Cycler (Perkin-Elmer Cetus). The polymerase chain reaction product was digested (*Bsp*HI/*Bam*HI) and cloned into an *Nco*I/*Bam*HI-digested pET9d vector, resulting in pLUW572, which was transformed into *E. coli* XL1 Blue and BL21(DE3). Sequence analysis on pLUW572 was done by the dideoxynucleotide chain termination method with a Li-Cor automatic sequencing system (model 4000L). Sequencing data were analysed using the computer program DNASTAR.

Overexpression of the phosphofructokinase gene in *E. coli*

An overnight culture of *E. coli* BL21(DE3) containing pLUW572 was used as a 1% inoculum in 1 litre of Luria Bertani medium with 50 µg/ml kanamycin. After growth for 16 h at 37°C, cells were harvested by centrifugation (2,200 × g for 20 min) and resuspended in 10 ml of 20 mM Tris/HCl buffer, pH 8.5. The suspension was passed twice through a French press (100 MPa), and cell debris was removed by centrifugation (10,000 × g for 20 min). The resulting supernatant was used for purification of the recombinant phosphofructokinase.

Purification of the recombinant phosphofructokinase

The *E. coli* cell-free extract was heated for 30 min at 80°C, and precipitated proteins were removed by centrifugation. The supernatant was filtered through a 0.45-µm filter and loaded onto a Q Sepharose column that was equilibrated with 20 mM Tris/HCl buffer, pH 8.5. Bound proteins were eluted by a linear gradient of NaCl (0 - 1 M in Tris/HCl buffer). Active fractions were pooled and desalted with 20 mM Tris/HCl buffer, pH 8.5, using a Centricon filter with a 30-kDa cut-off.

Protein concentration and purity

Protein concentrations were determined with Coomassie Brilliant Blue G250 as described before [Bradford, 1976] using bovine serum albumin as a standard. The purity of the enzyme was checked by SDS-PAGE as described before [Kengen *et al.*, 1993]. Protein samples for SDS-PAGE were heated for 5 min at 100°C in an equal volume of sample buffer (0.1 M citrate-phosphate buffer, 5% SDS, 0.9% 2-mercaptoethanol, 20% glycerol, pH 6.8).

Determination of enzyme activity

ADP-dependent phosphofructokinase activity was measured aerobically in stoppered 1-ml quartz cuvettes at 50°C as described before [Kengen *et al.*, 1994]. The assay mixture contained 100 mM MES buffer, pH 6.5, 10 mM $MgCl_2$, 10 mM fructose-6-phosphate, 0.2 mM NADH, 2.5 mM ADP, 3.9 U of glycerol 3-phosphate dehydrogenase, 11 U of triosephosphate isomerase, 0.23 U of aldolase, and 5 - 25 µl of enzyme preparation. The absorbance of NADH was followed at 340 nm (ε = 6.18 $mM^{-1}cm^{-1}$). Care was taken that the auxiliary enzymes were never limiting. Specific enzyme activities were calculated from initial linear rates and expressed in U/mg of protein. 1 U (unit) was defined as that amount of enzyme required to convert 1 µmol of fructose-6-phosphate per min. The activity of the enzyme in the reverse direction was measured in an assay containing 100 mM MES buffer, pH 6.5, 12.5 mM fructose-1,6-bisphosphate, 2.5 mM AMP, 0.5 mM NADP, 0.35 U of glucose-6-phosphate dehydrogenase, 1.4 U of phosphoglucose isomerase, and 5 - 25 µl of enzyme preparation. The absorbance of NADPH was followed at 340 nm (ε = 6.18 $mM^{-1}cm^{-1}$).

Molecular mass determination

The molecular mass of the partially purified phosphofructokinase from *P. furiosus* cell-free extract was determined on a Superdex 200 gel filtration column using 100 mM Tris/HCl buffer, pH 7.8, with 150 mM NaCl. The column was calibrated using the following standard proteins: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), and catalase (232 kDa).

Molecular mass determination of the purified recombinant phosphofructokinase was done by running PAGE gels at various acrylamide percentages (5, 6, 7, 8, 9, 10, 11, and 12%) as described before [Hedrick & Smith, 1968]. The following molecular mass standards were used: lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin monomer and dimer (66 and 132 kDa), and urease trimer and hexamer (272 and 545 kDa).

The subunit molecular mass of the purified recombinant protein was determined by SDS-PAGE, using a molecular mass standard mix of carbonic anhydrase (31.0 kDa), ovalbumin (45.0 kDa), serum albumin (66.2 kDa), and phosphorylase b (97.4 kDa).

pH optimum

The pH optimum of the phosphofructokinase was determined at 50°C in 200 mM Tris/maleate buffer over the pH range 5.0 - 8.0. Buffer pH values were adjusted at this temperature. Care was taken that the auxiliary enzymes were not limiting at the various pH values.

Substrate specificity

As possible phosphoryl group donors, ATP, GDP, GTP, pyrophosphate, phosphoenolpyruvate, acetylphosphate, tripolyphosphate, trimetaphosphate (each 2.5 mM), and polyphosphate (sodium phosphate glass type 35, 0.25 mg/ml) were used in the activity assay instead of ADP. The divalent cation requirement was tested by adding 10 mM $MnCl_2$, $CaCl_2$, $CoCl_2$, or $ZnCl_2$ instead of $MgCl_2$.

Kinetic parameters

Kinetic parameters were determined at 50°C by varying the concentration of ADP (0.0125 - 5 mM) or fructose-6-phosphate (0.1 - 10 mM) in the assay mixture in the presence of 10 mM fructose-6-phosphate or 2.5 mM ADP, respectively. Data were analysed by computer-aided direct fit to the Michaelis-Menten curve. Furthermore, the data were used to construct Hill plots (log (V/V_{max} - V) versus log S).

Allosteric effectors

Regulation of phosphofructokinase activity by possible allosteric modulators was investigated by adding adenine nucleotides (ATP, ADP, or AMP; 2, 5, and 10 mM), metabolites (glucose, pyruvate, phosphoenol-pyruvate, or citrate; 5 mM) or fructose-2,6-bisphosphate (0.1 and 1 mM) to the assay mixture. Furthermore, the effect of KCl and NaCl (30, 150 and 500 mM) on the enzyme activity was tested.

RESULTS

Purification of the phosphofructokinase from *P. furiosus* cell-free extract

Cell-free extracts of *P. furiosus* showed a phosphofructokinase activity of 0.038 U/mg. However, despite the use of various chromatographic techniques, we were unable to obtain a highly purified enzyme, because it tended to stick to other proteins, resulting in similar band patterns upon PAGE after each purification step. When applied to a hydrophobic interaction column, phosphofructokinase activity was completely lost. Moreover, the use of dye affinity chromatography was not successful; although the phosphofructokinase did bind to a number of the tested dye ligands, it could not be eluted specifically with ADP. Aspecific elution with NaCl did not result in loss of contaminating proteins. Consequently, following chromatography on five different columns, the enzyme was purified 80-fold to a specific activity of 3 U/mg but still contained several contaminating proteins (Figure 1).

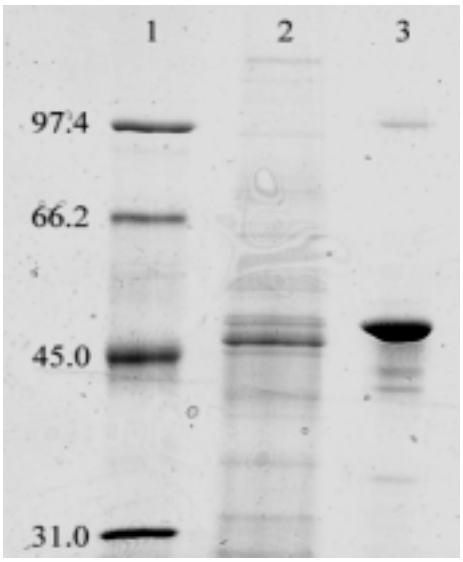

FIGURE 1 SDS-polyacrylamide gel electrophoresis of the phosphofructokinase from *P. furiosus*. Lane 1 contained a set of marker proteins with their molecular mass indicated (kDa). Lane 2 contained the partially purified phosphofructokinase from *P. furiosus* cell-free extract, and lane 3 contained purified recombinant phosphofructokinase. Proteins were stained with Coomassie Brilliant Blue R250.

Cloning of the phosphofructokinase gene

Using the previously obtained N-terminal amino acid sequence of the ADP-dependent glucokinase [Kengen *et al.*, 1995], a putative glucokinase gene was identified in the *P. furiosus* genome sequence. Expression of the gene in *E. coli* resulted in an ADP-dependent glucokinase activity of 20 U/mg in cell-free extracts at 50°C, confirming that the gene indeed encoded the glucokinase [Verhees *et al.*, 2002]. When the glucokinase gene, designated *glkA*, was used to search the *P. furiosus* genome, highest homology (25.7% nucleotide identity) was found with a 1365-base pair open reading frame predicted to encode a 455-amino acid protein. It was considered that this open reading frame might encode the ADP-dependent phosphofructokinase, and therefore the open reading frame was amplified by polymerase chain reaction and cloned into pET9d, resulting in plasmid pLUW572. DNA sequence analysis of pLUW572 confirmed the successful and faultless cloning of the open reading frame into pET9d (not shown).

Overexpression of the phosphofructokinase gene in *E. coli*

SDS-PAGE analysis of a cell-free extract of *E. coli* BL21(DE3) harbouring pLUW572 revealed an additional band of approximately 50 kDa, which corresponded with the calculated molecular mass (52.3 kDa) of the gene product. This band was absent in extracts of *E. coli* BL21(DE3) carrying the pET9d plasmid without insert. In a cell-free extract of *E. coli* BL21(DE3)

harbouring pLUW572, an ADP-dependent phosphofructokinase activity of 3.48 U/mg was measured at 50°C, confirming that indeed the *P. furiosus* phosphofructokinase gene, designated *pfkA*, had been cloned and expressed. The enzyme could be produced for up to 5% of the total *E. coli* cell protein without inducing gene expression by adding isopropyl-1-thio-β-D-galactopyranoside. Therefore, no attempts were made to optimise the overexpression.

Primary sequence comparison

On an amino acid level, the identity between the glucokinase and phosphofructokinase from *P. furiosus* was 21.1%. Comparison of the deduced amino acid sequence of the phosphofructokinase with those of proteins present in the GenBank data base showed high similarity with two hypothetical proteins from *Pyrococcus horikoshii* (PH1645, 75.2% identity; PH0589, 23.1% identity). Cloning and expression of the corresponding genes demonstrated that the proteins are an ADP-dependent phosphofructokinase and an ADP-dependent glucokinase, respectively (data not shown). Furthermore, 48.6% identity was found with a hypothetical protein from *Methano(caldo)coccus jannaschii* (MJ1604), which turned out to be an ADP-dependent phosphofructokinase [Verhees *et al*., 2001b]. Multiple sequence alignment showed several conserved regions throughout the five proteins (Figure 2). Comparison of the conserved regions with sequences present in the GenBank data base did not reveal additional similarities.

Purification and physical characterisation of the recombinant phosphofructokinase

The recombinant phosphofructokinase was easily purified by a heat incubation and anion exchange chromatography to at least 95% homogeneity as judged by SDS-PAGE (Figure 1). The specific activity of the purified protein was 88 U/mg at 50°C. On SDS-PAGE, the purified recombinant protein did not appear at the same height as the most abundant band in the partially purified *P. furiosus* fraction. However, because the phosphofructokinase activity of the partially purified *P. furiosus* cell-free extract is 3 U/mg, the enzyme represents only 3% of the total protein in the extract and can therefore not be the most dominant band in lane 2 of the SDS-PAGE gel.

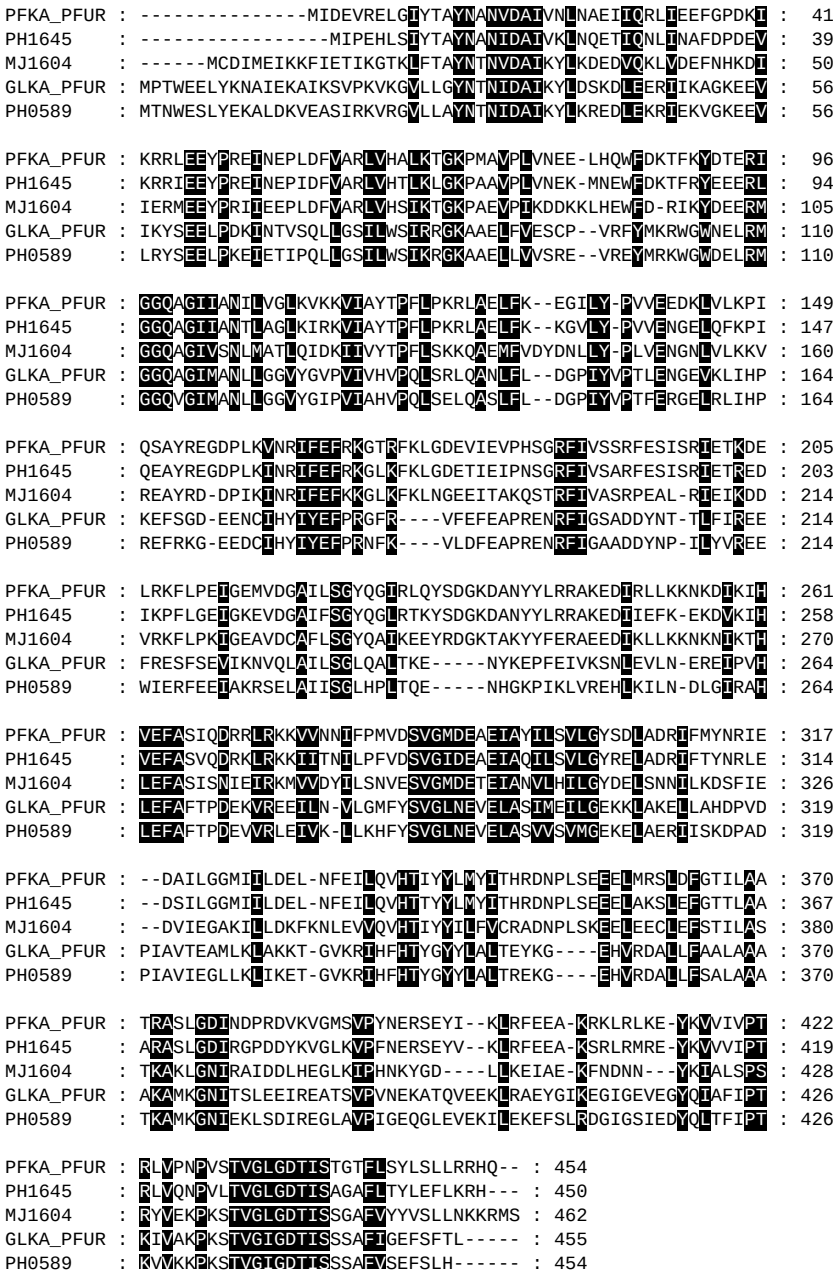

FIGURE 2 Multiple alignment of the deduced amino acid sequence of the *P. furiosus* ADP-dependent glucokinase and phosphofructokinase with the sequences of the hypothetical proteins from *P. horikoshii* and *M. jannaschii*, which were found to have high similarity with the phosphofructokinase. Gaps introduced for optimal alignment are marked by hyphens. Conserved regions are indicated as black boxes. PFKA_PFUR, ADP-dependent phosphofructokinase *P. furiosus* (accession number AF127909; Swiss-Prot); PH1645, putative ADP-dependent phosphofructokinase *P. horikoshii* (accession number 3258074; NCBI); MJ1604, putative ADP-dependent phosphofructokinase *M. jannaschii* (accession number 2128964; NCBI); GLKA_PFUR, ADP-dependent glucokinase *P. furiosus* (accession number AF127910; Swiss-Prot); PH0589, putative ADP-dependent glucokinase *P. horikoshii* (accession number 3256995; NCBI).

SDS-PAGE of the purified recombinant phosphofructokinase gave a single band at 52 kDa (Figure 1). The native molecular mass of the partially purified phosphofructokinase from *P. furiosus* cell-free extract, as determined by gel filtration chromatography, was approximately 180 kDa. This is in good agreement with the molecular mass determination of the purified recombinant phosphofructokinase. A native molecular mass of the phosphofructokinase of 179 kDa was calculated from the calibration curve (Figure 3), suggesting that the phosphofructokinase is a homotetramer. The phosphofructokinase showed activity between pH 5.5 and 7.0, with an optimum at pH 6.5 (data not shown).

Substrate specificity of the recombinant phosphofructokinase

The purified phosphofructokinase only showed activity in the forward direction. The enzyme showed highest activity with ADP as a phosphoryl group donor, which could be replaced by GDP, ATP, and GTP to a limited extent (Table 1). Divalent cations were required for activity of the enzyme, as shown by complete lack of activity in the presence of EDTA. Phosphofructokinase activity was highest in the presence of $MgCl_2$, followed by $CoCl_2$ (Table 1). The partially purified enzyme from *P. furiosus* cell-free extract showed the same substrate specificity pattern (data not shown).

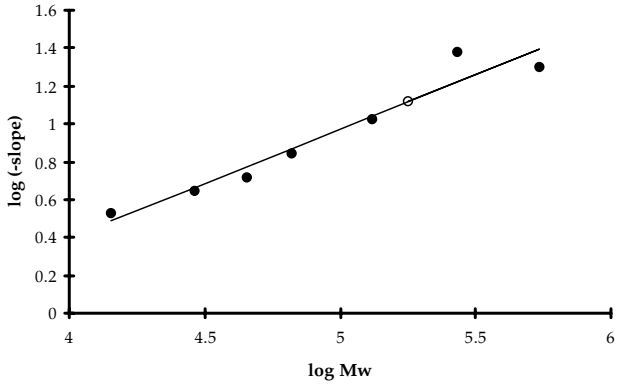

FIGURE 3 Calibration curve of molecular weight determination of the recombinant phosphofructokinase by native polyacrylamide gel electrophoresis. For each molecular weight marker protein, independent logarithmical plots were made of the relative mobility (R_f) against the acrylamide percentage of the gels. The slopes of these lines were plotted against the molecular weight of the marker proteins, depicted as filled circles. The slope of the phosphofructokinase was depicted as an open circle.

TABLE 1 Substrate specificity and cation dependence of the ADP-dependent phosphofructokinase from *P. furiosus*.

phosphoryl group donor	relative activity %	divalent cation	relative activity %
ADP	100	Mg^{2+}	100
GDP	28	Co^{2+}	81
ATP	<10	Mn^{2+}	43
GTP	<6	Ca^{2+}	8
phosphoenolpyruvate	ND[a]	Zn^{2+}	ND
pyrophosphate	ND		
tripolyphosphate	ND		
acetylphosphate	ND		
trimetaphosphate	ND		
polyphosphate	ND		

[a] ND, not detectable.

Kinetic parameters of the recombinant phosphofructokinase

The purified phosphofructokinase showed Michaelis-Menten kinetics at 50°C, with the following constants that were determined according to direct fit: K_m values of 2.3 ± 0.3 and 0.11 ± 0.01 mM for fructose-6-phosphate and ADP, respectively, and V_{max} values of 194 ± 13 and 150 ± 5 U/mg for fructose-6-phosphate and ADP, respectively. K_m values determined for the partially purified enzyme from *P. furiosus* cell-free extracts were in the same order of magnitude. Furthermore, Hill coefficients of 1.1 (fructose-6-phosphate) and 0.95 (ADP) were determined, indicative of noncooperative binding of the substrates to each subunit of the tetrameric enzyme.

Allosteric effectors of the recombinant phosphofructokinase

The addition of glucose, pyruvate, phosphoenolpyruvate, citrate, or fructose-2,6-bisphosphate did not show any effect on the phosphofructokinase activity. Both NaCl and KCl had a negative effect on the phosphofructokinase activity (42 and 43% activity in 300 mM NaCl and KCl, respectively).

Furthermore, the phosphofructokinase activity was negatively affected by the addition of ATP or AMP to the assay mixture. Because subsequent addition of $MgCl_2$ did not restore activity, the negative effect was not because of binding of Mg^{2+} to the ATP or AMP, resulting in lower availability of the ions for the substrate ADP. The addition of 5 mM ATP or AMP resulted in an increase in K_m values for ADP from 0.11 to 0.34 ± 0.02 or 0.41 ± 0.03 mM,

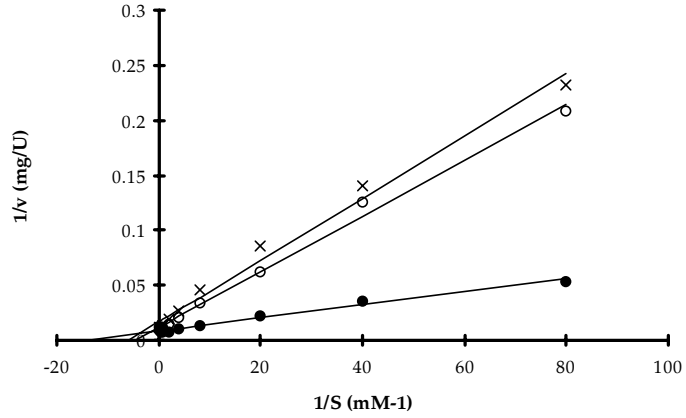

FIGURE 4 Lineweaver-Burk plot of recombinant *P. furiosus* phosphofructokinase with ADP as variable substrate, under normal assay conditions (•), in the presence of 5 mM ATP (o), or in the presence of 5 mM AMP (×).

respectively, whereas the V_{max} did not change (Figure 4). This indicates competitive inhibition of the phosphofructokinase by ATP and AMP. Apparently, the phosphofructokinase is not allosterically regulated by ATP, AMP, or any of the other tested compounds.

DISCUSSION

P. furiosus uses a modified Embden-Meyerhof pathway involving two novel-type kinases, *i.e.* an ADP-dependent glucokinase, which has previously been purified and characterised [Kengen *et al.*, 1995], and an ADP-dependent phosphofructokinase. In cell-free extracts of mass-cultured *P. furiosus* cells grown on starch, a phosphofructokinase activity of 0.038 U/mg was measured. Purification of the ADP-dependent phosphofructokinase from cell-free extracts of *P. furiosus* was hampered, because the enzyme tended to stick to other proteins, and both dye affinity and hydrophobic interaction chromatography could not be used in the purification. However, an alternative approach became available following the identification of the *P. furiosus pfkA* gene encoding the phosphofructokinase, which was successfully overexpressed in *E. coli*.

The recombinant phosphofructokinase was purified from *E. coli* to 95% homogeneity in a two-step purification. The specific activity of the purified protein was 88 U/mg at 50°C, which is approximately 2300-fold higher than the activity in crude cell-free extract of *P. furiosus* (0.038 U/mg). This suggests that the phosphofructokinase represents a very small fraction (0.043%) of the

total *P. furiosus* cell protein, which is unexpected for a catalytic enzyme present in an important metabolic pathway. However, using the experimentally determined relationship between activity and temperature (Q_{10} = 2 [Schäfer & Schönheit, 1992]), it can be calculated that the specific activity at 100°C would be 2816 U/mg. Furthermore, it has been calculated before that the specific activity of phosphofructokinase in cell-free extracts of *P. furiosus* is sufficiently high to sustain the glucose flux [Kengen *et al.*, 1994].

The ADP-dependent phosphofructokinase had a native molecular mass of 180 kDa and a subunit size of 52 kDa, in agreement with the deduced molecular mass of 52.3 kDa from the amino acid sequence. These data suggest that the phosphofructokinase has a tetrameric structure, which is most common for phosphofructokinases. ATP-dependent phosphofructokinases from bacteria and mammals are usually homotetramers with a subunit size of 33 and 85 kDa, respectively. Yeast phosphofructokinases show $\alpha_4\beta_4$ octameric structures with subunits of 112 and 118 kDa, whereas PP_i-dependent phosphofructokinases have been described to be monomers (110 kDa), homodimers (subunits of 48 - 55 kDa), homotetramers (subunits of 45 kDa), or heterotetramers (subunits of 60 and 65 kDa) [Fothergill-Gilmore & Michels, 1993].

The reaction catalysed by the phosphofructokinase was found to be irreversible. Therefore, *P. furiosus* needs a separate fructose-1,6-bisphosphate phosphatase to catalyse the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate during gluconeogenesis. Indeed, this enzyme has been detected in cell-free extract with a specific activity of 0.026 U/mg at 75°C [Schäfer & Schönheit, 1993]. The irreversibility of the phosphofructokinase reaction has also been described for ATP-dependent phosphofructokinases, although PP_i-dependent phosphofructokinases catalyse reversible reactions [Fothergill-Gilmore & Michels, 1993].

Apparent K_m values of 2.3 and 0.11 mM were found for fructose-6-phosphate and ADP, respectively. These values were determined at 50°C, which is much lower than the optimal growth temperature of *P. furiosus*. Because temperature can have a dramatic effect on K_m values [S.W.M. Kengen, unpublished observations], one has to realise that K_m values at the optimum growth temperature of 100°C could differ considerably from the data obtained in this study. Apparent K_m values at 55°C of the ADP-dependent phosphofructokinases from cell-free extracts of *Thermococcus celer* and *T. litoralis* were 2.5 and 4 mM, respectively, for fructose-6-phosphate and 0.2 and 0.4 mM, respectively, for ADP [Selig *et al.*, 1997]. However, the possible temperature effect makes it difficult to compare kinetic values of micro-

organisms with different optimal growth temperatures (100°C for *P. furiosus* and 85°C for both *Thermococcus* strains). For the purified PP_i-dependent phosphofructokinase from *T. tenax* (optimal growth temperature 85°C), much lower K_m values were found: 0.053 mM for fructose-6-phosphate and 0.023 mM for PP_i [Siebers *et al.*, 1998].

The ADP-dependent phosphofructokinase also showed activity with ATP, GTP, and GDP as phosphoryl group donors. In the case of ATP or GTP, however, the reaction product (ADP or GDP, respectively) is again an efficient phosphoryl group donor. Therefore, the relative activities with these compounds are probably overestimated. Furthermore, because of this fact, we were not able to determine kinetic values for ATP.

The phosphofructokinase was found to be inhibited by ATP and AMP through a competitive mechanism. In the case of ATP, this is not surprising, because ATP itself is a substrate and must therefore be able to bind to the catalytic site. In view of the role of phosphofructokinases in regulating the glycolytic pathway, it is surprising to see that ATP and AMP have the same (negative) effect on the activity of the phosphofructokinase. Allosterically regulated phosphofructokinases are usually inhibited by ATP but stimulated by AMP. ATP-dependent phosphofructokinases from *E. coli* and *Bacillus stearothermophilus* are allosterically activated by ADP and GDP and inhibited by phosphoenolpyruvate. Both yeast and mammalian phosphofructokinases are regulated by a large variety of effectors. Besides allosteric regulation by ATP and AMP, the enzymes are inhibited by citrate and activated by phosphate. Only mammalian enzymes are allosterically activated by fructose-1,6-bisphosphate. A very potent allosteric stimulator of eukaryotic phosphofructokinases is fructose-2,6-bisphosphate, which acts synergistically with AMP. This compound has been detected in most eukaryotes but never in prokaryotes [Fothergill-Gilmore & Michels, 1993]. Apparently, the ADP-dependent phosphofructokinase from *P. furiosus* is not allosterically regulated at all, and therefore it can not act as the major control point of the glycolytic pathway. Alternatively, the glyceraldehyde-3-phosphate ferredoxin oxidoreductase could be an important enzyme in control of the glycolysis of *P. furiosus* [van der Oost *et al.*, 1998]. The PP_i-dependent phosphofructokinase from *T. tenax* is not allosterically controlled either, nor does it function as the major control point of the glycolytic pathway of this organism [Siebers *et al.*, 1998].

Hill plot analysis indicated that the phosphofructokinase did not cooperatively bind either of the substrates ADP and fructose-6-phosphate, in contrast to the ATP-dependent phosphofructokinases from *E. coli* and *B.*

stearothermophilus, which were found to show co-operative binding to fructose-6-phosphate but not to ATP [Fothergill-Gilmore & Michels, 1993].

The assumption that the open reading frame related to the *glkA*, found in the *P. furiosus* genome, might encode the ADP-dependent phosphofructokinase was based on the observation that the N-terminal amino acid sequence of the glucokinase did not show any homology to known sugar kinases [Kengen *et al.*, 1995]. Furthermore, in the *P. furiosus* genome data base, no sequence could be found that showed significant homology to either gluco-, hexo-, or phosphofructokinases. Because both enzymes are ADP-dependent kinases, they could have identical ADP and sugar binding sites and might therefore be homologous to each other.

This hypothesis was confirmed when the expressed open reading frame indeed turned out to encode the ADP-dependent phosphofructokinase. Primary sequence analysis of the deduced amino acid sequence of the glucokinase and the phosphofructokinase showed that the proteins are significantly homologous and share several conserved regions. The functionally important residues for substrate binding that have been described for ATP- and PP_i-dependent phosphofructokinases [Siebers *et al.*, 1998] did, however, not seem to be present in any of the sequences of the ADP-dependent kinases, suggesting they represent a novel group of kinases. Altogether, these findings suggest that the glucokinase and the phosphofructokinase from *P. furiosus* are phylogenetically related. Further research is focused on scientific evidence for this suggestion.

ACKNOWLEDGEMENTS

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4

ADP-dependent phosphofructokinases in mesophilic and thermophilic methanogenic Archaea

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SUMMARY

Phosphofructokinase (PFK) is a key enzyme of the glycolytic pathway in all domains of life. Two related PFKs, ATP-dependent and PP_i-dependent PFK, have been distinguished in Bacteria and Eukarya, as well as in some Archaea. Hyperthermophilic Archaea of the order *Thermococcales*, including *Pyrococcus* and *Thermococcus* spp., have recently been demonstrated to possess a unique ADP-dependent PFK (ADP-PFK) that appears to be phylogenetically distinct. Here, we report the presence of ADP-PFKs in glycogen-producing members of the orders *Methanococcales* and *Methanosarcinales*, including both mesophilic and thermophilic representatives. To verify the substrate specificities of the methanogenic kinases, the gene encoding the ADP-PFK from *Methano(caldo)coccus jannaschii* was functionally expressed in *Escherichia coli*, and the produced enzyme was purified and characterised in detail. Compared to its counterparts from the two members of the order *Thermococcales*, the *M. jannaschii* ADP-PFK has an extremely low K_m for fructose 6-phosphate (9.6 µM), and it accepts both ADP and acetyl-phosphate as phosphoryl donors. Phylogenetic analysis of the ADP-PFK reveals it to be a key enzyme of the modified Embden-Meyerhof pathway of heterotrophic and chemolitho-autotrophic Archaea. Interestingly, uncharacterised homologs of this unusual kinase are present in several Eukarya.

INTRODUCTION

The Embden-Meyerhof pathway is the most common route for the degradation of glucose. While several small variations in this glycolytic pathway are known, major modifications have been demonstrated in *Pyrococcus furiosus* and other hyperthermophilic Archaea [de Vos *et al*., 1998], [Selig *et al*., 1997]. A combination of metabolic, biochemical, and genetic approaches have established that the pyrococcal glycolysis differs from the Embden-Meyerhof pathway by incorporating new conversions, novel enzymes, and unique control [Kengen *et al*., 1994], [Kengen *et al*., 1995], [Mukund & Adams, 1995], [Tuininga *et al*., 1999], [van der Oost *et al*., 1998]. First, the single-step conversion of glyceraldehyde-3-phosphate to 3-phospho (3P)-glycerate is catalysed by a uniquely controlled glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) instead of the two-step reaction catalysed by the conventional couple glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase [Mukund & Adams, 1995], [van der Oost *et al.*, 1998]. Second, instead of the classical ATP-dependent hexokinase, the pyrococcal pathway involves a novel ADP-dependent glucokinase (ADP-GLK) [Kengen *et al.*, 1995], [Koga *et al*., 2000]. Third, a novel nonallosteric ADP-dependent phosphofructokinase (ADP-PFK) replaces the more common ATP-PFK [Tuininga *et al.*, 1999].

The gene encoding an ADP-PFK was identified in the genome of *P. furiosus* and functionally expressed in *Escherichia coli*, and the encoded protein was thoroughly characterised [Tuininga *et al.*, 1999]. Primary structure comparison revealed the ADP-PFK to be a member of a novel enzyme family that did not show homology to known PFKs, which are monophyletic and include both ATP- and pyrophosphate (PP_i)-dependent enzymes. However, the ADP-PFK appeared to have significant similarity to the ADP-GLK from *P. furiosus*, suggesting that they belong to the same novel family of kinases. Recently, the crystal structure of the ADP-GLK from *Thermococcus litoralis* was solved. Unexpected structural similarity was recognised with members of the ribokinase family [Ito *et al*., 2001].

Initial analysis of the first sequenced archaeal genome, that of the hyperthermophilic Archaeon *Methanococcus* (*Methanocaldococcus*) *jannaschii* [Whitman *et al*., 2001], suggested the presence of several glycolytic-enzyme-encoding genes but indicated the absence of a gene encoding a classical PFK [Bult *et al*., 1996], [Selkov *et al*., 1997]. Hence, it was suggested that the PFK

from *M. jannaschii* could be ADP dependent and therefore undetectable in the sequence data [Selkov *et al.*, 1997]. Indeed, an ortholog (MJ1604) with 48% identity (on the amino acid level) to the *P. furiosus* ADP-PFK was found to be encoded by the *M. jannaschii* genome [Tuininga *et al.*, 1999]. The presence of this hypothetical ADP-PFK in *M. jannaschii* suggests the presence of a modified Embden-Meyerhof pathway in methanogenic Archaea as well. Previous studies on the genomic and enzyme levels indicated the presence of classical Embden-Meyerhof enzymes in Bacteria, Eukarya, and Archaea [Dandekar *et al.*, 1999]. However, no attention was given to enzymes involved in the modified Embden-Meyerhof pathway.

To obtain insight into the presence and function of ADP-PFKs in representatives of different phylogenetic lineages, we investigated their distribution on both the genomic and the functional level. Moreover, the gene encoding the ADP-PFK from *M. jannaschii* was overexpressed in *E. coli*, and the purified enzyme was thoroughly characterised. The results provided evidence for the presence of ADP-PFKs in both mesophilic and thermophilic Archaea and led us to propose an evolutionary model.

MATERIALS AND METHODS

Organisms and growth conditions

All micro-organisms were grown under an H_2-CO_2 atmosphere in 50 and 250 ml of medium, except that *Methanosaeta concilii* and *P. furiosus* were grown under an N_2-CO_2 atmosphere. *P. furiosus* (100°C) (DSM 3638) *Methanococcus igneus* (80°C) (DSM 5666), *M. jannaschii* (80°C) (DSM 2661), *Methanococcus maripaludis* (37°C) (DSM 2067), *Methanococcus thermolithotrophicus* (65°C) (DSM 2095), *Methanopyrus kandleri* (95°C) (DSM 6324), and *Methanobacterium thermoautotrophicum* Z-245 (65°C) (DSM 3720) [Kengen *et al.*, 1993] and *Methanosarcina mazei* (37°C) (DSM 2053) and *M. concilii* (37°C) (DSM 3671) [Stams *et al.*, 1993] were grown as described previously (optimum temperatures given in parentheses). *M. mazei* and *M. concilii* were supplemented with 50 mM methanol and 30 mM acetate, respectively. Nina Brunner (University of Essen, Essen, Germany) kindly provided cells of *Methanothermus fervidus* (DSM 2088), grown at 80°C. Cells of *Methanospirillum hungatei* (DSM 864), grown at 37°C, were kindly provided by Frank de Bok (Wageningen University, Wageningen, The Netherlands).

Preparation of cell extracts from methanogens

Cells were harvested from exponentially growing cultures by centrifugation (27,500 × g for 20 min) and suspended in 20 mM Tris/HCl (pH 7.5). The suspension was sonicated three times for 30 s each time. Cell debris was removed by centrifugation (16,000 × g for 15 min) and the resulting supernatant was used for enzyme measurements and protein determination. All manipulations in the preparation of cell extracts were performed anaerobically.

Enzyme activity assays in cell extracts

All enzyme assays were performed anaerobically in N_2-flushed stoppered 1-ml quartz cuvettes. Enzymatic activities were determined at temperatures close to the optimal growth temperature of the respective micro-organism, with a maximum of 80°C. However, when auxiliary enzymes from mesophilic sources were included in assays, the measurements were performed at 37°C (*M. maripaludis*, *M. mazei*, *M. concilii*, and *M. hungatei*) or 50°C (*P. furiosus*, *M. igneus*, *M. jannaschii*, *M. thermolithotrophicus*, *M. kandleri*, *M. thermoautotrophicum*, and *M. fervidus*), at which temperatures the heat-labile enzymes remained active and the activities of the thermostable enzymes could still be detected. The pH of buffers was adjusted at assay temperatures. Specific activities were calculated from linear rates and expressed in units per milligram of protein. One unit (U) was defined as the amount of enzyme required to convert 1 µmol of substrate per min.

PFK activity was measured by following the oxidation of NADH in a coupled assay with fructose-1,6-bisphosphate aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase. The assay mixture contained 100 mM morpholineethanesulphonic acid (MES) (pH 6.5), 10 mM $MgCl_2$, 0.2 mM NADH, 10 mM fructose-6-phosphate, 2.5 mM ATP, ADP, or acetyl-phosphate, 0.23 U of aldolase (from rabbit muscle), 11 U of triosephosphate isomerase (from rabbit muscle), 3.9 U of glycerol-3-phosphate dehydrogenase (from rabbit muscle), and 0.13 - 0.88 mg of protein. The absorbance of NADH was followed at 340 nm (ε = 6.18 $mM^{-1}cm^{-1}$).

Hydrogenase activity was measured in hydrogen-flushed cuvettes using methyl viologen as an artificial electron acceptor. The assay mixture contained 100 mM Tris/HCl (pH 7.8), 0.1 mM dithiothreitol, and 1 mM methyl viologen. To measure maximal hydrogenase activity, the buffer was slightly prereduced by adding ca. 1 µl of a freshly prepared 100 mM sodium

dithionite solution to the cuvette until an optical density at 578 nm of ca. 0.2 was reached. The reaction was started by adding 0.035 - 0.13 mg of protein. The reduction of methyl viologen was followed at 578 nm (ε = 9.7 $mM^{-1}cm^{-1}$). Protein concentrations were determined with Coomassie brilliant blue G250 as previously described [Bradford, 1976].

Genome analysis

Genome databases were screened for orthologs of modified and classical types of GLK and PFK, as well as of glyceraldehyde-3-phosphate dehydrogenase and glyceraldehyde-3-phosphate ferredoxin oxidoreductase genes, at the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov], update August 2001, using blastP (expect value [E] < 2e-04); at Genomes On Line Databases [http://igweb.integratedgenomics.com/GOLD], update, July 2001; and at Kyoto Encyclopedia of Genes and Genomes [http://www.genome.ad.jp/kegg], update July 2001. The *Sulfolobus solfataricus* genome was screened at Multipurpose Automated Genome Project Investigation [http://niji.imb.nrc.ca/sulfolobus/private/sulfolobus.html], update January 2001.

Multiple sequence alignment and tree construction

Sets of amino acid sequences were aligned with the ClustalX program (version 1.64b) and edited manually. A neighbour-joining tree was constructed with complete 16S and 18S rRNA sequences using the ARB software package [http://www.mikro.biologie.tumuenchen.de/pub/-ARB/documentation/arb.ps] and modified based on the model of [Woese *et al.*, 1990].

Cloning of the *pfkC* gene from *M. jannaschii*

An ortholog of the recently characterised *pfkC* (formerly called *pfk*A) gene from *P. furiosus* [Tuininga *et al.*, 1999] was detected in the *M. jannaschii* database (MJ1604). The following primer set was designed to amplify this *M. jannaschii pfkC* gene open reading frame by PCR: BG445 (5′-GCGCGCCATGGCATGTGATATTATGGAAATAA; sense) and BG446 (5′-GCGCGGATCCTTAGCTCATTCTTTTTTATTTAA; antisense); *Nco*I and *Bam*HI restriction sites are underlined. The 100-µl polymerase reaction mixture contained 100 ng of chromosomal *M. jannaschii* DNA, isolated as

described before [Sambrook *et al*., 1989]; 100 ng each of primers BG445 and BG446; 0.2 mM deoxynucleoside triphosphates; *Pfu* polymerase buffer; and 5 U of *Pfu* DNA polymerase and was subjected to 35 cycles of amplification (1 min at 94°C, 35 s at 50°C, and 3 min at 72°C) on a DNA thermal cycler (Perkin-Elmer Cetus). The PCR product was digested (*Nco*I/*Bam*HI) and cloned into an *Nco*I/*Bam*HI-digested pET9d vector, resulting in pLUW575, which was transformed into *E. coli* BL21(DE3). Sequence analysis of pLUW575 was done by the dideoxynucleotide chain termination method with a Li-Cor automatic sequencing system (model 4000L). Sequence data were analysed using the computer program DNASTAR.

Purification and characterisation of the *E. coli*-produced ADP-PFK from *M. jannaschii*

An overnight culture of *E. coli* BL21(DE3) harbouring pLUW575 was inoculated (1%) into 1 litre of Luria Bertani medium with 50 µg of kanamycin/ml. After growth for 16 h at 37°C, the cells were harvested by centrifugation (2,200 × g for 20 min) and resuspended in 10 ml of a 20 mM Tris/HCl buffer (pH 7.8). The suspension was passed twice through a French press (100 MPa), and cell debris was removed by centrifugation (10,000 × g for 20 min). The resulting supernatant was heat treated for 30 min at 80°C, and precipitated proteins were removed by centrifugation.

The cell extract was filtered through a 0.45-µm-pore-size filter and applied to a Q Sepharose fast-flow column (Amersham Pharmacia Biotech) that was equilibrated with a 20 mM Tris/HCl buffer (pH 7.8) containing 1 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate). ADP-PFK activity eluted at 0.3 M NaCl in a 125-ml gradient from 0 - 1 M NaCl. Active fractions were pooled and desalted by ultrafiltration using a 10 mM potassium phosphate buffer (pH 7.0). The desalted pool was applied to a Hydroxyapatite CHT5-1 column (Bio-Rad) that was equilibrated with 10 mM potassium phosphate buffer. The enzyme eluted in a 75-ml linear gradient (10 - 500 mM potassium phosphate) at 250 mM potassium phosphate. Active fractions were pooled, the buffer was changed for a 25 mM Tris/HCl buffer (pH 7.8) containing 1 mM CHAPS by ultrafiltration, and the pool was loaded onto a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) that was equilibrated in the same buffer. The enzyme eluted from the column at 0.3 M NaCl in a 20-ml linear gradient from 0 - 1 M NaCl. Fractions showing ADP-PFK activity were pooled and concentrated 16-fold to a final

volume of 460 µl. This concentrated pool was applied to a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) that was equilibrated with a 100 mM Tris/HCl buffer (pH 7.8) containing 100 mM NaCl, from which the protein eluted after 15 ml. The purity of the ADP-PFK was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The purified enzyme was characterised by determining its specific activity, molecular mass, pH optimum, substrate specificity, kinetic parameters, and allosteric effectors as described before [Tuininga *et al.*, 1999].

RESULTS

Genome analysis

To investigate the presence of genes that potentially encode enzymes involved in the modified Embden-Meyerhof pathway, we screened recent releases of complete and incomplete genome sequences of Archaea and thermophilic Bacteria (Table 1).

Orthologs of the novel GAPOR were identified in the genomes of all three *Pyrococcus* species (*P. furiosus*, *Pyrococcus horikoshii* and *Pyrococcus abyssi*) and in that of *M. jannaschii*. A classical NAD-dependent glyceraldehyde-3-phosphate dehydrogenase appeared to be present in all screened genomes. In *P. furiosus*, this glyceraldehyde-3-phosphate dehydrogenase is involved in gluconeogenesis, whereas GAPOR functions in the glycolytic direction [van der Oost *et al.*, 1998].

Orthologs of the ADP-GLK were identified only in the genomes of all three *Pyrococcus* species. Genes encoding classical ATP-dependent hexokinases were identified in *Halobacterium* sp. strain NRC-1, *Thermoplasma acidophilum*, *Aeropyrum pernix*, and the hyperthermophilic Bacteria *Thermotoga maritima*, *Aquifex aeolicus*, and *Thermus thermophilus*.

Finally, ADP-PFK orthologs were identified in the three *Pyrococcus* genomes, the *M. jannaschii* genome, and the genome of *M. mazei* Gö1. Remarkably, both an ATP-PFK and a PP_i -PFK ortholog were identified in the genome of *T. maritim*a, whereas in the closely related *A. aeolicu*s, only an ATP-PFK ortholog was identified.

Interestingly, homologs of ADP-dependent sugar kinases (12 to 17% identity to the archaeal kinases) were identified in several eukaryotic genome

TABLE 1 Enzymes of the classical and modified Embden-Meyerhof pathways encoded in the different genomes of Archaea and (hyper)thermophilic Bacteria.

genome[a]	accession number						
	ATP-GLK	ADP-GLK	ATP-PFK	ADP-PFK	PP_iPFK	GAPDH[b]	GAPOR
Pfu		AF127910		AF127909		PF1729232	AAC70892
Pho		PH0589		PH1645		PH1830	PH0457
Pab		PAB0967		PAB0213		PAB0257	PA1315
Mja				MJ1604		MJ1146	MJ1185
Mma				present[c]		present	
Afu						AF1732	
Mth						MT1009	
Hal	AAG20664					AAG18725	
Tac	TA0825					TA1103	
Sso						SSO0528	
Ape	APE2091		APE0012[d]			APE0171	
Tma	TM1469		TM0209		TM0289	TM0688	
Aae	AQ1496		AQ1708			AQ1065	
Tth	present		present			present	

[a] Genome analyses were performed on the following organisms: Euryarchaea, Pfu, *P. furiosus*; Pho, *P. horikoshii*; Pab, *P. abyssi*; Mja, *M. jannaschii*; Mma, *M. mazei*; Afu, *A. fulgidus*; Mth, *M. thermoautotrophicum*; Hal, *Halobacterium* sp. strain NRC-1; Tac, *T. acidophilum*; Crenarchaea, Sso, *S. solfataricus*; Ape, *A. pernix*; Bacteria, Tma, *T. maritima*; Aae, *A. aeolicus*; Tth, *T. thermophilus*.

[b] GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[c] Present, present in the genome but not yet annotated.

[d] APE0012 was detected using the N-terminal amino acid sequence of the ATP-PFK from *Desulfurococcus amylolyticus* [Hansen & Schönheit, 2000] and was recently experimentally confirmed [Ronimus *et al*., 2001b].

sequences, *i.e.* those of *Drosophila melanogaster* (AAF49769), *Caenorhabditis elegans* (T32780), *Mus musculus* (BAB27619), and *Homo sapiens* (AAH06112) (query, ADP-GLK [AF127910]; blastP, E < 1e-07); no homologous sequence was identified in yeast. Based on the recently described ADP-GLK structure of *T. litoralis* [Ito *et al*., 2001], a multiple sequence alignment of the identified homologs was created showing the conservation of several structurally important active- site residues in the ADP-dependent sugar kinases and their eukaryal homologs (Figure 1). The *C. elegans* amino acid sequence was omitted from the alignment because the C-terminal part of its sequence was fused with a putative transacylase, and therefore, important residues conserved at the C-terminus might have been lost.

Distribution of ADP-PFK activity in methanogens

The identification of orthologs of the ADP-PFK in *M. jannaschii* and *M. mazei* (Table 1) suggested that the modified Embden-Meyerhof pathway, as first described in the fermentative hyperthermophilic Archaeon *P. furiosu*s,

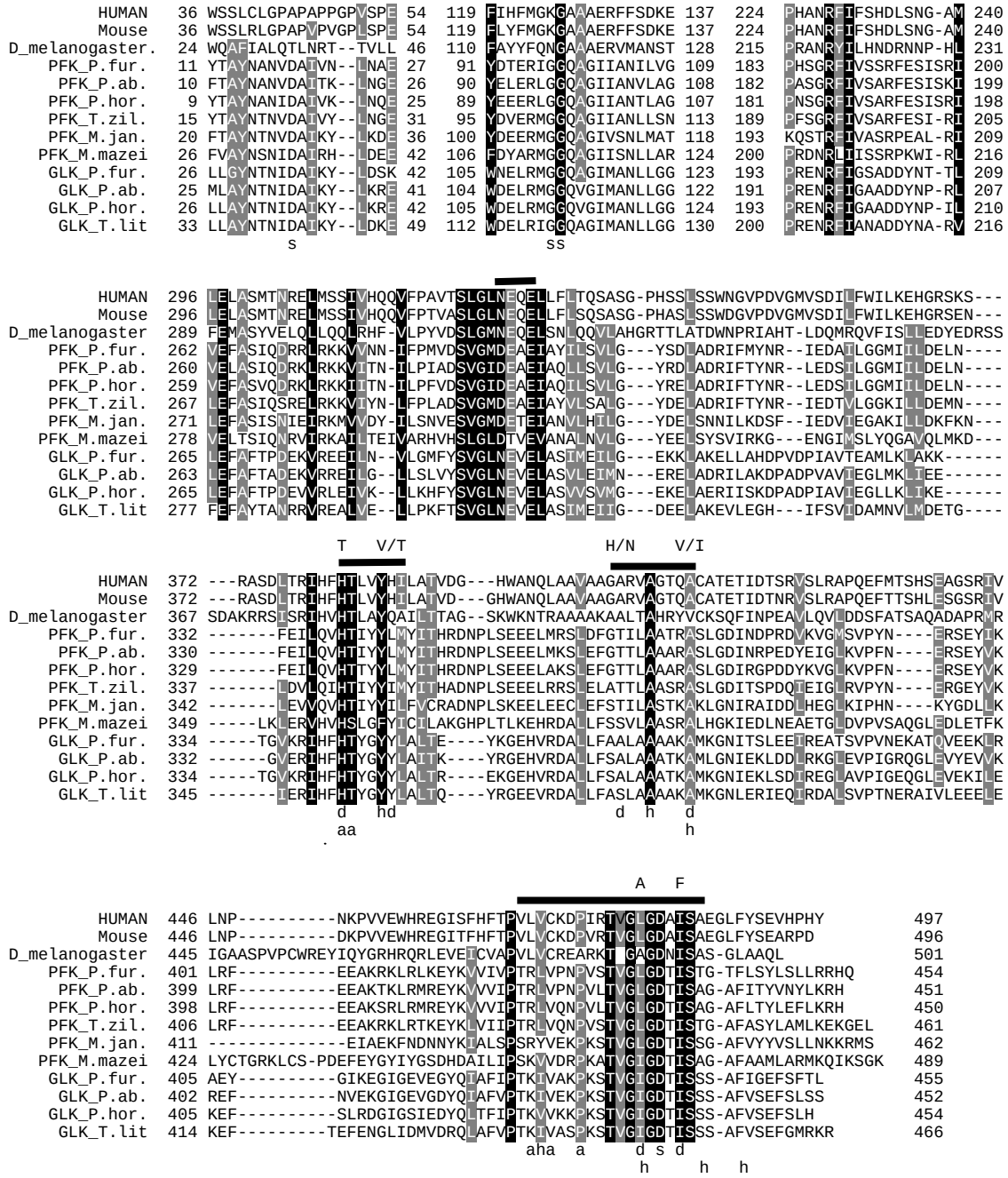

FIGURE 1 Multiple alignment of ADP-dependent kinase homologs. Gaps introduced for optimal alignment are marked by hyphens. Conserved residues are shaded black. Residues that are present in at least one eukaryal sequence and in the majority of the archaeal sequences are shaded grey. Nucleotide binding loops are indicated by bars above the alignment. a, residues that form hydrogen bonds with ADP; d, residues involved in the discrimination between ADP and ATP (the residues in these positions specific for ATP-dependent ribokinase and adenosine kinase are indicated above the alignment); h, hydrophobic residues that form a pocket with the approximate shape of the adenosine moiety; s; sugar binding and/or activation residues [Ito *et al.*, 2001]. The sequences have the following accession numbers: Human, hypothetical *H. sapiens* protein, AAH06112; Mouse, hypothetical *M. musculus* protein, BAB27619; D_melanogaster, hypothetical protein, AAF49769; PFK_P.fur, *P. furiosus* ADP-PFK, AF127909; PFK_P.ab., putative *P. abyssi* ADP-PFK PAB0213, D75170; PFK_P.hor., putative *P. horikoshii* ADP-PFK PH1645, E71044; PFK_T.zil., *T. zilligii* ADP-PFK, AAF97356; PFK_M.jan., *M. jannaschii* ADP-PFK MJ1604,

C64500; PFK_M.mazei, putative *M. mazei* ADP-PFK [A. Johann, personal communication]; GLK_P.fur, *P. furiosus* ADP-GLK, AF127910; GLK_P.ab., putative *P. abyssi* ADP-GLK PAB0967, B75058; GLK_P.hor., putative *P. horikoshii* ADP-GLK PH0589, A71174; GLK_T.lit., *T. litoralis* ADP-GLK.

may also be present in methanogens. This prompted us to determine PFK activity using ADP and ATP as the phosphoryl group donors in a wide range of methanogenic species belonging to various taxonomic groups. As a reference enzyme, hydrogenase activity was measured as well.

ADP-PFK activity was detected in cell extracts of the thermophilic methanogens *M. jannaschii*, *M. igneus*, and *M. thermolithotrophicus* and in the mesophilic methanogens *M. maripaludis* and *M. mazei* (Table 2). The presence of an ATP-PFK activity (5.6 mU/mg) in *M. maripaludis* had been reported previously [Yu *et al*., 1994]. However, we could detect only activity of an ADP-PFK (6.1 mU/mg) but not that of an ATP-PFK in *M. maripaludi*s. An ADP-PFK activity was also detected in the mesophilic *M. concilii*. In that organism, a high ATP-PFK activity was also detected. Since *M. concilii* is known to possess high adenylate kinase activity [Jetten *et al*., 1989], the detected ADP-PFK activity might be the result of the combined action of the adenylate kinase and the ATP-PFK activity. In *M. thermolithotrophicu*s, both ADP-PFK and ATP-PFK activities were detected.

TABLE 2 ADP-PFK activities in Archaea. Enzyme activities were determined as described in "Materials and Methods". No PFK activity could be detected in *M. thermoautotrophicum*, *M. kandleri*, *M. fervidus*, and *M. hungatei* extracts.

micro-organism	growth temperature °C	hydrogenase activity *U/mg*	ADP-PFK activity *mU/mg*	ATP-PFK activity *mU/mg*
P. furiosus	100	NM[a]	150	2.6
T. litoralis[b]	85	NM	200	NM
M. jannaschii	80	28	21	ND
M. igneus	80	67	16	ND
M. thermolithotrophicus	65	130	18	3.8
M. maripaludis	37	23	2.7	ND
M. mazei	37	140	6.1	ND
M. concilii	37	ND[c]	29	38

[a] NM, not measured.
[b] data from [Selig *et al*., 1997].
[c] ND, not detectable.

Overexpression of the *M. jannaschii pfkC* gene in *E. coli*

To gain insight into the substrate specificity of the methanogic ADP-PFK orthologs, we compared the properties of the enzyme of *M. jannaschii* to those of *P. furiosus* ADP-PFK. For this purpose, the *M. jannaschii pfkC* gene (MJ1604) was PCR amplified and cloned into pET9d, resulting in plasmid pLUW575. DNA sequence analysis of pLUW575 confirmed that the cloned *pfkC* gene showed the expected sequence. SDS-PAGE analysis of a cell extract of *E. coli* BL21(DE3) harbouring pLUW575 revealed an additional band of 51.5 kDa, which corresponded to the calculated molecular mass (53.4 kDa) of the gene product (not shown). This band was absent in cell extracts of *E. coli* BL21(DE3) carrying the empty pET9d vector that also showed no ADP-PFK activity. In a cell extract of *E. coli* BL21(DE3) harbouring pLUW575, an ADP-PFK activity of 0.8 U/mg was measured at 50°C, confirming that the cloned *M. jannaschii pfkC* gene indeed encoded an ADP-PFK. The enzyme could be produced up to 10% of total soluble cell protein after 16 h of cultivation at 37°C without inducing gene expression by adding isopropyl-1-thio-β-D-galactopyranoside.

Characteristics of the *M. jannaschii* ADP-PFK

The *E. coli*-produced *M. jannaschii* ADP-PFK was purified to homogeneity. The native molecular mass of the enzyme, as determined by native PAGE at various acrylamide percentages was approximately 50.1 kDa, indicating that the *M. jannaschii* ADP-PFK is a monomer (not shown).

The purified enzyme had a specific activity of 8.2 U/mg at 50°C at the optimum pH of 6.5, only in the direction of phosphorylation. Apart from ADP, acetyl-phosphate could serve as an efficient phosphoryl group donor to the enzyme (Table 3). Divalent cations were required for activity, as indicated by complete lack of activity in the presence of EDTA. ADP-PFK activity was highest in the presence of $CaCl_2$, followed by $MgCl_2$ (Table 3). Both KCl and NaCl had negative effects on the ADP-PFK activity (84 and 88% activity in 500 mM KCl and NaCl, respectively). Furthermore, the enzyme activity was negatively affected by the addition of ATP or AMP to the assay mixture (53 and 24% activity in 10 mM ATP and AMP, respectively). However, the addition of fructose-2,6-bisphosphate, pyruvate, glucose, phosphoenol-pyruvate, or citrate to the assay mixture had no effect on the activity. The enzyme showed Michaelis-Menten kinetics at 50°C, with the following constants, which were determined according to a computer-aided direct fit

TABLE 3 Phosphoryl group donor and cation dependence of the ADP-PFK from *M. jannaschii*. Standard enzyme assays were done at 50°C, except that phosphoryl group donors or cations were varied as described in "Materials and Methods".

phosphoryl group donor	relative activity %	divalent cation	relative activity %
ADP	100	Mg^{2+}	100
GDP	1.4	Ca^{2+}	120
ATP	0.3	Co^{2+}	78
GTP	8.1	Mn^{2+}	54
acetyl-phosphate	83	Zn^{2+}	ND[a]
polyphosphate	ND		
phosphoenolpyruvate	ND		
pyrophosphate	ND		

[a] ND, not detectable, *i.e.* the activity was less than 0.3% of the activity under optimal conditions.

using the Michaelis-Menten equation: apparent K_m values of 0.0096 ± 0.0007 and 0.49 ± 0.13 mM for fructose 6-phosphate and ADP, respectively, and apparent V_{max} values of 11.2 ± 0.3 and 9.59 ± 0.74 U/mg for fructose 6-phosphate and ADP, respectively. For acetyl-phosphate as a phosphoryl group donor, an apparent K_m value of 11.9 ± 1.8 mM and an apparent V_{max} of 14.4 ± 1.0 U/mg at 50°C were determined. In a Hill plot, the kinetic data of fructose-6-phosphate, ADP, and acetyl-phosphate showed noncooperative binding of the substrates (not shown).

DISCUSSION

Following the discovery of ADP-PFK activity in *P. furiosus* [Kengen *et al.*, 1994] and characterisation of this novel enzyme [Tuininga *et al.*, 1999], ADP-PFK activity has been detected in various members of the order *Thermococcales* [Ronimus *et al.*, 1999a], [Selig *et al.*, 1997]. The presence of a glycolytic pathway in methanogens has recently been proposed based on (i) enzyme analyses of *M. maripaludis* [Yu *et al.*, 1994] and (ii) analysis of the genome sequence of *M. jannaschii*, which revealed several glycolytic orthologs [Bult *et al.*, 1996]. However, no PFK gene was detected [Selkov *et al.*, 1997]. Here, we demonstrate the functional presence of ADP-PFKs in methanogenic Archaea, analyse their distribution, and describe the unique catalytic properties of the purified enzyme from *M. jannaschii*.

The recent characterisation of the amino acid sequence of the *P. furiosus* and *Thermococcus zilligii* ADP-PFK [Ronimus *et al.*, 2001a], [Tuininga *et al.*, 1999] resulted in the identification of orthologs in the genomes of both chemolithoautotrophic (*M. jannaschii* and *M. mazei*) and heterotrophic (*P. abyssi* and *P. horikoshii*) Archaea (Table 1). These data already suggested that a modified Embden-Meyerhof pathway, present in *P. furiosu*s, might also be operational in methanogens. In addition, we detected PFK activity in the methanogens *M. jannaschii*, *M. thermolithotrophicus*, *M. igneus*, and *M. maripaludis*, of the order *Methanococcales*, and *M. mazei*, of the order *Methanosarcinales* (Table 2). Although ADP-PFK activity could be detected in *M. concilii* extracts, this activity was probably the result of the concerted action of ATP-PFK activity and high adenylate kinase activity [Jetten *et al.*, 1989]. The high ADP-PFK activity (18 mU/mg) and relatively low ATP-PFK activity (3.8 mU/mg) detected in *M. thermolithotrophicus* might indicate that two types of PFKs, *i.e.* ADP- and ATP-dependent, are present in that organism. On the other hand, the *P. furiosus* PFK was shown to be active with ADP and to some extent with ATP [Tuininga *et al.*, 1999]. Therefore, the ADP-PFK and ATP-PFK activities detected in *M. thermolithotrophicus* might also be the result of a single enzyme with affinity for both ATP and ADP. In all other methanogens investigated, neither ADP-PFK nor ATP-PFK activity was detected.

Apart from the analysis of an ADP-PFK, the modified Embden-Meyerhof pathway in *P. furiosus* also differs from the conventional Embden-Meyerhof pathway at the level of glyceraldehyde-3-phosphate. The conversion of glyceraldehyde 3-phosphate by GAPOR has also been detected in *Thermococcus* spp. [Selig *et al.*, 1997] and *M. jannaschii* [van der Oost *et al.*, 1998], [G.J. Schut & J. van der Oost, personal communication]. This observation confirms predictions from the genome analyses (Table 1) that the glycolytic pathway in *M. jannaschii* resembles that in *P. furiosu*s, not only in the ADP dependency of the PFK but also in regard to the conversion of glyceraldehyde-3-phosphate by GAPOR.

The activities of ADP-PFK and GAPOR found in cell extracts of *M. jannaschii* grown under standard autotrophic conditions (H_2 and CO_2), are an order of magnitude lower (PFK, 21 mU/mg; GAPOR, 100 mU/mg) than those in cell extracts of *P. furiosus* grown on saccharides (PFK, 150 mU/mg; GAPOR, 1,800 mU/mg) [Kengen *et al.*, 1995], [van der Oost *et al.*, 1998]. This suggests that, at least under the investigated conditions, the modified Embden-Meyerhof pathway is less prominent in *M. jannaschii* than in *P. furiosu*s. Since the pathway in methanogens is assumed to be used for glycogen degradation during starvation, the enzyme activities might be

induced in starved cells. Under starvation, the mesophilic Archaeon *M. maripaludis* has been reported to degrade its glycogen storage, resulting in the release of glucose-1-phosphate, which is converted to glucose-6-phosphate to enter the Embden-Meyerhof pathway [Yu *et al.*, 1994]. Moreover, glycogen has been reported to be a reserve polysaccharide in *Methanosarcina* spp. and *Methanosaeta* spp. [Murray & Zinder, 1984], [Pellerin *et al.*, 1987]. Likewise, the detected ATP-PFK activity in *M. concilii* and the ADP-PFK activity in *Methanococcus* spp. and *M. mazei* presumably play a role in the degradation of glycogen. All methanogens in which no PFK activity could be detected, such as *M. thermoautotrophicu*m, *M. kandler*i, *M. fervidu*s, and *M. hungatei*, appear to lack the capacity to synthesise and degrade glycogen.

Because of the different physiologies of heterotrophic and chemolithoautotrophic organisms, a comparison of the properties of the ADP-PFKs from both types of organisms was of interest. Therefore, the gene encoding the ADP-PFK from *M. jannaschii* (MJ1604) was expressed in *E. coli*, and the protein was purified and characterised in detail. The biochemical characteristics of this protein were compared to those of the ADP-dependent enzymes from *P. furiosus* [Tuininga *et al.*, 1999] and *T. zilligii* [Ronimus *et al.*, 1999a] (Table 4). The *M. jannaschii* enzyme is the only ADP-PFK studied that was able to use acetyl-phosphate as a phosphoryl group donor. It remains unclear whether the specificity for acetyl-phosphate is a specific physiological adaptation of methanogens or a nonspecific result of a minor deviation in the substrate binding pocket of the *M. jannaschii* enzyme. The latter explanation appears to be the more likely, since no acetyl-phosphate-dependent PFK activity was detected in the other methanogens used in this study, including the acetoclastic ones (data not shown).

The affinity of the *M. jannaschii* enzyme for fructose 6-phosphate is extremely high, and its catalytic efficiency (k_{cat}/K_m) of 1,038 $mM^{-1}s^{-1}$ is the highest reported to date. Normally, the apparent K_m for the phosphoryl group donor is lower than that for the cosubstrate fructose-6-phosphate, presumably a reflection of the average intracellular concentrations of both substrates [Ronimus *et al.*, 1999a]. This contrasts with the properties of the *M. jannaschii* enzyme, which shows a 50-fold lower apparent K_m for fructose-6-phosphate than for ADP. This might reflect different intracellular concentrations of fructose-6-phosphate in the glycogen-degrading *M. jannaschii* and the sugar-fermenting *P. furiosus* and *T. zilligii*.

TABLE 4 Biochemical comparison of ADP-PFKs. Enzyme assays were done at 50°C as described in Materials and Methods or as specified elsewhere.

parameter	substrate	*P. furiosus*[a]	*T. zilligii*[b]	*M. jannaschii*
native molecular mass (kDa)		180	200	51.5
subunit molecular mass (kDa)		52	53.9	53.4
allosteric regulation		no	no	no
apparent K_m (mM)	fructose-6-phosphate	2.3	3.77	0.0096
	ADP	0.11	0.11	0.49
	acetyl-phosphate	ND[c]	ND	11.9
	fructose-1,6-bisphosphate	ND	12.5	ND
	AMP	ND	0.56	ND
apparent V_{max} (U/mg)	fructose-6-phosphate	194	197	11.2
	ADP	150	243	9.59
	acetyl-phosphate	ND	ND	14.4

[a] data from [Tuininga *et al.*, 1999].
[b] data from [Ronimus *et al.*, 1999a], [Ronimus *et al.*, 2001a].
[c] ND, not detectable, *i.e.* the activity was less than 0.3% of the activity under optimal conditions.

Our data extend the previously noted presence of ADP-PFKs in members of the order *Thermococcales* to their abundant presence in members of the closely related order *Methanococcale*s. Furthermore, we show that PFKs are present in all tested glycogen-consuming methanogens of the genera *Methanococcus*, *Methanosarcina*, and *Methanosaeta*. The first two genera harbour ADP-PFKs, whereas *M. concilii* most likely contains an ATP-PFK rather than ADP-PFK activity. The presence of activity of an ADP-PFK in mesophilic Archaea and that of ATP-PFK and PP_i -PFK in hyperthermophilic Archaea [Hansen & Schönheit, 2000], [Ronimus *et al.*, 1999b], [Siebers *et al.*, 1998] indicates that ADP dependency is not essential for this glycolytic conversion at higher temperatures (Figure 2).

Recently, the crystal structure of the ADP-dependent glucokinase from *T. litoralis* has been solved [Ito *et al.*, 2001]. Because of its distinct primary structure, it came as a surprise that the ADP-GLK structure had significant structural similarity to the ATP-dependent ribokinase family. Cocrystallisation with ADP elucidated the position of the nucleotide binding site. Remarkably, minor modifications in amino acid composition appear to result in a clear preference for either ADP or ATP in the different kinases [Ito *et al.*, 2001]. The majority of the residues that were found to interact directly with ADP in the *T. litoralis* ADP-GLK are conserved in all ADP-dependent sugar kinase homologs, including the eukaryal sequences (Figure 1). The size of the side chain of residue 353 (*M. jannaschii* ADP-PFK numbering) seems to

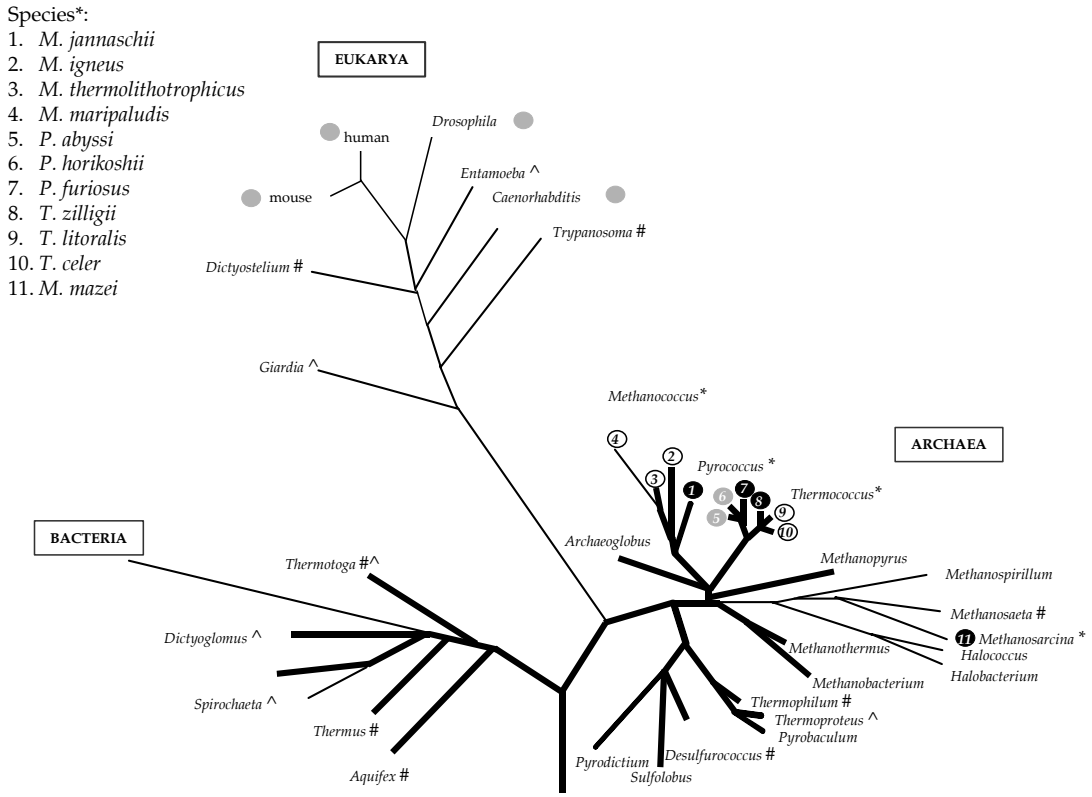

FIGURE 2 Phylogenetic tree based on 16S and 18S rRNA sequences. The tree was constructed based on complete 16S and 18S rRNA sequences using the ARB software package. The thick lines represent the (hyper)thermophilic lineages, and the thin lines represent the mesophilic lineages. PFK activity or related sequences identified in the different species on the genomic and/or functional level are marked as follows: *, ADP-PFK; #, ATP-PFK; ^, PP_i -PFK; ○, ADP-PFK activity present; ●, ADP-dependent (sugar) kinase homolog present; ●, both ADP-PFK activity and homologous gene identified.

be important for the ADP-dependent kinases to lose their ATP-dependent activity, as it excludes a nucleotide to adopt the proper position for donating the γ-phosphate [Ito *et al.*, 2001]. However, ATP can be phosphorylated to some extent by several ADP-PFKs (*P. furiosus* [Tuininga *et al.*, 1999], *M. jannaschii*, and *T. zilligii* [Ronimus *et al.*, 1999a]). Interestingly, in the last two sequences a Leu or Ile residue has replaced a Tyr residue. ATP-dependent kinases of the ribokinase family in general have residues with small side chains (Val or Thr) at this position (Figure 1) [Ito *et al.*, 2001]. The eukaryal sequences have more bulky His or Gln residues at the same position. Moreover, adjacent to Ile353 (*M. jannaschii* ADP-PFK numbering), there is a well-conserved Tyr residue (Tyr352) (except for the Phe residue in *M. mazei*) (Figure 1). In all known members of the ATP-dependent ribokinase family, an extra residue is located between Tyr352 and Ile353 (not shown). This elongation of one residue may result in a rearrangement of the main chain to

create an appropriate shape for the recognition of the α-phosphate of ATP in these ATP-dependent enzymes [Ito *et al.*, 2001]. This elongation is absent in the ADP-dependent sugar kinases and their eukaryal homologs. Ile444, close to the catalytic base Asp442 (*M. jannaschii* ADP-PFK numbering) seems to be one of the key residues that determine ADP specificity. These two residues are conserved in all ADP-PFK homologs, as well as in the eukaryal homologs (Figure 1). ATP-dependent ribokinases show a Phe residue at the corresponding position (Figure 1) [Ito *et al.*, 2001]. Hydrophobic amino acids forming a pocket for the right shape of the adenosine moiety are conserved in the eukaryal sequences as well (Figure 1), suggesting that the identified homologs in Eukarya are most likely kinases, with a possible preference for ADP as a phosphoryl donor. Unfortunately, no ADP-GLK/glucose-6-phosphate cocrystals are available at the moment that would allow identification of the sugar binding site of the ADP-dependent sugar kinases. Nevertheless, important residues involved in the binding of sugars in members of the ribokinase family [Ito *et al.*, 2001] seem to be conserved in the ADP-dependent sugar kinases (Figure 1). These residues (Asp28 and Gly106-Gly107 of the *M. jannaschii* ADP-PFK) are conserved in all archaeal homologs but not in the eukaryal homologs. Hence, the structure-based sequence comparisons suggest that the eukaryal homologs are presumably (ADP-dependent) kinases but most likely do not phosphorylate similar sugar substrates.

A phylogenetic tree of the eukaryal and archaeal ADP-dependent (sugar) kinase homologs (not shown) is in good agreement with the dendrogram based on 16S and 18S rRNA sequences (Figure 2), suggesting natural inheritance rather than horizontal gene transfer. This suggests that ADP-dependent kinases may have evolved before the separation of Eukarya and Archaea and, if present in the last common ancestor, were lost from Bacteria. Such a loss may also have occurred in the Crenarchaea and among the Euryarchaea in *Archaeoglobus* (see Addendum), non-glycogen-consuming methanogens, and deeply rooted halophiles (Figure 2). Probably because of their specific physiological characteristics (no Embden-Meyerhof pathway), the selective pressure to maintain these kinases was lost in these organisms. Only the heterotrophic order *Thermococcales*, as well as the glycogen-degrading orders *Methanococcales* and *Methanosarcinales*, are found to contain a functional variant of the Embden-Meyerhof pathway and, as such, benefit from harbouring an ADP-PFK.

The observed presence of ADP-PFK activity and the corresponding genes in the phylogenetically closely related *Pyrococcus* spp. and

Methanococcus spp., as well as in the deeply branched *M. mazei*, suggests that the specific function of ADP-dependent sugar phosphorylation originated at least before the branching of *Thermococcales* and *Methanococcales* and that gene duplication in the order *Thermococcales* has led to two specific enzymes, *i.e.* ADP-PFK and ADP-GLK. The acquired sugar kinases and the gaining of specific glycosyl hydrolases and sugar transporters by *Pyrococcus* spp. [Ettema *et al*., 2001] probably enabled these organisms to ferment sugars. The ADP-PFKs described here most likely function as a key step in a central metabolic pathway. Functional analysis of the eukaryal homologs will be the next step to gain more insight into the evolution of this enzyme family.

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ADDENDUM

While this report was being evaluated, an ADP-GLK and an ADP-PFK activity were described in the starch-degrading *Archaeoglobus fulgidus* strain 7324 [Labes & Schönheit, 2001]. The corresponding genes have not yet been identified. In the genome sequence of type strain VC16, no genes encoding ADP-GLK or ADP-PFK could be identified [Klenk *et al*., 1997].

5

Pyruvate kinase and phosphoenolpyruvate synthase from *Pyrococcus furiosus* and their role in the modified Embden-Meyerhof pathway

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submitted for publication

SUMMARY

Pyrococcus furiosus uses a modified Embden-Meyerhof pathway for growth on sugars. The ATP yield of this pathway appears to be too low to explain the growth yield of the organism. Recently, it was suggested that the organism might use the reversible phosphoenolpyruvate (PEP) synthase in glycolytic direction, thereby gaining additional ATP. However, the organism also possesses a pyruvate kinase. In this study, the role of both enzymes was investigated.

In cell-free extracts of *P. furiosus*, no difference in specific enzyme activity was observed between growth on pyruvate or on either of the sugars cellobiose or maltose. Whereas pyruvate kinase activity remained the same during exponential and stationary phase, the activity of the PEP synthase was considerably higher in the stationary phase, which suggests a role of the enzyme in increasing the ATP yield of the glycolysis during starvation. The PEP synthase and the *E. coli*-produced pyruvate kinase from *P. furiosus* were purified and characterised. The PEP synthase is an unusual 2000-kDa multimeric protein, whereas the pyruvate kinase has a normal 187-kDa tetrameric structure. Opposite to previously reported values, the experimentally determined $\Delta G^{0\prime}$ value of the PEP synthase reaction at 80°C was found to be -8.75 kJ/mol in the glycolytic direction, indicating that in *P. furiosus* this reaction is thermodynamically feasible. Kinetic studies of both enzymes revealed allostericity of the pyruvate kinase for its substrate ADP. Furthermore, addition of 2.5 mM AMP to the pyruvate kinase assay lead to an increase of the $S_{0.5}$ for ADP from 1.17 to 1.65 mM, suggesting that in case of a high intracellular AMP level the PEP synthase, that has an apparent K_m for AMP of 1.0 mM, is favoured.

We present a hypothesis in which the modified Embden-Meyerhof pathway of *P. furiosus* is regulated at the level of PEP by the integrated activities of pyruvate kinase and PEP synthase in glycolytic direction. The relative activities of the enzymes were suggested to be dependent on the energy level of the cell. The catabolic role of the PEP synthase allows the conservation of an extra ATP in the pathway, but it also recycles the AMP that is formed by the two ADP-dependent sugar kinases that are active in the first part of the pathway.

INTRODUCTION

The hyperthermophilic Archaeon *Pyrococcus furiosus* uses a modified Embden-Meyerhof pathway for growth on sugars. In this pathway, a number of novel enzymes are involved. The sugar kinases that catalyse the first steps of the pathway, *i.e.* glucokinase and phosphofructokinase, use ADP instead of ATP as phosphoryl group donor [Kengen *et al*., 1995], [Tuininga *et al*., 1999]. Furthermore, the conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate is catalysed by a tungsten-containing glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) [Mukund & Adams, 1995]. This conversion appears to be not linked to substrate level phosphorylation, thus lowering the ATP yield of the modified Embden-Meyerhof pathway. Apparently, in this glycolysis no net ATP is produced between glucose and pyruvate, thus the only energy-yielding step of the pathway is found in the conversion of acetyl-CoA to acetate, catalysed by acetyl-CoA synthase [Mai & Adams, 1996], [Glasemacher *et al*., 1997]. Based on the stoichiometry of this pathway, it could be expected that growth on sugars or on pyruvate would yield the same amount of energy per mole of substrate utilised. However, in growth yield studies done in batch cultures, it was shown that the growth yield per mol of ATP produced (Y_{ATP}) of *P. furiosus* on maltose and cellobiose is twice as high as that on pyruvate, suggesting an additional site of energy conservation between glucose and pyruvate [Kengen & Stams, 1994b].

Recently, it was suggested that an additional energy-yielding reaction might be the conversion of phosphoenolpyruvate (PEP) into pyruvate. This reaction is usually catalysed by pyruvate kinase (EC 2.7.1.40), that produces ATP from ADP (reaction 1).

$$PEP + ADP + H^+ \rightarrow pyruvate + ATP \qquad (1)$$

In *P. furiosus*, however, the reaction might be catalysed by PEP synthase (EC 2.7.9.2) in a reversal of its gluconeogenic reaction, thereby producing ATP from AMP, thus generating two instead of one energy-rich phosphate bonds in this step [Sakuraba *et al*., 1999], [Sakuraba *et al*., 2001] (reaction 2).

$$PEP + AMP + P_i + 2\ H^+ \leftrightarrow pyruvate + ATP + H_2O \qquad (2)$$

Although the specific activity of the purified PEP synthase was described to be 44 times higher in glycolytic than in gluconeogenic direction

[Sakuraba *et al.*, 1999] and the transcription of the PEP synthase encoding gene is enhanced in the presence of maltose [Sakuraba *et al.*, 2001], the catalytic efficiency of the enzyme is at least 100-fold higher in gluconeogenic direction than in glycolytic direction [Hutchins *et al.*, 2001]. Thus, the physiological role of the PEP synthase is not clear yet. Furthermore, the presence of a pyruvate kinase in the organism [Schäfer & Schönheit, 1992], [Sakuraba *et al.*, 1999] raises the question why the organism possesses a pyruvate kinase if it can use the PEP synthase in glycolytic direction, thereby producing extra ATP.

In this paper we describe the purification and characterisation of both the PEP synthase and the pyruvate kinase from *P. furiosus* in order to get more insight into their respective role in glycolysis. Furthermore, we investigated the influence of the growth substrate of the organism on the activity of both enzymes and measured the equilibrium constant and $\Delta G^{0\prime}$ value of the PEP synthase.

MATERIALS AND METHODS

Materials

Lactate dehydrogenase (EC 1.1.1.27; type II from rabbit muscle), myokinase (EC 2.7.4.3; from rabbit muscle) and pyruvate kinase (EC 2.7.1.40; type II from rabbit muscle) were obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), hexokinase/glucose-6-phosphate dehydrogenase (EC 2.7.1.1/1.1.1.49; from yeast) was obtained from Roche Molecular Biochemicals (Almere, The Netherlands). *Pfu* turbo polymerase was purchased from Stratagene (La Jolla, CA, USA). All other chemicals were of analytical grade.

Pyrococcus furiosus (DSM 3638) was obtained form the German Collection of Micro-organisms (Braunschweig, Germany). *Escherichia coli* XL-1 Blue and *E. coli* BL21(DE3) were obtained from Stratagene (La Jolla, CA, USA). The expression vector pET24d was obtained from Novagen (Madison, WI, USA).

Organisms and growth conditions

To determine the influence of the growth substrate on the activity of pyruvate kinase and PEP synthase, *P. furiosus* was cultured in stoppered 500-

ml serum bottles, containing 250 ml of artificial seawater, as previously described [Kengen *et al*., 1993]. Cellobiose (5 mM), maltose (10 mM), or pyruvate (40 mM) was used as substrate and cells were grown in a rotary shaker at 95°C. Cultures were transferred at least five times on the same substrate before preparing cell-free extracts. Growth was followed by measuring the production of hydrogen gas by GC analysis.

For purification of the enzymes, *Pyrococcus furiosus* (DSM 3638) was mass-cultured (200 litres) in an N_2-sparged artificial seawater medium supplemented with Na_2WO_4 (10 µM), yeast extract (1 g/litre), and vitamins using potato starch (8 g/litre) as a substrate as described before [Tuininga *et al.*, 1999].

E. coli XL1 was used as a host for the construction of pET24d derivatives and *E. coli* BL21(DE3) was used as an expression host. Both strains were grown in Luria Bertani medium with 50 µg/ml kanamycin in a rotary shaker at 37°C.

Preparation of cell-free extract from *P. furiosus*

From 250-ml cultures, cells were harvested in the log phase (after 9 h of growth) and the stationary phase (after overnight growth). In both cases, the cultures were centrifuged at 16,000 × g for 15 min, the cells were resuspended in 1 ml 20 mM Tris/HCl pH 7.8 and sonicated three times 30 sec on ice. Cell debris was removed by another centrifugation step (16,000 × g for 15 min) and supernatant was used for enzyme activity measurements.

P. furiosus cells from a 200-litre culture were harvested as described before [Tuininga *et al.*, 1999]. Cell-free extract was prepared by suspending cells in 2 volumes (w/v) of 50 mM Tris/HCl pH 7.8 with 10 µg/ml DNase I and passing through a French press at 100 MPa. Cell debris was removed by ultracentrifugation at 100,000 × g for 1 h at 10°C. The supernatant was used for purification of the enzymes.

Purification of the PEP synthase from *P. furiosus*

All steps were done without protection against oxygen. Cell-free extract was brought to 35% ammonium sulphate saturation (1.5 M) at 0°C for 2 hours. After centrifugation the supernatant was filtered through a 0.2 µm filter and applied to a 30-ml Phenyl Sepharose column equilibrated with 100 mM Tris/HCl pH 7.7 containing 1.5 M ammonium sulphate. Following a 240-ml linear gradient from 1.5 - 0 M ammonium sulphate, the PEP synthase

eluted at ca. 200 mM ammonium sulphate. Fractions containing PEP synthase activity were pooled and desalted by ultrafiltration using 100 mM Tris/HCl pH 7.7.

The PEP synthase pool was loaded on a Mono Q HR 5/5 column, equilibrated with 100 mM Tris/HCl pH 7.7. The PEP synthase eluted at ca. 300 mM NaCl in a linear 60-ml gradient from 0 - 1 M NaCl. Because some activity was observed in the flowthrough, this was applied to the column again, using the same conditions. In this second step, PEP synthase eluted at the same NaCl concentration and no activity was found in the flowthrough. Active fractions of both trials were pooled and concentrated by ultrafiltration.

The concentrated pool was applied to a 33-ml (1.2 * 29 cm) Sephacryl S-400 column, equilibrated with 100 mM Tris/HCl pH 7.7 containing 100 mM NaCl. Active fractions from this column were pooled, concentrated by ultrafiltration and this concentrated pool was used for characterisation of the enzyme.

Cloning and overexpression of the pyruvate kinase gene

The pyruvate kinase gene that was identified in the *P. furiosus* data base (gene number PF1188) [www.ncbi.nlm.nih.gov], was amplified by PCR, using primers BG559 (5'-GCGCGTCATGAGAAGGGTGAAGCTCCCATCT, sense) and BG560 (5'-GCGCGGATCCTCAAGCTATTTGGAATATCTTAAT-TGAG, antisense) with *Bsp*HI and *Bam*HI restriction sites underlined. By introduction of the *Bsp*HI restriction site, the N-terminal Val residue was replaced by a Met. The 100-µl polymerase chain reaction mixture contained 10 ng of *P. furiosus* DNA, isolated as described before [Sambrook *et al*., 1989], 100 ng each of primer BG559 and BG560, 0.2 mM dNTPs, *Pfu* PCR buffer, and 5 units (U) of *Pfu* turbo polymerase and was subjected to 30 cycles of amplification (1 min at 94°C, 1 min at 60°C, and 2 min at 72°C) on a DNA Thermal Cycler (Perkin-Elmer Cetus). The PCR product was digested (*Bsp*HI/*Bam*HI) and cloned into an *Nco*I/*Bam*HI-digested pET24d vector, resulting in pWUR134, which was transformed into *E. coli* XL1 and BL21(DE3).

An overnight culture of *E. coli* BL21(DE3) containing pWUR134 was used as a 1% inoculum in 500 ml of Luria Bertani medium with 50 µg/ml kanamycin. After growth at 37°C until an OD_{600} of approximately 0.8, expression of the gene was induced by adding 0.1 mM IPTG. After overnight growth at 37°C, cells were harvested by centrifugation (19000 × g for 30 min) and resuspended in 10 ml 20 mM Tris/HCl buffer, pH 7.8. The suspension

was passed through a French press at 100 MPa, and subsequently centrifuged (31000 × g for 30 min). The supernatant was designated soluble fraction. The pellet was resuspended in 10 ml 20 mM Tris/HCl buffer pH 7.8, and designated insoluble fraction. Because highest pyruvate kinase activity was found in the insoluble fraction, attempts were made to solubilise the pyruvate kinase from this fraction.

Purification of the *E. coli*-produced pyruvate kinase

To solubilise the pyruvate kinase activity from the insoluble fraction, 1-ml samples of the fraction were diluted 10 times in 20 mM Tris/HCl buffer pH 7.8 containing 20 mM CHAPS, 1% Triton X-100, 20% glycerol, or no detergent, respectively. After stirring of the suspensions for 1, 2, 4, and 8 h at room temperature, the samples were centrifuged. Pyruvate kinase activity was measured in the supernatant and the resuspended pellet. Best results were obtained with 20% glycerol and therefore this was used to solubilise the pyruvate kinase from the remaining insoluble fraction.

The insoluble fraction was diluted 10 times in 20 mM Tris/HCl buffer pH 7.8 containing 20% glycerol (final concentration). The suspension was stirred for 16 h at room temperature and subsequently centrifuged for 20 min at 19,000 × g. The 150-ml supernatant was heated for 30 min at 80°C. Denaturated proteins were removed by centrifugation (19,000 × g for 20 min). The supernatant was concentrated approximately 3 times to 45 ml by ultrafiltration and loaded onto a 68-ml Q Sepharose column equilibrated with 100 mM Tris/HCl buffer pH 7.8. Proteins were eluted in a gradient of 0 - 0.5 mM NaCl in 3.5 column volumes and 0.5 - 1 M NaCl in 0.5 column volumes. Pyruvate kinase activity eluted in a very broad peak, and therefore the fractions containing activity were pooled, desalted, and loaded onto a Mono Q HR 5/5 column, equilibrated with 100 mM Tris/HCl buffer pH 9.0. A linear gradient of 60 ml from 0 - 1 M NaCl was applied. Active fractions were pooled, concentrated and loaded onto a Superdex 200 gelfiltration column equilibrated with 100 mM Tris/HCl buffer pH 7.8 containing 100 mM NaCl. Fractions containing pyruvate kinase activity were pooled and the purified protein was used for enzyme characterisation.

Protein concentration and purity

Protein concentrations were determined with Coomassie Brilliant Blue G250 as described before [Bradford, 1976]. The purity of the enzymes was

checked by SDS-PAGE as described before [Kengen *et al.*, 1993]. Protein samples for SDS-PAGE were diluted 2:1 with sample buffer and heated for 10 min at 100°C.

Determination of enzyme activity

All enzyme assays were done aerobically at different temperatures, depending on the presence of auxiliary enzymes. Specific activities were calculated from initial linear rates and expressed in units (U) per mg of protein. One unit was defined as the amount of enzyme required to convert 1 µmol of substrate per min.

PEP synthase activity was measured in both directions. To measure activity in the PEP-forming direction, a discontinuous assay was used that was modified from [Eyzaguirre *et al.*, 1982]. The 1-ml assay mixture contained 100 mM Tricine/KOH (pH 8.0), 20 mM $MgCl_2$, 2 mM DTT, 40 mM NH_4Cl (pH 8.0), 5 mM pyruvate, 2 mM ATP, and 25 µl of enzyme preparation. The assay mixture was incubated at 85°C for 20 min. Every 5 min a 25-µl sample was taken and immediately put on ice. The phosphate concentration in the samples was determined with a malachite green assay, by adding 200 µl freshly prepared malachite green reagent and 775 µl water to the 25-µl sample. After 10 min at room temperature, the OD_{630} was measured and compared to a phosphate calibration curve of 0 - 2 mM phosphate. The reagent consisted of 2.5 ml 7.5% ammonium molybdate, 0.2 ml Tween 20, and 10 ml colouring solution (150 ml water, 30 ml concentrated H_2SO_4, and 0.22 g malachite green). Phosphate formation was strictly dependent on pyruvate and ATP. No significant phosphate formation due to PEP hydrolysis caused by thermal instability could be detected (maximally 0.0027 mM per min when 0.5 mM of phosphoenolpyruvate was added at the start of the reaction).

For measurement of PEP synthase activity in the pyruvate-forming direction, a continuous assay was done in stoppered 1-ml quartz cuvettes at 50°C. Pyruvate formation was coupled to the oxidation of NADH by lactate dehydrogenase. The assay mixture contained 100 mM MES buffer (pH 6.5), 10 mM $MgCl_2$, 25 mM KH_2PO_4, 0.2 mM NADH, 5 mM AMP, 5 mM PEP, 13.4 U of lactate dehydrogenase, and 25 µl of enzyme preparation. The reaction was followed by monitoring NADH oxidation at 340 nm (ε = 6.18 $mM^{-1}cm^{-1}$). A drawback of this assay is that the NADH oxidase activity present in cell-free extracts of *P. furiosus* [Ward *et al.*, 2001] caused a background decrease of NADH, thereby making accurate PEP synthase activity determination in crude extracts difficult. This background NAD(P)H-oxidising activity also

interfered in an alternative assay with hexokinase and glucose-6-phosphate dehydrogenase. Because of the high affinity of the NADH oxidase for oxygen, the problem could not be solved by doing the assays anaerobically.

Therefore, a discontinuous assay at 85°C was used alternatively. The 1-ml assay mixture contained 100 mM Tris/HCl buffer (pH 7.8), 20 mM $MgCl_2$, 2.5 mM PEP, 2.5 mM AMP, 12.5 mM KH_2PO_4, and 25 µl of enzyme preparation. Eppendorf tubes containing the assay mixture were incubated at 85°C during 30 min. Every 5 min, a 50-µl sample was taken and directly cooled on ice. The pyruvate concentration in the samples was measured in a separate assay. The assay mixture contained 100 mM Tris/HCl buffer (pH 7.8), 0.125 mM NADH, 20 µl of sample, and 13.4 U of lactate dehydrogenase. The change in OD_{340} upon addition of the lactate dehydrogenase was measured and compared to that of a calibration curve of pyruvate (0 - 5 mM).

Pyruvate kinase activity was measured in a continuous assay coupling the formation of pyruvate to the oxidation of NADH by lactate dehydrogenase. The assay was done in stoppered 1-ml quartz cuvettes at 50°C. The assay mixture contained 100 mM Tricine/KOH buffer (pH 8.0), 20 mM $MgCl_2$, 0.2 mM NADH, 2.5 mM AMP, 2.5 mM PEP, 13.4 U of lactate dehydrogenase, and 5 - 25 µl of enzyme preparation. The reaction was followed by monitoring NADH oxidation at 340 nm ($\varepsilon = 6.18\ mM^{-1}cm^{-1}$). As in the continuous PEP synthase assay, the NADH-oxidising activity present in cell-free extracts of *P. furiosus* caused a background decrease of NADH, thereby making accurate pyruvate kinase activity determination in crude extracts difficult.

To measure pyruvate kinase activity at a more physiological temperature of 85°C, a discontinuous assay was used, that was similar to the discontinuous PEP synthase assay in the pyruvate-forming direction, as described above. In this assay, the AMP was replaced with 2.5 mM ADP and phosphate was omitted.

Determination of enzymatic properties

The molecular mass of the purified pyruvate kinase was determined on a Superdex 200 gel filtration column. The column was calibrated using the following standard proteins: ribonuclease (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (2,000 kDa).

The molecular mass of the purified PEP synthase was roughly determined by electron microscopy. The purified protein was dialysed against distilled water to obtain a protein solution with a Tris concentration below 0.01 mM. The suspension was deposited on a 400-mesh copper TEM grid covered with a carbon-strengthened formvar membrane and stained with 2% uranyl acetate. Samples were examined in a Philips type CM12 transmission electron microscope.

The pH optimum of the pyruvate kinase was determined at 50°C in 50 mM Tris/maleate buffer over the pH range 5.0 - 8.0. Buffer pH values were adjusted at 50°C. Care was taken that the lactate dehydrogenase was not limiting at the various pH values.

The divalent cation requirement of the pyruvate kinase was tested by replacing the $MgCl_2$ in the enzyme assay with 10 mM of $CaCl_2$, $CoCl_2$, $MnCl_2$, or $ZnCl_2$.

Kinetic parameters of the PEP synthase for ATP and pyruvate were determined using the discontinuous enzyme assay at 85°C. The concentration of ATP was varied (0.01 - 5 mM) in the presence of 5 mM pyruvate and the concentration of pyruvate was varied (0.05 - 5 mM) in the presence of 2 mM ATP. After incubation for 5 min, the reaction was stopped and the activity was measured by phosphate determination as described above. In a pilot experiment it was confirmed that the activity was linear during this incubation time. Data were analysed by computer-aided fit to the Michaelis-Menten curve.

Kinetic parameters of the pyruvate kinase were determined at 50°C by varying the concentration of ADP (0.05 - 10 mM) or phosphoenolpyruvate (0.05 - 10 mM) in the assay mixture in the presence of 2 mM phospho-enolpyruvate or 2.5 mM ADP, respectively. Data were analysed by computer-aided direct fit to the Michaelis-Menten curve and to a sigmoidal curve. Furthermore, the data were used to construct Hill plots (log (V/V_{max} - V) versus log S).

Regulation of pyruvate kinase activity by possible allosteric effectors was tested by adding ATP, AMP (2.5 mM), or phosphorylated glycolytic intermediates (glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate; 2 - 4 mM) to the assay mixture. Furthermore, the effect of KCl and $CaCl_2$ (10 - 20 mM) on the enzyme activity was tested. Regulation of PEP synthase activity in glycolytic direction was tested by adding ATP, ADP, or fructose-1,6-bisphosphate to the assay mixture, using the same concentrations as for the pyruvate kinase.

Determination of equilibrium constant and $\Delta G^{0''}$ value

The equilibrium constant of the PEP synthase reaction was determined by incubating the enzyme at 80°C for 20 min in the presence of the reactants and products involved in the reaction (2), until an equilibrium was attained.

$$PEP + AMP + P_i + 2\ H^+ \leftrightarrow pyruvate + ATP + H_2O \qquad (2)$$

The 400-µl assay mixture contained 100 mM Tricine/KOH buffer pH 8.0, 20 mM $MgCl_2$, 2 mM DTT, 40 mM NH_4Cl (pH 8.0), 1 mM pyruvate, 1 mM ATP, 5 mM PEP, 5 mM AMP, 20 mM KH_2PO_4, and 5 µl of the purified PEP synthase. In a second approach, the initial concentrations of the reactants and products was changed so that the equilibrium was attained from the opposite direction. In this assay, concentrations of 5 mM pyruvate, 5 mM ATP, 1 mM PEP, and 1 mM AMP were used and phosphate was omitted. At various points in time, the reaction was stopped by adding 50 µl 50% perchloric acid and neutralised by adding 550 µl 2 M Tris/HCl buffer pH 8.0. The samples were kept on ice until the concentrations of the reactants and products were determined in separate assays.

For pyruvate determination, a 40-µl sample was added to a 1-ml assay containing 100 mM Tris/HCl buffer (pH 8.0), 0.125 mM NADH, and 13.4 U of lactate dehydrogenase. The change in OD_{340} after addition of the lactate dehydrogenase was compared to that of a calibration curve of pyruvate (0 - 5 mM).

PEP was determined in a 1-ml assay containing 100 mM HEPES/KOH buffer (pH 7.5), 2.5 mM ADP, 0.125 mM NADH, 20 µl of sample, 13.4 U of lactate dehydrogenase, and 3.6 U of pyruvate kinase. The change in OD_{340} after addition of the pyruvate kinase was compared to that of a calibration curve of phosphoenolpyruvate (0 - 10 mM).

The concentration of ATP was determined in a 1-ml assay containing 100 mM Tris/HCl buffer (pH 8.0), 10 mM $MgCl_2$, 5 mM glucose, 0.125 mM NADP, 40 µl sample, 1.4 U of hexokinase and 0.7 U of glucose-6-phosphate dehydrogenase. The change in OD_{340} after addition of the hexokinase/ glucose-6-phosphate dehydrogenase mixture was compared to that of a calibration curve of ATP (0 -5 mM).

Phosphate concentration was measured using the malachite green assay as described above. The concentration of AMP was not determined, but the change in concentration of AMP and PEP were assumed to be equal.

From the concentrations determined in an equilibrium situation, the equilibrium constant K_{eq}'' at pH 8.0 was calculated using equation 3:

$$K_{eq}'' = [\text{pyruvate}]*[\text{ATP}]*[H_2O] / [\text{PEP}]*[\text{AMP}]*[\text{phosphate}]*[H^+]^2 \quad (3)$$

Although water acts as a reactant, its concentration was taken as unity and therefore omitted from equation 3. Furthermore, since the reaction takes place in a buffered environment, the proton concentration was assumed to be constant during the reaction and was therefore also omitted from the equation. Thus, the equilibrium constant at pH 8.0 was calculated according to equation 4:

$$K_{eq}'' = [\text{pyruvate}]*[\text{ATP}] / [\text{PEP}]*[\text{AMP}]*[\text{phosphate}] \quad (4)$$

The $\Delta G^{0''}_{obs}$ value at pH 8.0 was calculated from this constant as $-RT \ln K_{eq}''$.

RESULTS

PEP synthase and pyruvate kinase activity on different substrates

To investigate whether the enzymes are induced by growth on pyruvate, cellobiose or maltose, activity of pyruvate kinase and PEP synthase in the glycolytic direction, *i.e.* the pyruvate-forming direction, was measured at 85°C in cell-free extracts from cells grown on the respective substrates, both in exponential and in stationary phase (Figure 1). There was no obvious difference in activity of the enzymes between the glycolytic (cellobiose, maltose) and the gluconeogenic (pyruvate) substrates. In the exponential phase, the activities of pyruvate kinase ranged from 0.072 to 0.11 U/mg and the PEP synthase varied between 0.025 and 0.053 U/mg. In the stationary phase the pyruvate kinase activity was between 0.10 and 0.17 U/mg, whereas the PEP synthase activities varied between 0.17 and 0.26 U/mg. Based on these results no conclusions can be drawn on the role of both enzymes in glycolysis. It is however obvious that the ratio between the activities of both enzymes in glycolytic direction changed during growth. During the exponential phase the pyruvate kinase activity was 1.4 - 3.6 times higher than the PEP synthase activity, while the opposite was found for the stationary phase, when the PEP synthase was 1.4 - 2.6 times higher. In cells harvested

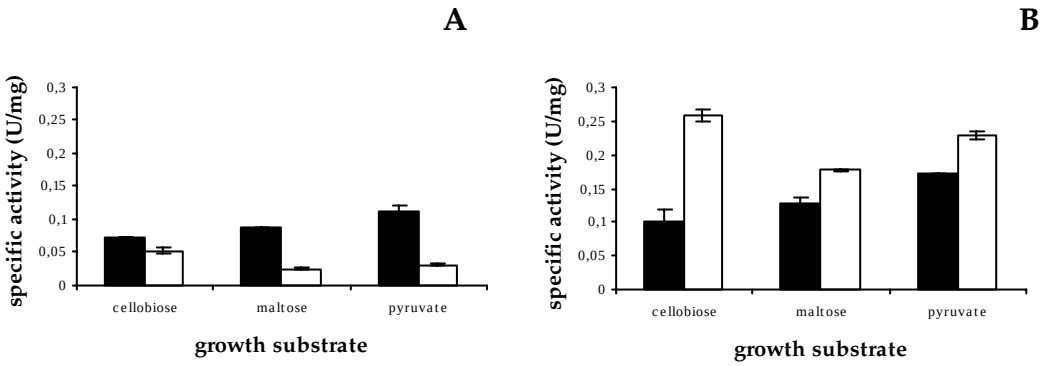

FIGURE 1 Specific activity of pyruvate kinase and PEP synthase in glycolytic direction in cell-free extracts of *P. furiosus* grown on different substrates. Activities were measured aerobically at 85°C in discontinuous assays, as given in "Materials and Methods". For each growth substrate, the bars indicate the specific activity in glycolytic direction of pyruvate kinase (black) and PEP synthase activity in glycolytic direction (white). A. Cells harvested after 9 h of growth (exponential phase). B. Cells harvested after 16 h of growth (stationary phase).

during exponential growth, the pyruvate kinase activity was only slightly higher than in cells from the stationary phase, whereas the PEP synthase activity was much higher in the stationary-phase cells than in the exponential-phase cells. Apparently, the pyruvate kinase is constitutively present throughout the growth curve, whereas the PEP synthase activity increases at the end of the growth.

Additionally, the PEP synthase activity was measured in the gluconeogenic direction, *i.e.* the PEP-forming direction. In all tested extracts, in exponential phase as well as in stationary phase, the PEP synthase activity in gluconeogenic direction was at least twelve-fold higher than in glycolytic direction (data not shown).

Purification and characterisation of the PEP synthase

The PEP synthase was purified under aerobic conditions, because no loss of activity was observed upon storage at 4°C or -20°C under air. During initial purification, it was observed that the PEP synthase eluted in the void volume of a Superdex 200 gel filtration column, indicating a very large native molecular mass of the protein. Therefore, a Sephacryl S-400 column specific for purification of proteins between 20 and 8000 kDa was included in the purification procedure. From this 33-ml column, the PEP synthase eluted after approximately 14 ml. Unfortunately, we were not able to determine the molecular mass of the PEP synthase from its elution volume because no calibration proteins are available for this type of gelfiltration column. A rough

estimate based on the elution volume would suggest a molecular mass between 1000 and 8000 kDa.

After four purification steps, the protein was purified to ca. 80% homogeneity (not shown). This purified PEP synthase was used for further characterisation.

Physical characterisation of the PEP synthase

Electron microscopy of the purified PEP synthase showed globular particles with an approximate diameter of 17.5 nm, with a central cavity (Figure 2). This configuration is highly similar to the structure of the multimeric archaeal PEP-synthase (MAPS) from *Staphylothermus marinus*. Additional STEM mass measurements of the MAPS protein showed a native molecular mass of ca. 2000 kDa [Cicicopol *et al*., 1994], [Harauz *et al*., 1996], [Cicicopol *et al*., 1999]. Since the subunit molecular mass of the PEP synthase from both *Staphylothermus marinus* and *P. furiosus* is ca. 93 kDa, we assume that the *P. furiosus* PEP synthase has a similar 24-subunit multimeric structure with a native molecular mass of ca. 2000 kDa.

Purification of the pyruvate kinase

Despite the use of several chromatographic techniques, we were not able to purify the pyruvate kinase from cell-free extract of *P. furiosus*, because it tended to stick to other proteins, resulting in similar band patterns in SDS-PAGE after each purification step (not shown). When the protein was applied to a Hydroxyapatite or a hydrophobic interaction column, the activity of the

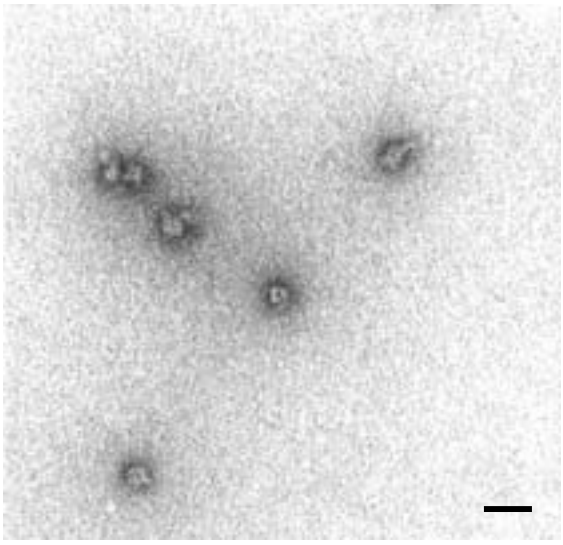

FIGURE 2 Electron micrograph of purified PEP synthase from *P. furiosus* negatively stained with uranyl acetate. The bar indicates a size of 50 nm.

enzyme was completely lost. Therefore, these columns could not be used for purification.

Alternatively, the pyruvate kinase gene of *P. furiosus* was cloned into a pET24d vector, resulting in pWUR134, which was transformed into *E. coli* XL1 and BL21(DE3). Both the soluble and the insoluble fraction of cells harvested from an *E. coli* BL21 (DE3) culture containing plasmid pWUR134 were analysed on SDS-PAGE. The insoluble fraction revealed an additional band of approximately 50 kDa, which corresponded with the calculated molecular mass (53 kDa) of the gene product. The soluble fraction showed a faint extra band at the same position. In both fractions, thermoactive pyruvate kinase activity was measured at 50°C, which was absent in extracts of *E. coli* BL21 (DE3) harbouring the pET24d plasmid without insert. Although the specific activities in the soluble and the insoluble fraction were comparable (9.0 and 16.5 U/mg, respectively), the total activity in the soluble fraction (81 U) was much lower than that in the insoluble fraction (1766 U) and therefore the latter fraction was used for purification of the pyruvate kinase.

Although most of the pyruvate kinase was present in the insoluble fraction, the enzyme was active, indicating a correct folding. Therefore, attempts were made to solubilise the enzyme as was described before for purification of an α-amylase from *Pyrococcus woesei* expressed in *E. coli* [Linden *et al*., 2000]. Stirring in buffer without detergent as well as in buffer with Triton X-100 increased the activity of pyruvate kinase in the soluble fraction by ca. 40%, while stirring with glycerol and CHAPS nearly doubled the soluble pyruvate kinase activity after 8 hours. Therefore, the remaining insoluble fraction was stirred with glycerol overnight.

Approximately 25% of the pyruvate kinase could be solubilised from the insoluble fraction by overnight stirring in buffer containing 20% glycerol and subsequent centrifugation. The pyruvate kinase was subsequently purified by heat incubation, anion exchange chromatography and gel filtration chromatography to at least 95% homogeneity (not shown).

Characterisation of the *E. coli*-produced pyruvate kinase

SDS-PAGE of the purified recombinant pyruvate kinase gave a single band around the expected size of 53 kDa (not shown). The native molecular mass of the protein as determined by gel filtration chromatography was approximately 187 kDa, indicating that the pyruvate kinase is a homotetramer.

The specific activity of the purified pyruvate kinase was 41.4 U/mg at 50°C. The enzyme showed activity between pH 6.0 and 8.0, with an optimum pH of 6.5 (data not shown). The pyruvate kinase required divalent cations for activity, as shown by complete lack of activity in the presence of EDTA. Highest activity was found in the presence of $CoCl_2$, followed by $MgCl_2$ and $MnCl_2$ (Table 1). Although $CoCl_2$ gave a much higher activity than $MgCl_2$, the assays were done in the presence of $MgCl_2$, being physiologically the most important cofactor.

Kinetic parameters of the PEP synthase and pyruvate kinase

The PEP synthase showed Michaelis-Menten kinetics for both pyruvate and ATP. Apparent K_m values were calculated as 0.49 ± 0.06 mM for pyruvate and 0.48 ± 0.08 mM for ATP. Kinetic parameters of the PEP synthase in glycolytic direction were previously described by [Sakuraba *et al.*, 1999] and [Hutchins *et al.*, 2001]. Table 2 shows the K_m and V_{max} values of the PEP synthase for both directions.

The pyruvate kinase showed Michaelis-Menten kinetics for PEP, whereas a sigmoidal saturation curve was obtained with ADP, indicating positive cooperativity of this substrate (Figure 3). Table 2 shows the K_m or $S_{0.5}$ values, V_{max} values, and Hill coefficients for both substrates of the pyruvate kinase.

Allosteric effectors of the PEP synthase and pyruvate kinase

Addition of 2.5 mM ATP to the PEP synthase assay resulted in a 33% lower activity. The addition of 2.5 mM ADP had no significant effect. Furthermore, the enzyme was slightly inhibited by the presence of 4 mM fructose-1,6-bisphosphate.

TABLE 1 Divalent cation requirement of the pyruvate kinase. Enzyme assays were done at 50°C as described under "Materials and Methods". 100% activity corresponds to a specific activity of 41.4 U/mg, in the presence of Mg^{2+}.

divalent cation	relative activity %
Mg^{2+}	100
Co^{2+}	183
Mn^{2+}	79
Ca^{2+}	1
Zn^{2+}	ND[a]

[a] ND, not detectable.

TABLE 2 Kinetic constants of pyruvate kinase and PEP synthase of *P. furiosus*. Activity assays were done as described in "Materials and Methods". Pyruvate kinase assays were done at 50°C, PEP synthase assays were done at 85°C.

substrate		pyruvate kinase	PEP synthase	
PEP	K_m (mM)	0.35 ± 0.04	0.40[a]	1.0[b]
	V_{max} (U/mg)	42.3 ± 0.95	0.14[a]	13.3[b]
ADP	$S_{0.5}$ (mM)	1.17 ± 0.04		
	V_{max} (U/mg)	43.4 ± 0.6		
	Hill coefficient	1.59 ± 0.08		
AMP	K_m (mM)		1.00[a]	0.10[b]
	V_{max} (U/mg)		0.095[a]	
phosphate	K_m (mM)		38.4[a]	35[b]
	V_{max} (U/mg)		0.13[a]	
pyruvate	K_m (mM)	0.49 ± 0.06	0.11[a]	
	V_{max} (U/mg)	28.4 ± 1.1	17.1[a]	0.3[b]
ATP	K_m (mM)	0.48 ± 0.08	0.39[a]	
	V_{max} (U/mg)	33.8 ± 1.8	14.4[a]	

[a] Data from [Hutchins *et al.*, 2001] determined at 80°C.
[b] Data from [Sakuraba *et al.*, 1999] determined at 50°C.

None of the tested possible allosteric effectors, *i.e.* glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate, had a significant effect on pyruvate kinase activity at saturating concentrations of the substrates. Furthermore, addition of KCl had no effect, indicating that the enzyme is independent of K^+ ions. However, addition of $CaCl_2$ had an inhibitory effect on pyruvate kinase activity (27% remaining activity at 20 mM).

Because the pyruvate kinase showed a sigmoidal saturation curve for ADP, the effect of AMP and ATP on the enzyme activity was investigated in more detail. Upon the addition of 2.5 mM AMP or ATP to the activity assay, the shape of the saturation curve of ADP changed (Figure 3). In the presence of AMP, the $S_{0.5}$ for ADP increased from 1.17 to 1.65 mM, whereas the V_{max} value did not change. However, in the presence of ATP, the V_{max} value decreased from 43.4 to 34.4 U/mg while the $S_{0.5}$ values remained practically unchanged.

Determination of equilibrium constant and $\Delta G^{0\prime\prime}$ values of PEP synthase

PEP synthase was incubated at 80°C in the presence of PEP, pyruvate, phosphate, AMP, and ATP. In two separate assays, with a high and a low starting concentration of PEP respectively, an incubation time of 20 min was

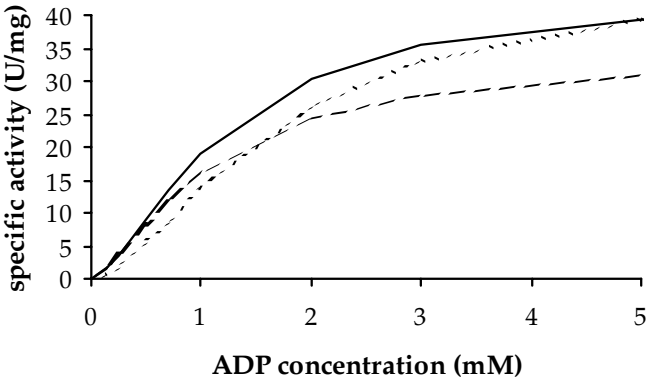

FIGURE 3 ADP saturation curve of the recombinant pyruvate kinase of *P. furiosus*. Pyruvate kinase activity was measured at 50°C as described under "Materials and Methods". _____ without additions, ……. in presence of 2.5 mM AMP, ----- in presence of 2.5 mM ATP.

enough to obtain an equilibrium situation (Figure 4). From the concentrations determined in the equilibrium state, the equilibrium constant K_{eq}'' and the $\Delta G^{0\prime\prime}_{obs}$ value were calculated. Both assays resulted in similar values for K_{eq}'' (22.9 and 16.6; average 19.75 M^{-1}) and $\Delta G^{0\prime\prime}_{obs}$ (-9.2 and -8.3; average -8.75 kJ/mol), indicating that a true equilibrium was established. Remarkably, in contrast to data in literature ($\Delta G^{0\prime}$ of -12 kJ/mol in gluconeogenic direction) [Eyzaguirre *et al.*, 1982], [Thauer *et al.*, 1977], the $\Delta G^{0\prime\prime}_{obs}$ was negative in glycolytic direction, indicating that the PEP synthase reaction is thermodynamically feasible in this direction at 80°C.

The equilibrium constant at 80°C for pyruvate kinase was impossible to determine, because the equilibrium lies too far towards pyruvate formation to accurately measure PEP and pyruvate concentrations in the reaction vial. This indicates that the $\Delta G^{0\prime\prime}$ value for the pyruvate kinase reaction is negative in the direction of pyruvate formation, as was described in literature [Thauer *et al.*, 1977].

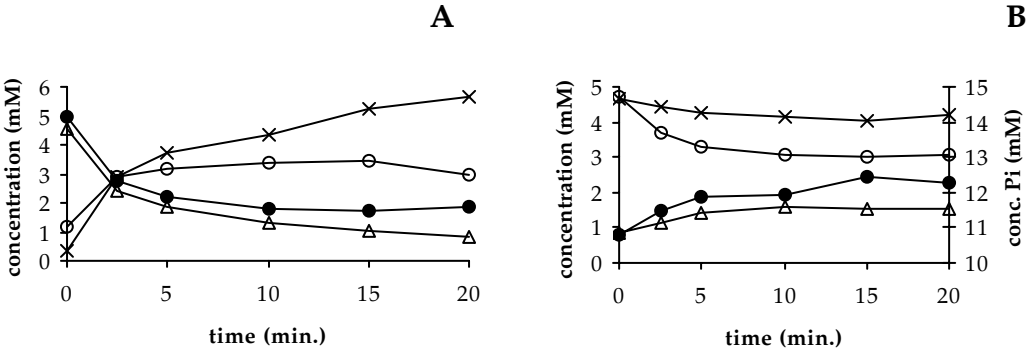

FIGURE 4 Equilibrium of *P. furiosus* PEP synthase reaction at 80°C. The equilibrium was approached from a high PEP and phosphate concentration (A) and from a high pyruvate and ATP concentration (B). In both cases the reactants were indicated as follows: pyruvate (•), PEP (o), ATP (Δ), and phosphate (×).

DISCUSSION

In this study, we describe the purification and characterisation of the PEP synthase and the pyruvate kinase from *P. furiosus*. Both enzymes are able to convert PEP into pyruvate, but whereas pyruvate kinase catalyses a virtually irreversible reaction, the PEP synthase reaction is known to be reversible. Usually, PEP synthase is active in gluconeogenesis, converting pyruvate into PEP at the expense of ATP. However, if the enzyme would be physiologically active in glycolysis, as was suggested before [Sakuraba *et al.*, 1999], this would have important consequences for the bioenergetics of the organism. In that case pyruvate and ATP would be produced from PEP, phosphate and AMP, thus two energy-rich phosphate bonds would be created in one step. The free energy of hydrolysis of both phosphate bonds in ATP is equal [Thauer *et al.*, 1977] and therefore, the conversion of 1 AMP into 1 ATP corresponds to the formation of 2 ATP from 2 ADP. Thus, the suggested physiological activity of the PEP synthase in glycolysis would increase the ATP yield of the pathway from 0 to 2 ATP per glucose converted (Figure 5). This increase in the yield of ATP could account for the extra ATP-conserving site suggested by [Kengen & Stams, 1994b] that is needed to explain the differences in molar growth yields reported for growth of *P. furiosus* on pyruvate and sugars. However, if the organism can gain extra energy by using the PEP synthase in glycolytic direction, the function of the pyruvate kinase is not clear. This enzyme was detected in cell-free extracts of *P. furiosus* [Schäfer & Schönheit, 1992], [Sakuraba *et al.*, 1999] and can be expected to play its classical role in the Embden-Meyerhof pathway. In addition, if *P. furiosus* can gain extra energy by using the PEP synthase in this direction, it is unclear why not all other organisms do so. To clarify these points, we purified and characterised both enzymes and investigated their role in the glycolytic pathway of *P. furiosus*.

In cell-free extracts of *P. furiosus* grown on cellobiose, maltose, and pyruvate, no significant difference was observed in the activities of pyruvate kinase and PEP synthase. This suggests that both enzymes are constitutively expressed instead of being induced by growth on either sugars or pyruvate, and does not allow to draw any conclusions on the physiological role of either of the enzymes. However, the activity of the PEP synthase was much higher in gluconeogenic than in glycolytic direction, which indicates an anabolic rather than a catabolic role of the enzyme. Remarkably, when enzyme activities were compared between cells harvested during exponential growth

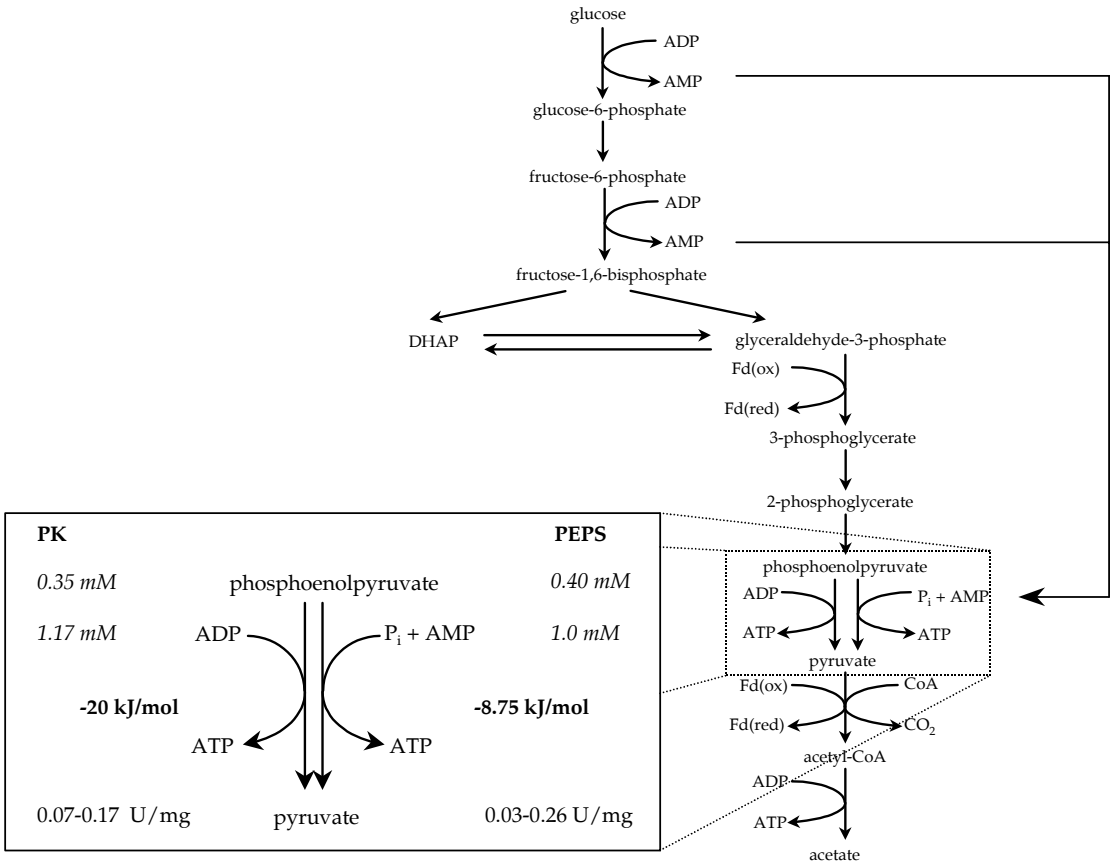

FIGURE 5 Modified Embden-Meyerhof pathway in *P. furiosus* with indicated fate of AMP. Inset: comparison of pyruvate kinase (PK) and PEP synthase (PEPS). Apparent K_m and $S_{0.5}$ values for PEP, ADP and AMP are indicated in italics. In bold: $\Delta G^{0\prime}$ for pyruvate kinase from literature and $\Delta G^{0\prime\prime}{}_{obs}$ for PEP synthase. Specific activities in cell-free extracts are given in normal font.

and cells harvested at the end of the growth curve, the activity of the pyruvate kinase appeared to be the same during both growth phases, whereas the PEP synthase activity was much higher in the stationary than in the exponential growth phase. This suggests a role of the latter enzyme in case of a lower energy state of the cell.

The apparent constitutive activity of the PEP synthase was confirmed by recent analysis of a whole-genome DNA microarray of *P. furiosus*. When relative transcript levels in maltose-grown cells were compared to those in peptide-grown cells, both pyruvate kinase and PEP synthase genes were expressed at comparable levels under both growth conditions. For the PEP synthase this was confirmed by activity measurements [Schut *et al.*, 2003]. This finding is in contrast with previous observations [Robinson & Schreier, 1994], [Sakuraba *et al.*, 2001] that during growth on maltose transcription of PEP synthase is increased compared to that during growth on peptides, which would indicate that the gene encoding PEP synthase is regulated at the transcriptional level and that the PEP synthase probably functions in the

glycolytic direction. Remarkably, transcription was not enhanced upon addition of the glucose dimers trehalose and cellobiose or the glucose polymer laminarin [Sakuraba *et al.*, 2001]. Trehalose and cellobiose are like maltose taken up by the cell through identified transport systems [Koning *et al.*, 2001], [Koning *et al.*, 2002] and intracellularly converted into glucose that subsequently enters the glycolysis, whereas laminarin is extracellularly degraded into oligomers that are subsequently taken up and converted into glucose [Gueguen *et al.*, 1997]. Thus, all four substrates are eventually converted into glucose and therefore, it could be expected that addition of trehalose, cellobiose or laminarin to the growth medium would have the same effect as the addition of maltose on transcription of the PEP synthase encoding gene. The apparent lack of regulation by the other three substrates implies that the effect of maltose might be an artefact rather than a solid piece of evidence for the suggested glycolytic role of the enzyme.

In order to study the PEP synthase in more detail, the enzyme was purified from cell-free extract of *P. furiosus* grown on starch. The PEP synthase was shown to have a globular structure with a diameter of approximately 17.5 nm. It was assumed that the PEP synthase had a similar structure as the previously described structure of the multimeric archaeal PEP-sythase homologue (MAPS) from the hyperthermophilic Archaeon *Staphylothermus marinus*, *i.e.* 24 93-kDa subunits that are arranged in six groups of four, adding up to a native molecular mass of ca. 2000 kDa [Cicicopol *et al.*, 1994], [Harauz *et al.*, 1996], [Cicicopol *et al.*, 1999]. Previously, the *P. furiosus* PEP synthase was described to have a molecular mass higher than 670 kDa [Sakuraba *et al.*, 1999], and to be an octomer of 690 kDa and partly present as an inactive larger complex of ca. 1600 kDa [Hutchins *et al.*, 2001]. However, in our experiments only the active 2000-kDa oligomer was found. The reason for this discrepancy remains unclear.

Most PEP synthases have a dimeric or in some cases tetrameric structure and it is not clear why the PEP synthases of *P. furiosus* and *S. marinus* have a different oligomeric structure. An explanation might be that both enzymes are active at high temperature, and have a more stabilised enzyme configuration. However, many other enzymes from hyperthermophiles have been described to have mono-, di-, or tetrameric structures, just like their couterparts active at moderate temperatures. It might also be that the unusual multimeric structure is related to its anticipated catabolic function in *P. furiosus*, although *S. marinus* is unable to grow on carbohydrates [Fiala *et al.*, 1986] and therefore its PEP synthase most likely functions only in the anabolic direction.

Besides the PEP synthase, also the pyruvate kinase of *P. furiosus* was investigated. The gene encoding the pyruvate kinase was cloned and expressed in *E. coli* and the resulting protein was purified. The protein had a native molecular mass of approximately 187 kDa and a subunit size of approximately 53 kDa, suggesting that the protein is a homotetramer. This corresponds to the situation in almost all other organisms, where pyruvate kinases have been described as tetramers, although also heterotetrameric, monomeric and dimeric pyruvate kinases are known [Fothergill-Gilmore & Michels, 1993].

When the possibility of a physiological role of the PEP synthase in the glycolytic direction is discussed, the thermodynamics of the reaction in both directions become very important. The Gibbs free energy change ($\Delta G^{0\prime}$) of the PEP synthase reaction has previously been calculated as -12 kJ/mole at 25°C in the direction of PEP formation, thus the reaction is thermodynamically feasible in gluconeogenesis under standard conditions [Eyzaguirre *et al.*, 1982], [Thauer *et al.*, 1977]. Based on this $\Delta G^{0\prime}$ value it is highly unlikely that a PEP synthase would be active in the opposite direction, *i.e.* pyruvate formation. This would only be possible if the intracellular concentrations of PEP, phosphate and AMP would be much higher than those of pyruvate and ATP, which would force the equilibrium in the direction of pyruvate formation. Remarkably, our results indicate a $\Delta G^{0\prime\prime}_{obs}$ of +8.75 kJ/mole in the direction of PEP formation, or -8.75 kJ/mole in the pyruvate-forming direction , which implies that the reaction is thermodynamically feasible in glycolytic direction. This is more consistent with a role for the enzyme in glycolysis than in gluconeogenesis.

In a previous study on the characterisation of the PEP synthase from *P. furiosus*, the equilibrium constant was calculated as 1.07, with a $\Delta G^{0\prime}$ value of -0.17 kJ/mol [Sakuraba *et al.*, 1999]. Although these data lead to the same conclusion that the PEP synthase reaction in *P. furiosus* is thermodynamically feasible in glycolytic direction, some remarks should be placed to the data. Firstly, the equilibrium reaction was done at 50°C, which is far from the physiological temperature of the organism. Secondly, a large error was introduced by measuring the concentration of only one of the reactants and subsequently deriving the other concentrations from the assumed stoichiometry of the reaction. Thirdly, a concentration of 55.6 M of water, which is usually taken as unity, was included in the calculation. Altogether, recalculation of the data presented by [Sakuraba *et al.*, 1999] resulted in a equilibrium constant of 0.019 and a $\Delta G^{0\prime}$ value of +10.6 kJ/mol. The discrepancies in these results prompted us to investigate the thermodynamics

of the reaction by measuring the equilibrium constant at a more physiological temperature of 80°C, leading to the $\Delta G^{0\prime\prime}_{obs}$ value as described here.

The spectacular difference between our empirical data determined at 80°C and the data described in literature (25°C) can be explained by the difference in temperature. In general, large errors are introduced when data determined at 25°C and pH 7 are applied to systems at elevated temperature. For example, chemical reaction rates increase with temperature and the solubility of some gases decreases with temperature while other gases become more soluble at higher temperature. Furthermore, the neutrality of a solution changes from pH 7 at room temperature to pH 6.1 at 100°C. Therefore, a pH value of 7 does not reflect neutrality at high temperatures [Amend & Shock, 2001]. Thus, thermodynamic data that were determined at 25°C cannot simply be compared to data determined at high temperatures. Our results show that under the circumstances used in the experiments, the $\Delta G^{0\prime\prime}_{obs}$ for the PEP synthase reaction is negative in the glycolytic direction, indicating that in *P. furiosus*, the reaction of PEP synthase in this direction is thermodynamically feasible at 80°C.

Regarding the effect of possible regulators, the pyruvate kinase from *P. furiosus* showed slight modifications as compared to its known counterparts from other organisms. While bacterial pyruvate kinases are usually activated by fructose-1,6-bisphosphate [Muñoz & Ponce, 2003], the enzyme from *P. furiosus* is slightly inhibited by this glycolytic intermediate. The pyruvate kinases from *A. fulgidus*, *A. pernix*, and *P. aerophilum* are not affected by fructose-1,6-bisphosphate or AMP [Johnsen *et al*., 2003]. The inhibition by ATP that was shown for the *P. furiosus* enzyme, on the other hand, is common for bacterial pyruvate kinases [Fothergill-Gilmore & Michels, 1993]. The pyruvate kinase from *P. furiosus* was independent of K^+ ions, which was also described for the pyruvate kinases from the Archaea *T. tenax*, *A. fulgidus*, *A. pernix*, and *P. aerophilum* [Schramm *et al*., 2000], [Johnsen *et al*., 2003]. The observed inhibition of PEP synthase activity in glycolytic direction by the addition of ATP or fructose-1,6-bisphosphate is difficult to compare to literature data, since most studies with PEP synthases were done in gluconeogenic direction.

To make a comparison between the PEP synthase and the pyruvate kinases, kinetic data were determined for both enzymes. The kinetic studies on the PEP synthase in gluconeogenic direction showed that the enzyme has K_m values of 0.49 mM for pyruvate and 0.48 mM for ATP, respectively. These values are in the same range as the ones described by [Hutchins *et al*., 2001] (0.11 mM for pyruvate and 0.39 mM for ATP). In a study by [Sakuraba *et al*.,

1999] kinetic data were only determined in glycolytic direction. The PEP synthase from the thermophilic methanogen *Methanothermobacter thermoautotrophicus* showed K_m values of 0.04 mM for pyruvate and 0.7 mM for ATP [Eyzaguirre *et al.*, 1982]. For the PEP synthase from *E. coli*, K_m values of 0.08 mM for pyruvate and 0.08 mM for ATP were determined [Chulavatnatol & Atkinson, 1973], which are much lower than the values obtained for the *P. furiosus* enzyme.

More important for the comparison of the PEP synthase and the pyruvate kinase are the kinetic data for both enzymes in the glycolytic direction. The kinetics for the PEP synthase have been described before [Hutchins *et al.*, 2001], [Sakuraba *et al.*, 1999] and therefore, these data were used for comparison of the two enzymes. While the K_m values for PEP (0.40 and 1.0 mM, respectively) and for phosphate (38.4 and 35 mM, respectively) are in the same range between the two studies, the K_m value for AMP as determined by [Hutchins *et al.*, 2001] is a factor 10 higher than that determined by [Sakuraba *et al.*, 1999] (1.0 and 0.10 mM, respectively). An explanation for this difference might be found in the temperature used for the experiments, namely 50°C [Sakuraba *et al.*, 1999] and 80°C [Hutchins *et al.*, 2001]. Apparently, the K_m values for PEP and phosphate are less influenced by the temperature than that of AMP. The kinetic data for the pyruvate kinase were determined at 50°C.

The K_m values for PEP for both enzymes are in the same range, 0.35 and 0.4 (or 1.0) mM for the pyruvate kinase and the PEP synthase, respectively. This means that in an *in vivo* situation, both enzymes have the same affinity for their common substrate. For the phosphoryl group acceptor (ADP in the case of pyruvate kinase and AMP for PEP synthase), the K_m values are more difficult to compare since the values for the PEP synthase deviate by a factor 10. Compared to the data from [Hutchins *et al.*, 2001], the K_m values are in the same range, *i.e.* 1.17 mM for pyruvate kinase and 1.00 mM for PEP synthase, but the K_m value for PEP synthase determined by [Sakuraba *et al.*, 1999] is only 0.10 mM, leaving it difficult to draw any conclusion on the relative affinities of the enzymes for their substrates. Besides, to predict which enzyme is the most active *in vivo*, it is important to know the intracellular concentrations of the nucleotides. Therefore, from the K_m values that were determined by us and by others, it can not be concluded which of the two enzymes functions in glycolysis.

Together with the K_m values, also V_{max} values were determined. The V_{max} values for the PEP synthase in gluconeogenic direction that were determined in our study are higher (28.4 and 33.8 U/mg for pyruvate and

ATP, respectively) than the values previously determined (17.1 and 14.4 U/mg for pyruvate and ATP, respectively [Hutchins *et al.*, 2001]). These differences can be explained by the different temperatures used for activity measurements, namely 80°C for the experiments done by [Hutchins *et al.*, 2001] and 85°C in our experiments. When a Q_{10} of 2 [Schäfer & Schönheit, 1992] is assumed for the PEP synthase, the data mentioned by [Hutchins *et al.*, 2001] would lead to values of 25.7 U/mg for pyruvate and 21.6 U/mg for ATP at 85°C, which are more comparable to our data. In the study by [Sakuraba *et al.*, 1999], no apparent V_{max} values were calculated, but only specific enzyme activities of the purified enzyme were measured at 50°C in both direction. Although the V_{max} values described by [Hutchins *et al.*, 2001] were much higher in gluconeogenic direction than in glycolytic direction, the opposite holds for the activities reported by [Sakuraba *et al.*, 1999]. The reason for the discrepancy between these results remains unclear, but the temperature used for activity measurements in the latter study (50°C) is far from the physiological temperature of the organism, which might influence the results.

For comparison between the V_{max} values for the pyruvate kinase and the PEP synthase in glycolytic direction, again the data previously reported by [Hutchins *et al.*, 2001] were used. It is clear that the V_{max} values of the pyruvate kinase (42.3 U/mg for PEP and 43.4 U/mg for ADP) are much higher than those of the PEP synthase in glycolytic direction (0.14 U/mg for PEP, 0.095 U/mg for AMP, and 0.13 U/mg for phosphate). This effect becomes even larger when the Q_{10} value of 2 is applied to the V_{max} data of the pyruvate kinase, that were determined at 50°C. At 80°C, the V_{max} values would become 338 U/mg for PEP and 347 U/mg for ADP. However, from the V_{max} values *per se* it may not be concluded which of the enzymes has the highest activity in either direction, since the activity in the cell is also dependent on the expression level of the protein. Therefore, the specific activities of the enzymes in cell-free extract, as described above, give more information than the apparent V_{max} data.

An important finding in the present study is the fact that the pyruvate kinase is allosteric for ADP, suggesting that the enzyme may be a site for regulation of the pathway. However, while most bacterial pyruvate kinases are allosteric for phosphoenolpyruvate [Fothergill-Gilmore & Michels, 1993], the *P. furiosus* enzyme is allosteric for ADP and shows Michaelis-Menten kinetics for phosphoenolpyruvate. The pyruvate kinase from the hyperthermophilic Archaeon *Thermoproteus tenax* is allosteric for its substrate PEP, but its activity is not influenced by ATP or AMP. Therefore, the *T. tenax* enzyme was designated "weakly regulated" [Schramm *et al.*, 2000]. The

pyruvate kinases from three hyperthermophilic Archaea (*Archaeoglobus fulgidus*, *Aeropyrum pernix*, and *Pyrobaculum aerophilum*) showed sigmoidal saturation kinetics with both substrates, PEP and ADP [Johnsen *et al.*, 2003]. Remarkably, the pyruvate kinase is the first enzyme of the glycolytic pathway of *P. furiosus* that shows allosteric regulation, which could have consequences for the regulation of the complete pathway. Whereas in the classical Embden-Meyerhof pathway the major control point is generally considered to be the phosphofructokinase that is therefore allosterically regulated, the ADP-dependent phosphofructokinase of *P. furiosus* is non-allosteric and can therefore not be considered as the major control point in glycolysis [Tuininga *et al.*, 1999].

The effect of AMP on the kinetics of the pyruvate kinase has important consequences for the bioenergetics and the regulation of the pathway. Namely, in the presence of AMP, the affinity of the pyruvate kinase for ADP decreased, *i.e.* the $S_{0.5}$ changed from 1.17 to 1.65 mM. This means that when the energy charge of the cell decreases, that is when the concentration of AMP increases, the activity of the pyruvate kinase would decrease. The PEP synthase that is also present in the cell and that has the same affinity for the common substrate PEP, would be able to convert a larger portion of the available PEP to pyruvate, thereby producing extra ATP and decreasing the AMP concentration. In this way, *P. furiosus* has a novel site of regulation for its glycolytic pathway.

The suggested role of the PEP synthase in glycolytic direction would seem feasible in view of the AMP that is produced by the two ADP-dependent kinases that catalyse the first steps of the pathway [Kengen *et al.*, 1995], [Tuininga *et al.*, 1999]. In the classical Embden-Meyerhof pathway, the kinases convert ATP into ADP, which is regenerated into ATP by the pyruvate kinase. In the modified Embden-Meyerhof pathway of *P. furiosus*, the AMP could be regenerated into ATP in one step by the activity of the PEP synthase in glycolytic direction (Figure 5). This might also be the answer to the question why not all organisms use the PEP synthase in glycolytic direction, since usually there is no AMP produced in the glycolytic pathway and therefore no need to regenerate AMP into ATP. Apparently, AMP seems to play a novel role in regulation of the glycolysis in *P. furiosus*.

Alternatively, AMP can be regenerated by the activity of adenylate kinase, that converts AMP with ATP into 2 ADP, which subsequently can be used by the ADP-dependent kinases. Adenylate kinase activity has been detected in cell-free extracts of *P. furiosus* [Schäfer & Schönheit, 1991], but its specific activity of 0.045 U/mg at 55°C is probably not high enough to sustain

the glucose flux that was calculated as 0.6 µmol glucose/min/mg protein at 90°C [Kengen *et al*., 1994]. Using a Q_{10} value of 2 [Schäfer & Schönheit, 1992], it can be calculated that the specific activity of the adenylate kinase at 90°C (0.54 U/mg) would not be enough to regenerate the 2 ADP that are produced per glucose converted (*i.e.* 1.2 µmol ADP/min/mg). Thus, not all AMP can be regenerated by the adenylate kinase, which favours a role for the PEP synthase in AMP regeneration.

On the genetic level, another indication of a glycolytic role for the PEP synthase was found. This was given by an inverted repeat that was identified in the genome sequence of *P. furiosus*. The 15-nucleotide inverted repeat, designated *Pyrococcus*-Specific Repeat (PSR) was exclusively found in promotor sequences of genes encoding proteins involved in uptake and degradation of α-linked sugars. It was found in all genes encoding enzymes of the modified Embden-Meyerhof pathway. Remarkably, the only exception is the pyruvate kinase gene, that apparently lacks the PRS-element. Moreover, in the gene encoding PEP synthase the element is also present. The position and the structure of the PRS-element suggest that it is involved in negative control of gene expression, and might represent a specific site for regulation of the glycolytic pathway. The observation that the PEP synthase gene harbours the PRS-element indicates that the gene is regulated in the same way as glycolytic enzymes, thereby suggesting a catabolic role for the PEP synthase [Verhees, 2002], [Verhees *et al*., 2003].

To summarise the above-mentioned data, we can conclude that the suggested physiological role of the PEP synthase in glycolysis is indeed possible. With regard to the $\Delta G^{0''}_{obs}$ value, the reaction is thermodynamically feasible in glycolytic direction at 80°C. The specific activities of the PEP synthase and the pyruvate kinase in cell-free extracts do not clearly indicate whether the PEP synthase has a role in the glycolysis. However, from transcriptional analysis it can be concluded that the transcription of the gene encoding PEP synthase is induced by growth on maltose, although this might be a specific effect of maltose rather than a sugar-related effect. Furthermore, the promoter sequence identified in all glycolytic genes except pyruvate kinase was also present in the PEP synthase, which is another indication that the gene is regulated in the same way as the glycolytic genes. From the kinetic data obtained for the pyruvate kinase and the PEP synthase alone it can not be concluded which of the enzymes plays a role in the glycolysis, but the effect of AMP on the activity of pyruvate kinase, which favours the activity of PEP synthase, is a clear indication that both enzymes play a role in the glycolysis and its regulation.

Based on the evidence presented here, we suggest the following hypothesis. Both PEP synthase and pyruvate kinase play a certain role in the glycolysis of *P. furiosus*, that is based on the energy demand of the cell. Whereas the PEP synthase is regulated on the genetic level, the pyruvate kinase is allosterically regulated. When enough energy is present in the cell, indicated by a relatively low AMP concentration, the pyruvate kinase is the most important enzyme catalysing the conversion of PEP to pyruvate. However, when the AMP concentration increases - by the activity of the two ADP-dependent sugar kinases - the activity of the pyruvate kinase decreases and the PEP synthase becomes more important in the conversion of PEP to pyruvate. In that situation, AMP is converted into ATP in one step, thereby increasing the ATP yield of the glycolytic pathway. This hypothesis would provide *P. furiosus* with an elegant site of regulation for the glycolysis and for the amount of energy that can be obtained from the pathway under different circumstances.

The above-mentioned hypothesis has important consequences for the bioenergetics of the glycolytic pathway in *P. furiosus*, since the activity of either of the enzymes leads to different ATP yields from the complete pathway. Therefore, to be able to make proper bioenergetic calculations it is important to know which of the enzymes is responsible for what fraction of the conversion in different situations. If a genetic system would become available for *P. furiosus*, knock-out mutants could be constructed for either of the enzymes, and comparison of the bioenergetics of both mutants could give essential information on the role of the two enzymes in the glycolytic pathway of *P. furiosus*.

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6

Growth yield studies of *Pyrococcus furiosus* in continuous culture

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SUMMARY

The bioenergetics of the glycolytic pathway of the hyperthermophilic Archaeon *Pyrococcus furiosus* were investigated using chemostat cultures with cellobiose and pyruvate as the limiting carbon sources. The growth yield data obtained in this study are comparable to those obtained in batch cultures of *P. furiosus* on cellobiose, maltose and pyruvate, which confirms the previous suggestion that an additional ATP-conserving site might be present in the glycolysis. Maximal growth yields and maintenance coefficients were calculated. The obtained values were somewhat different from previously reported values. Furthermore, an experiment using cysteine-free growth medium indicated that alanine, which is one of the end products of the fermentative metabolism, can also be produced directly from cysteine, possibly enzymatically by a cysteine desulfurase. The adenylate energy charge was determined in steady-state culture and although the ADP concentration appeared to be lower than in a reference *E. coli* culture, the energy charge of both organisms was comparable.

INTRODUCTION

The hyperthermophilic Archaeon *Pyrococcus furiosus* can grow on a variety of carbon compounds, such as di- and polysaccharides, peptone, tryptone and pyruvate. For growth on di- and polysaccharides it uses a modified Embden-Meyerhof pathway for its sugar metabolism. In this pathway, two novel ADP-dependent sugar kinases [Kengen *et al.*, 1995], [Tuininga *et al.*, 1999] and a novel glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) [Mukund & Adams, 1995] are important modifications from the classical glycolysis.

The presence of the ADP-dependent kinases has no consequences for the bioenergetics of the glycolytic pathway, since the free energy of hydrolysis of ADP and ATP is equal [Thauer *et al.*, 1977]. Furthermore, *P. furiosus* possesses an adenylate kinase that converts the AMP formed by the kinases into ADP at the expense of ATP [Schäfer & Schönheit, 1991]. Therefore, the net cost of both kinase reactions is 1 ATP per reaction per sugar molecule converted, as in the classical Embden-Meyerhof pathway. However, the presence of GAPOR does have important bioenergetic consequences. This enzyme converts glyceraldehyde-3-phosphate into 3-phosphoglycerate in only one step, apparently without energy conservation by substrate-level phosphorylation, whereas in the classical Embden-Meyerhof pathway 1 ATP is formed in this conversion. Because of this difference, no net ATP appears to be produced in the pathway between glucose and pyruvate. Apparently, the only ATP-producing step in the sugar metabolism is the conversion of acetyl-CoA into acetate. Therefore, energy yield (Y_{ATP}) values for growth on sugars and pyruvate could be expected to be in the same range. For growth in batch cultures, however, this was shown to be untrue. It appeared that the Y_{ATP} on pyruvate is 9 - 10 g/mol whereas it is 18 - 22 g/mol for disaccharides. Therefore it was suggested that an extra ATP-conserving site could be present in the glycolytic pathway [Kengen & Stams, 1994b].

A possible site for this extra energy conservation is the conversion of phosphoenolpyruvate to pyruvate. This reaction is usually catalysed by pyruvate kinase, thereby producing ATP from ADP, but recently it was suggested that in *P. furiosus* the reversible PEP synthase might have a physiological role in the glycolytic direction, thereby producing ATP from AMP in one step [Sakuraba *et al.*, 1999], [Tuininga *et al.*, 2003]. Since the free energy of hydrolysis of both phosphate bonds in ATP is equal [Thauer *et al.*, 1977], the conversion of 1 AMP into 1 ATP corresponds to the formation of 2

ATP from 2 ADP. Thus, the suggested physiological activity of the PEP synthase in glycolysis could increase the energy yield of the pathway from 0 to maximally 2 ATP per glucose converted. However, whether the PEP synthase indeed plays a role in the glycolytic pathway is still a matter of debate.

Very recently, another energy-conserving system was described in *P. furiosus*. In this single-enzyme system, the reduced ferredoxin that is produced in the modified Embden-Meyerhof pathway is reoxidised by a membrane-bound hydrogenase, which reduces protons to produce hydrogen. The hydrogenase also pumps protons across the membrane, thereby creating a transmembrane proton gradient, that can be used by an ATP synthase to produce ATP [Sapra *et al*., 2003]. Calculations on the stoichiometry of this energy-conserving system indicated that an additional 1.2 mol of ATP can be formed per mol of glucose converted, which would decrease the Y_{ATP} during growth on sugars to 11.1 g/mol ATP. This value is much more consistent with the values reported for other organisms. Whether this system is of any importance in pyruvate-grown cells remains to be established.

In batch cultures, alanine has been identified as an end product of sugar fermentation. Formation of alanine was proposed to function as a means of discarding the electrons generated during sugar metabolism [Kengen & Stams, 1994a]. Recently, it was suggested that *P. furiosus* could also produce alanine directly from cysteine [H. Haaker, personal communication] probably by the activity of a cysteine desulfurase [Mihara & Esaki, 2002]. This would imply that part of the alanine found in the culture medium should be left out of the carbon balances for the glycolysis.

Thus, the bioenergetics of the modified Embden-Meyerhof pathway of *P. furiosus* are still not completely understood. Therefore, in this study the bioenergetics of the pathway were investigated using energy-limited continuous cultures with either cellobiose or pyruvate as energy and carbon source. For this purpose, continuous culture is better suited than batch culture, because in the former system the circumstances are kept constant. Steady-state fermentation patterns and growth yields were determined at various dilution rates. Using these data, maximum growth yields and maintenance coefficients were calculated. To test the possibility of direct alanine formation from cysteine, an experminent was done in which cysteine was omitted from the growth medium. Furthermore, intracellular adenylate levels were measured in steady-state cells grown on cellobiose, from which the adenylate energy charge was calculated.

MATERIALS AND METHODS

Organisms and growth medium

Pyrococcus furiosus DSM 3638 was obtained from the German Collection of Micro-organisms (Braunschweig, Germany). The growth medium for growth of *P. furiosus* was modified from [Krahe *et al*., 1996] and contained per litre: NaCl, 30 g; L-cysteine hydrochloride, 0.5 g; KH_2PO_4, 1.4 g; $(NH_4)_2SO_4$, 1.3 g; $MgSO_4$, 2.5 g; $CaCl_2$, 0.066 g; Na_2WO_4 solution (100 mM), 0.1 ml; yeast extract, 1 g; resazurin solution (0.5 mg/ml), 1 ml; trace element solution, 1 ml; and vitamin solution, 1 ml. Trace element solution (per litre): $FeCl_2.4H_2O$, 2 g; H_3BO_3, 0.05 g; $ZnCl_2$, 0.05 g; $CuCl_2$, 0.03 g; $MnCl_2.4H_2O$, 0.05 g; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05 g; $AlCl_3$, 0.05 g; $CoCl_2.6H_2O$, 0.05 g; $NiCl_2$, 0.05 g; EDTA, 0.5 g; and concentrated HCl, 1 ml. Vitamin solution (per liter): D-biotin, 2 mg; folic acid, 2 mg; thiamine, 5 mg; riboflavin, 5 mg; pyridoxine, 10 mg; cyanocobalamin, 5 mg; nicotinamide, 5 mg; p-aminobenzoic acid, 5 mg; DL-α-lipoic acid, 5 mg; and D-panthothenic acid, 5 mg. As a limiting carbon source, either 2 mM cellobiose or 5 mM pyruvate was added. A stock solution of cellobiose was autoclaved separately and added to the autoclaved medium. Stock solutions of cysteine and pyruvate were added through a 0.2 µm sterile filter after autoclaving the medium.

Escherichia coli strain NCTC 9002 was obtained from our laboratory's culture collection and grown aerobically in Luria Bertani medium in a rotary shaker at 37°C.

Batch precultures

For inoculation of the chemostat, *P. furiosus* was grown in 50-ml batch cultures in stoppered 120-ml serum bottles, flushed with N_2/CO_2 (80:20). The above-mentioned medium was used, with addition of 1 g per litre $NaHCO_3$. Cellobiose (5 mM) or pyruvate (40 mM) was used as a carbon source. Cultures were incubated overnight at 95°C in a rotary shaker and transferred at least five times on the same substrate before using them to inoculate the fermentor.

Continuous culture

Chemostat experiments were done in a FairMenTec (Göttingen, Germany) fermentor with a 1-litre working volume. Anaerobic conditions

were maintained by sparging the fermentor with nitrogen (flow rate 7.3 litre per hour) and the fermentor was stirred at ca. 300 rpm. The temperature in the culture vessel was maintained at 90°C by using a water jacket and a water bath at 95°C. The pH of the culture was maintained at neutral pH, which is 7.0 at room temperature and ca. 6.2 at 90°C [Amend & Shock, 2001] by controlled addition of 1 M NaOH. The pH electrode was calibrated at room temperature and autoclaved in the fermentor.

After inoculation of the fermentor with 50 ml of a fresh overnight culture of *P. furiosus*, the fermentor was operated as a batch fermentor for several hours. When hydrogen production from the culture was observed, the medium pump was started. At least five volume changes were allowed before steady state, which was maintained for at least two volume changes before increasing the medium flow rate. Dilution rates of 0.1, 0.15, 0.2, 0.25, 0.35, and 0.45 h^{-1} were used.

To test whether *P. furiosus* can produce alanine directly from cysteine, a chemostat experiment was done in which cysteine was omitted and the medium was reduced with titanium(III) citrate instead of cysteine. A titanium citrate solution, made as described before [Zehnder & Wuhrmann, 1976], was added to the fermentor to a final concentration of 75 µM. A dilution rate of 0.2 h^{-1} was applied as described above. After steady state was obtained on 2 mM cellobiose, the usual concentration of cysteine was added to the fermentor and to the medium vessel and titanium citrate was omitted from the incoming medium. Fermentation products and biomass concentrations were determined at steady state with and without cysteine as described above.

Analysis of substrates and fermentation products

At regular time intervals, 2-ml samples were taken from the chemostat and centrifuged. Supernatants were stored at -20°C until analysis. Mono- and disaccharides and organic acids in the supernatant were analysed by HPLC as described before [Kengen & Stams, 1994a]. Alanine was analysed on an ion-exchange column as previously described [Kengen & Stams, 1994a].

Hydrogen production was measured by gas chromatographic analysis of 0.2-ml samples that were taken from the gas phase of the chemostat. The amount of CO_2 formed was assumed to equal the amount of C_2-products formed.

Determination of growth yields

Growth yields were calculated from the optical density of culture samples at 600 nm (OD_{600}), using the experimentally determined linear relationship of 185 mg of protein per litre for an OD_{600} of 1 [Kengen & Stams, 1994a]. The protein data were converted to biomass dry weight data in molar units, assuming the protein/cell dry weight ratio to be 50%, the formula of the organic fraction of the biomass of bacterial cells to be $C_5H_7O_2N$ (M = 113), and the organic fraction to comprise 90% of the cell dry weight, as described before [Kengen & Stams, 1994a].

Alternatively, biomass was determined by total organic carbon (TOC) measurement on a Model 700 TOC analyser (OI Analytical, College Station, TX, USA).

Bioenergetic calculations

For calculation of bioenergetic parameters, the yield and maintenance model of Pirt [Pirt, 1975] was used (equation 1).

$$q_E = \mu / Y_{max} + m \qquad (1)$$

In this equation, q_E is the metabolic quotient for the energy source, μ is the growth rate, which is the same as the dilution rate under steady-state conditions, Y_{max} is the maximal growth yield, and m is the maintenance coefficient.

For several dilution rates (D), or growth rates (μ), the growth yield (Y) was calculated in g cell dry weight/mol glucose equivalent. From these data, the metabolic quotient q_E was calculated as D/Y. In a linear plot of q_E against μ, the slope equals the reciprocal maximal growth yield ($1/Y_{max}$) and the intercept on the ordinate equals the maintenance coefficient (m) [Pirt, 1975].

Measurement of adenylate energy charge

Steady-state cells of *P. furiosus* grown on cellobiose were harvested from the chemostat by injection of 20-ml samples immediately from the fermentor into liquid nitrogen (-196°C). After evaporation of excess nitrogen, the frozen culture was collected and stored at -20°C until use. As a reference, material was collected from an exponentially growing batch culture of *E. coli* in the same manner.

Adenosine nucleotide concentrations were determined by bioluminescence using a Luminac Biocounter M 2500 and luciferin-luciferase from a Microbial Biomass Kit (Celsis BV, Landgraaf, the Netherlands), following the manufacturer's instructions. Instead of extraction with supplied LuminEX reagent, the frozen cultures were extracted with ethanol by adding 2 g of ice-cold 96% ethanol to 1 g of frozen culture material. The suspension was diluted 10 times with autoclaved distilled water before measurement of the ATP content. Control experiments indicated that endogenous adenylate kinase was inactivated by this treatment. Residual ethanol concentrations did not inhibit the bioluminescence reaction. To prevent contamination with ATP, the distilled water and buffer (50 mM HEPES pH 7.0) used for dilutions were autoclaved.

ADP concentrations were determined after conversion of the ADP to ATP in a 1-ml assay containing 10 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate (PEP), 2.5 units (U) of pyruvate kinase, and 100 µl of sample. The assay mixture was incubated at room temperature for 15 min before measurement. For conversion of AMP to ATP, the same assay was used with addition of 5 U of adenylate kinase. Both auxiliary enzymes were obtained as a lyophilised powder and were resuspended in 50 mM HEPES pH 7.0. Concentrations of ATP, ADP, and AMP were calculated from calibration curves (0 - 1 µM) of the respective adenylates treated in the same manner as the culture samples, including extraction with 96% ethanol.

RESULTS AND DISCUSSION

Continuous culture

In this study, we describe the cultivation of *P. furiosus* in continuous culture. After autoclaving the fermentor with growth medium, the pH of the medium appeared to have dropped dramatically. Therefore, before inoculation of the fermentor the pH was adjusted by adding 1 M NaOH to the medium. While doing so, a salt precipitation was formed in the medium, which made the biomass determination based on optical density measurement less accurate and less reproducible. For biomass calculations, the amount of precipitate was assumed to be stable during the experiments and subtracted from the measured optical densities. In pilot experiments the medium described by [Kengen *et al.*, 1993] was used, but this medium gave even more problems with precipitation. Therefore the medium described by

[Krahe *et al.*, 1996] was used for our experiments. The main difference between the two media is the lower amounts of magnesium salts in the latter medium (2.5 instead of 3.4 g per litre $MgSO_4$, and $MgCl_2$ was completely omitted), which were thought to be important components of the precipitate.

The presence of yeast extract in the growth medium introduces an imperfection to the growth yield calculations since *P. furiosus* is able to grow on yeast extract [Fiala & Stetter, 1986]. However, growth of the organism on yeast extract alone is so weak [S.W.M. Kengen, unpublished results] that the fraction of biomass resulting from growth on yeast extract can be neglected in the calculations. In previous batch experiments, a defined medium was used, as described before [Raven & Sharp, 1997]. However, we were unable to obtain reasonable growth of *P. furiosus* in this medium (data not shown), which prompted us to use an undefined medium instead.

As an alternative to optical density measurements, biomass concentrations were also determined with total organic carbon (TOC) measurement. However, due to the fact that yeast extract is present in the medium, which contains a lot of organic carbon, the medium showed such a high background TOC value that accurate determination of the biomass was not possible. To avoid such problems, cells should have been washed in a salt-containing buffer prior to TOC measurement. In our experiments, culture samples were taken from the fermentor and directly stored at -20°C. Unfortunately, after storage significant lysis of the cells was observed, which made washing of the cells and subsequent TOC determination impracticable. Therefore, biomass was determined by optical density measurement as described above.

When the hydrogen production and OD_{600} in the fermentor were stable, the concentration of the growth substrate in the samples was reduced to zero, indicating that the cultures were carbon limited. In the cellobiose experiment, the concentration of glucose in the medium was measured to make sure that all cellobiose was taken up by the cells instead of being hydrolysed extracellularly. Indeed, no glucose could be detected in the medium, confirming that the data did not need to be corrected for extracellularly hydrolysed cellobiose.

Fermentation patterns

During steady state on cellobiose and pyruvate, at each dilution rate samples were taken to determine product concentrations. The fermentation

data for the chemostat cultures at the various dilution rates are summarised in Table 1.

The carbon balances obtained for the experiment on cellobiose were too high for all dilution rates, indicating that one of the products was overestimated in the calculations. This observation might be explained by an overestimation of the biomass concentration in the culture due to the formation of the salt precipitate in the fermentor. However, the carbon balances were especially too high at the lower dilution rates, where the biomass concentration was the lowest. Another explanation could be found in the alanine concentrations, which were higher than expected for chemostat culture. The suggested role of alanine production in batch culture, *i.e.* the removal of electrons produced in the glycolytic pathway [Kengen & Stams, 1994a], is expected not to be necessary in a fermentor that is continuously sparged with nitrogen in order to remove the produced hydrogen. However, besides being formed as a product from the glycolytic pathway, alanine might be directly formed from cysteine [H. Haaker, personal communication]. If this is true, the alanine that is formed directly from cysteine (which could be all or a part of the observed alanine) should not be taken as a product of the glycolytic pathway and should therefore be left out of the carbon balance. To investigate this possibility, a cysteine-free experiment was done (see below).

TABLE 1 Fermentation patterns of *P. furiosus* grown in continuous culture on 2 mM of cellobiose or 5 mM of pyruvate, at several dilution rates. The concentrations of the products are expressed in mM, the biomass concentration in mg dry weight per litre, the molar growth yield in g dry weight per mol substrate utilised and the Y_{ATP} in g dry weight per mol ATP produced, assuming that one mol of ATP is synthesised per mol of acetate produced. All variables were measured in triplicate. Carbon balances for growth on cellobiose were corrected for the amount of alanine produced directly from cysteine (see text). The corrected values are given in parentheses.

substrate	dilution rate	acetate	alanine	biomass	carbon balance	Y	Y_{ATP}
	h^{-1}	*mM*	*mM*	*mg/l*		*g/mol*	*g/mol*
cellobiose	0.1	7.77	1.23	85	1.27 (1.21)	42.4	10.9
	0.15	7.02	1.51	109	1.25 (1.19)	54.4	15.5
	0.2	6.23	1.7	121	1.19 (1.14)	60.3	19.4
	0.25	5.57	1.54	131	1.11 (1.05)	65.6	23.6
	0.35	5.56	1.34	151	1.11 (1.06)	75.6	27.2
	0.45	5.39	1.26	165	1.11 (1.05)	82.6	30.6
pyruvate	0.1	4.31	0.39	40	1.05	8.02	9.30
	0.15	3.01	0.75	34	0.84	6.73	11.2
	0.2	2.85	0.77	33	0.83	6.75	11.6
	0.25	2.59	0.84	31	0.81	6.54	11.9

The carbon balances obtained for the chemostat on pyruvate appeared to be too low for all but the lowest dilution rate. Remarkably, also the alanine concentrations measured in the pyruvate experiment were much lower than those measured in the cellobiose experiment. This pattern could be expected since fewer reducing equivalents are formed during growth on pyruvate as compared to growth on disaccharides, and therefore less alanine would be needed to serve as an electron sink.

Remarkably, in the cellobiose experiment, the biomass concentration increased substantially when the dilution rate increased, whereas in the pyruvate experiment the biomass concentration slightly decreased at higher dilution rates. An explanation for this difference was not found.

When the averages of the concentrations as listed in Table 1 were compared to those obtained for growth of *P. furiosus* in batch culture [Kengen & Stams, 1994b], some differences were observed. For both cellobiose and pyruvate, the average acetate concentrations per mol of substrate utilised were slightly higher than in batch (1.4 - 1.9 compared to 1.1 - 1.4 for cellobiose and 0.5 - 0.9 compared to 0.7 for pyruvate in continuous and batch culture, respectively). However, the concentrations of alanine showed the opposite trend, namely lower in the continuous culture than in batch culture (0.3 - 0.4 compared to 0.4 - 0.5 for cellobiose and 0.08 - 0.17 compared to 0.2 - 0.3 for pyruvate in continuous and batch cultures, respectively). This difference is not surprising because of the suggested role for alanine production as an electron sink, as explained above.

The amount of acetate produced in both cellobiose and pyruvate cultures tended to decrease when the dilution rate (or growth rate) increased, however at the higher dilution rates the acetate concentrations remained stable (Figure 1). The shape of the curves suggests that a shift in metabolism occurs around a dilution rate of 0.25 h^{-1} for cellobiose and 0.15 h^{-1} for pyruvate. However, no extra product that could account for the decrease in

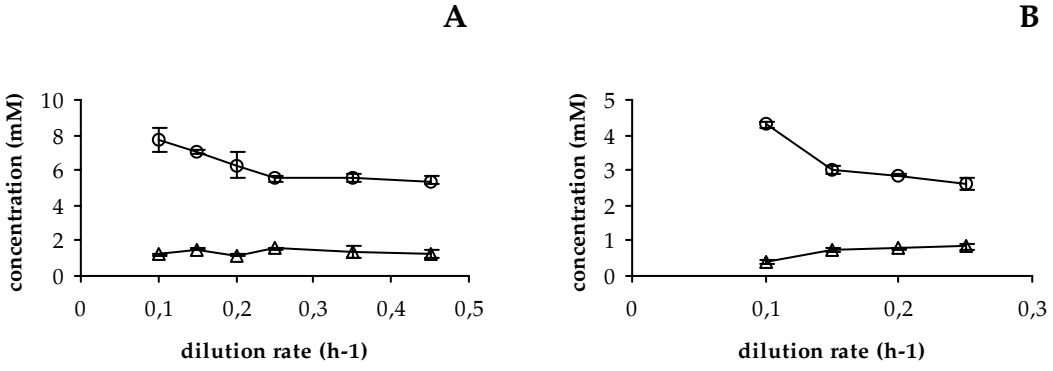


FIGURE 1 Concentrations of acetate (O) and alanine (Δ) in continuous culture of *P. furiosus* grown on cellobiose (A) or pyruvate (B) at several dilution rates.

acetate could be detected in any of the samples. Alanine concentrations remained practically stable throughout the range of dilution rates, which is an indication that alanine is partly produced directly from cysteine instead of being used as an electron sink for reducing equivalent produced in the glycolytic pathway.

Growth yields

Using the determined concentrations of biomass and acetate, growth yields (Y and Y_{ATP}) were calculated (Table 1). For calculation of the Y_{ATP} values it was assumed that only 1 ATP is produced in the pathway, namely at the level of acetate. Therefore, the concentration of ATP produced in the culture was assumed to be equal to the measured acetate concentration. Remarkably, the Y_{ATP} values for growth on cellobiose increased from 10.9 g/mol ATP at the lowest dilution rate (0.1 h^{-1}) to 30.6 g/mol ATP at the highest dilution rate (0.45 h^{-1}). In the chemostats on pyruvate, however, the Y_{ATP} values were much more stable throughout the various dilution rates, although they also slightly increased from 9.3 to 11.9 g/mol ATP. The reason for this difference between the results on cellobiose and pyruvate was unclear. Generally, a Y_{ATP} value of 10.5 is considered to be average for fermentative organisms [Bauchop & Elsden, 1960]. Thus, the values found for growth on pyruvate are within the range of normal values, whereas the values obtained for growth on cellobiose are too high for all dilution rates except the lowest one tested. Assuming one extra ATP produced per glucose converted, the Y_{ATP} values for growth on cellobiose would decrease by a factor two to values between 5.5 and 15.3. The former value is low compared to the average value and the latter value is still too high. A reason for this discrepancy might be an overestimation of biomass concentrations due to the formation of the precipitate as described above.

When our data were compared to those obtained for batch cultures of *P. furiosus* [Kengen & Stams, 1994b], some striking similarities and differences were observed. Both the Y and Y_{ATP} values for the pyruvate cultures are similar to those obtained in batch culture, namely Y values of 6.54 - 8.02 g/mol for continuous culture and 6.15 - 6.63 g/mol for batch culture and Y_{ATP} values of 9.30 - 11.9 g/mol and 8.66 - 10.2 g/mol for continuous and batch culture, respectively. Unfortunately, for cellobiose the values are more difficult to compare since our data show a wide variation. However, for the lowest dilution rate of the continuous culture a Y value of 42.4 g/mol and a Y_{ATP} value of 10.9 g/mol were obtained, whereas in batch cultures Y values of

37.9 - 51.7 g/mol corresponded to Y_{ATP} values of 18.0 - 21.2 g/mol. Whether this difference might be explained by a difference in metabolism in batch and continous culture or by an error in the calculations remains to be established.

The Y_{ATP} values obtained for the pyruvate culture remained stable over the range of dilution rates, while the Y_{ATP} values for the cellobiose culture increased considerably with increasing dilution rate. The lowest value of 10.9 g/mol ATP, corresponding to the lowest dilution rate, is comparable to the average Y_{ATP} found for many other fermentative organisms. However, at the highest dilution rate a value of 30.6 g/mol was calculated. This increase can totally be accounted for by the increasing biomass concentrations (85 to 165 mg/l between 0.1 and 0.45 h^{-1}) that were obtained for the increasing dilution rates. It is therefore tempting to explain the increasing Y_{ATP} values by an overestimation of the biomass concentrations, possibly due to the observed precipitates, as explained above. However, at the higher dilution rates, where the highest biomass and Y_{ATP} values were calculated, the carbon balances fit better (1.11) than at the lower dilution rates (1.27).

In the pyruvate culture, the effect of the dilution rate was much less, partly due to the fact that the highest dilution rates used for the cellobiose experiment (0.35 and 0.45 h^{-1}) were for technical reasons not applied to the pyruvate culture. However, the biomass concentration slightly decreased (from 40 to 31 mg/l) and the Y_{ATP} value slightly increased (from 9.30 to 11.9 g/mol) at increasing dilution rates. These Y_{ATP} values are well within the range of those from other fermentative micro-organisms. This confirms the previously described results that in cellobiose batch cultures of *P. furiosus* too high values of Y_{ATP} were obtained compared to pyruvate cultures and compared to the average Y_{ATP} values calculated for other organisms [Kengen & Stams, 1994b]. Therefore, the previous suggestion that an extra ATP-conserving step should be present in the glycolytic pathway of *P. furiosus*, is confirmed by our chemostat results.

Bioenergetic calculations

From the growth yields that were determined at various dilution rates, metabolic quotients q_E were calculated as D/Y. In Figure 2 these data were plotted against the dilution rate D, which equals the growth rate μ. Y_{max} and m values for both growth substrates were calculated (Table 2). The maximal growth yield on cellobiose was 55.9 g/mol, whereas on pyruvate it was 5.9 g/mol. These values are highly comparable to the growth yield values

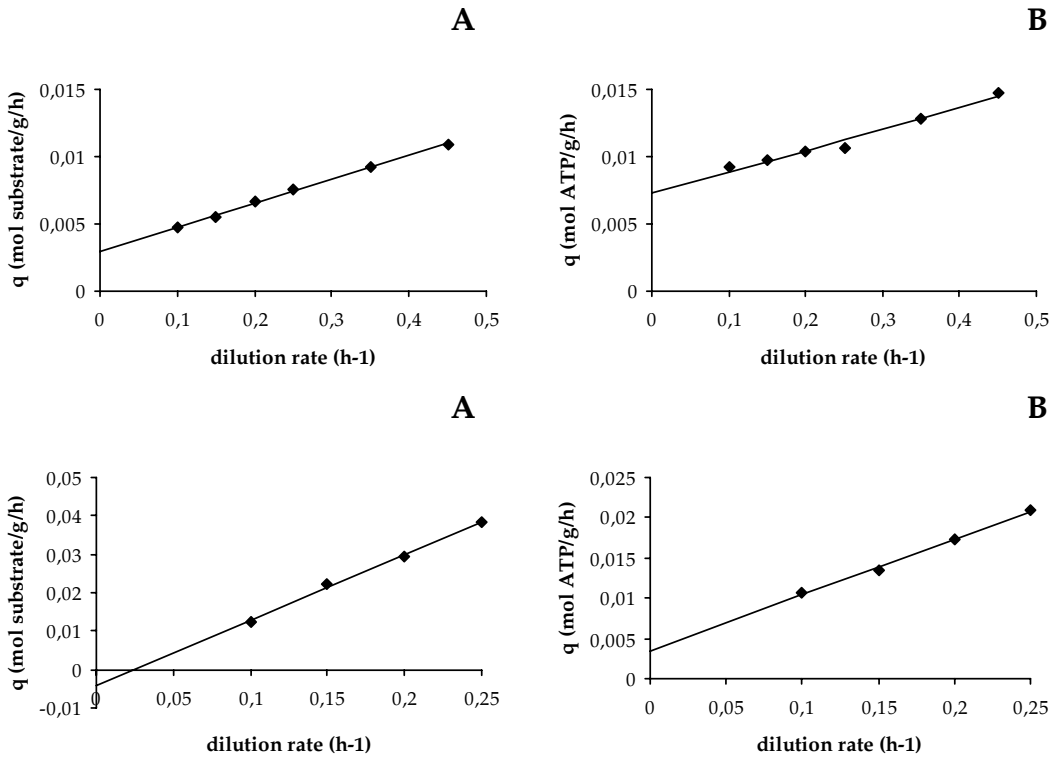


FIGURE 2 Data regression for determination of true growth yield and maintenance coefficient for *P. furiosus* grown in continuous culture on cellobiose (top) or pyruvate (bottom). A: data based on glucose equivalent or pyruvate, B: data based on ATP.

calculated for batch cultures of *P. furiosus* on cellobiose and pyruvate of ca. 47 and 6.4 respectively [Kengen & Stams, 1994b].

The maintenance coefficient was calculated to be 2.9 mmol glucose equivalent per g dry weight per hour on cellobiose and -4.0 mmol pyruvate per g dry weight per hour on pyruvate. Because a negative maintenance coefficient is non-existent, the data were also expressed per mol ATP produced, assuming 1 mol of ATP produced per mol of acetate (Figure 2 and Table 2). Doing so, the maximal growth yield on cellobiose became 62.5 g dry weight per mol of ATP and on pyruvate 14.5 g dry weight per mol of ATP. The maintenance coefficients expressed in mmol ATP per g per hour (m_{ATP}) were 7.2 and 3.5 mmol ATP per g dry weight per hour on cellobiose and pyruvate, respectively. This difference between the two growth substrates is remarkable. When the suggested extra ATP-conserving step in the glycolysis as described above was taken into accout, *i.e.* if one extra ATP would be included in the calculations, the $Y_{max\ ATP}$ for growth on cellobiose would become 31.1 instead of 62.5 g dry weight per mol ATP, which is still a twice as high as the value (14.5 g/mol ATP) for pyruvate, and the m_{ATP} would become 14.4 mmol/g/h for cellobiose, which is even more deviant from the value obtained for pyruvate (3.5 mmol/g/h).

TABLE 2 Calculation of maximal growth yield (Y_{max}) and maintenance coefficient (m) from chemostat data. Y_{max} and and $Y_{max\ ATP}$ were expressed in g dry weight per mol glucose equivalent, pyruvate or ATP, m and m_{ATP} were expressed in mmol glucose equivalent, pyruvate or ATP per g dry weight per hour.

substrate	Y_{max} *g/mol*	$Y_{max\ ATP}$ *g/mol*	m *mmol/g/h*	m_{ATP} *mmol/g/h*
cellobiose	55.9	62.5	2.9	7.2
pyruvate	5.9	14.5	-4.0	3.5

In a previous study, bioenergetic values were calculated for *P. furiosus* grown in continuous culture with and without sulphur using maltose as the limiting carbon source [Schicho *et al*., 1993]. Y_{max} and $Y_{max\ ATP}$ values of 25.7 g per mol glucose equivalent and 12.8 g per mol ATP, respectively, were determined in cultures without sulphur, which are much lower than the values obtained from our data. The maintenance coefficients m and m_{ATP} that were determined as 6.8 mmol glucose equivalent per g per hour and 13.7 mmol ATP per g per hour, respectively, are both a factor 2 higher than our values for growth on cellobiose. A reason for this discrepancy could be that maltose instead of cellobiose was used in the previous study, although previous results did not show any difference between the two disaccharides [Kengen & Stams, 1994b].

Cysteine-free chemostat

Because of the unexpectedly high alanine concentration in the chemostat, an experiment was done in which the medium was reduced with titanium citrate instead of cysteine. Table 3 shows the data obtained from the cysteine-free chemostat experiment, before and after addition of cysteine to the culture. Unfortunately, not all cellobiose was utilised in the cysteine-free

TABLE 3 Data obtained from the cysteine-free chemostat experiment, before and after addition of cysteine to the culture. Values per mM of cellobiose converted are given in parentheses.

parameter	without cysteine	with cysteine
cellobiose converted (*mM*)	1.6	2.0
acetate produced (*mM*)	3.6 (2.25)	5.2 (2.6)
alanine produced (*mM*)	0.38 (0.24)	0.90 (0.45)
biomass concentration (*mg/l*)	140 (87.5)	123 (61.5)
carbon balance	0.91	0.96
Y (*g/mol cellobiose*)	87.1	61.2
Y_{ATP} (*g/mol ATP*)	38.7	23.8

part of the experiment, and therefore the culture was not carbon-limited. Therefore, the hydrogen pressure in the fermentor is expected to be lower in the cysteine-free part of the experiment, which could influence the formation of alanine. Nevertheless, some conclusions can be drawn from this experiment.

Firstly, it is obvious that also in the cysteine-free culture alanine was produced, indicating that the alanine observed in the cysteine-containing experiments cannot completely be explained by a direct formation from cysteine.

Secondly, the observation that more alanine per glucose converted is produced in the cysteine-containing culture than in the cysteine-free culture indicates that alanine can indeed be directly produced from cysteine, possibly by a cysteine desulfurase, an enzyme that has been described for several bacteria [Mihara & Esaki, 2002]. In *P. furiosus*, a cysteine desulfurase has not yet been described nor has a corresponding gene been identified in the genome database. However, in the highly related species *Pyrococcus abyssi* a gene encoding a designated "probable cysteine desulfurase" was identified [www.ncbi.nlm.nih.gov]. A BLAST search of this protein sequence to the genome sequence of *P. furiosus* yielded a 89% identical protein, designated nifS protein, which is in fact a cysteine desulfurase [Mihara & Esaki, 2002]. Therefore, it can be concluded that also in *P. furiosus* a cysteine desulfurase is present. However, before conclusions can be drawn, this should be confirmed by enzyme activity measurements in cell-free extract of *P. furiosus*.

Furthermore, the results of this experiment have consequences for the growth yield data derived from the previous experiments. In the cysteine-free experiment, 0.38 mM alanine was produced from 1.6 mM of cellobiose, which corresponds to 0.24 mol of alanine per mol cellobiose converted. In the cysteine-containing medium, 0.90 mM alanine was found when 2 mM cellobiose was converted, of which 0.43 mM was apparently directly formed from cysteine. This portion of the alanine should be subtracted from the amount of alanine found in the samples and be left out of the carbon balance for the glycolysis, resulting in lower carbon balances (Table 1).

Because the same circumstances were used in this experiment as in the experiment with increasing dilution rates, the same fermentation pattern was expected as in the previous experiment at the same dilution rate. However, only 5.2 instead of 6.23 mM acetate and 0.90 instead of 1.7 mM alanine was produced, whereas the biomass concentration and the Y were in the same range. Since the carbon balance was lower and the Y_{ATP} somewhat higher

than in the previous experiment, probably the acetate (and alanine) concentration was underestimated.

Adenylate energy charge of *P. furiosus* and *E. coli*

The concentrations of ATP, ADP and AMP that were determined in a steady-state culture of *P. furiosus* grown on cellobiose and an exponentially growing *E. coli* culture are listed in Table 4. From these concentrations, the adenylate energy charge was calculated as ([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]). Because the cell numbers in the samples were not determined, the concentrations were calculated in µM in the culture and not corrected for differences in cell numbers. Therefore, the intracellular concentrations cannot be compared between *P. furiosus* and *E. coli*.

However, the data indicate that the adenylate energy charge of *P. furiosus* and *E. coli* is in the same range under these growth conditions. The values of 0.53-0.59 seem low compared to the adenylate energy charge of at least 0.8 that is generally considered to be maintained in growing cells. However, for *E. coli*, also a value of 0.59 has been described [Lundin & Thore, 1975].

Remarkably, in *P. furiosus*, the ADP concentration is almost twice as high as the ATP concentration, whereas in *E. coli* both concentrations are practically equal. Previously it was suggested that the intracellular ATP concentration in *P. furiosus* might be very low, which was based on the very high affinity of the galactokinase for its substrate ATP and the ADP-dependence of the glucokinase and the phosphofructokinase [Verhees *et al.*, 2002]. Our results show that indeed in *P. furiosus* the intracellular concentration of ATP is lower than that of ADP, albeit only by a factor 2. Therefore, it cannot be concluded that in *P. furiosus* ADP acts as the main energy carrier, like ATP does in most other organisms.

TABLE 4 Adenylate concentrations and adenylate energy charge in *P. furiosus* and *E. coli*. Concentrations were measured as described under "Materials and Methods".

concentration	*P. furiosus*	*E. coli*
[ATP] (*µM in culture*)	0.47	0.92
[ADP] (*µM in culture*)	0.72	0.90
[AMP] (*µM in culture*)	0.37	0.49
adenylate energy charge	0.53	0.59

Concluding remarks

The results from our experminents in continuous culture, *i.e.* the Y_{ATP} for growth on cellobiose is higher than that on pyruvate, are comparable to those obtained in batch culture [Kengen & Stams, 1994b], which confirms the previous suggestion that an additional ATP-producing site might be present in the glycolytic pathway of *P. furiosus*. A possible site for extra ATP-generation might be the activity of the PEP synthase in glycolytic direction [Tuininga *et al*., 2003]. However, due to technical problems biomass concentrations could not be accurately determined, making the calculated growth yield data less reliable. Furthermore, from our data it became clear that alanine, besides its function as an electron sink, can be produced directly from cysteine, that is present in the growth medium. The adenylate energy charge of cellobiose-grown cells of *P. furiosus* appeared to be comparable to that of exponentially growing *E. coli* cells.

However, much remains unclear in the bioenergetics of the glycolytic pathway of *P. furiosus*. To obtain better insight, more research is needed in which the uptake mechanisms of the substrates should also be taken into account. In calculations presented in previous studies, energy needed for uptake of substrates was not taken into account because at that time nothing was known about the uptake mechanisms. Recently, an ABC transport system for cellobiose was characterised [Koning *et al*., 2001], which indicates that transport of cellobiose into the cell is an energy-consuming process that requires ATP, thus decreasing the amount of ATP that is available for growth and maintenance. It is not known yet how much ATP is involved in this process, nor how pyruvate is transported into the cell and whether pyruvate uptake also is an energy-consuming process. Growth of *P. furiosus* on pyruvate is stimulated at higher pyruvate concentration, which suggests that uptake of pyruvate is a passive transport process [S.W.M. Kengen, unpublished results].

Furthermore, the explanation of the difference between growth yield data on pyruvate and disaccharides need not be found in the glycolytic pathway itself. The presence of an energy-conserving system, consisting of a membrane-bound hydrogenase, was calculated to account for an additional 1.2 mol of ATP per mol of glucose converted, which would decrease the Y_{ATP} during growth on sugars [Sapra *et al*., 2003]. Whether this system is of any importance in pyruvate-grown cells remains to be established.

7

Summary and concluding remarks

Over the past decade, much research has been done on so-called "extremophiles", *i.e.* organisms that grow under extreme conditions like high or low pH, high or low temperature or high salt concentration. Especially thermophiles and hyperthermophiles (growing optimally at temperatures above 80°C) have received a lot of attention, since they are considered to be the closest relatives to the last common ancestor of all life on earth [Woese *et al*., 1990] and because of the potential application of their enzymes in many industrial processes [van den Burg, 2003].

One of the most intensively studied hyperthermophilic Archaea is *Pyrococcus furiosus*, that uses a modified Embden-Meyerhof pathway during growth on sugars. In this pathway, two ADP-dependent sugar kinases and a tungsten-containing glyceraldehyde-3-phosphate ferredoxin oxidoreductase are present. These modifications from the classical Embden-Meyerhof pathway have important consequences for the bioenergetics of the organism. This thesis describes the research that was done on the enzymology and the bioenergetics of the modified Embden-Meyerhof pathway in *P. furiosus*. The bioenergetically important enzymes of the pathway were purified and characterised and chemostat studies were done to get more insight into the bioenergetics of *P. furiosus*.

In **Chapter 1** a general introduction to the subject is given. Recent studies on the sugar degradation pathways of several hyperthermophiles have indicated that many hyperthermophilic micro-organisms use a modified pathway. Some of them show the same modifications as in *P. furiosus*, whereas also other modifications have been described.

Chapter 2 describes the purification and characterisation of the first enzyme of the modified Embden-Meyerhof pathway, the ADP-dependent glucokinase. The level of this enzyme and the subsequent glycolytic enzymes, *i.e.* phosphoglucose isomerase and ADP-dependent phosphofructokinase appeared to be closely regulated by the substrate. The ADP-dependent glucokinase was purified to homogeneity. The oxygen-stable enzyme was composed of two identical subunits and was highly specific for ADP, which could not be replaced by ATP, phosphoenolpyruvate, GDP, pyrophosphate, or polyphosphate. D-Glucose could be replaced only by 2-deoxy-D-glucose, albeit with a low efficiency. The K_m values for D-glucose and ADP were 0.73 and 0.033 mM, respectively. An optimum temperature of 105°C and a half-life of 220 min at 100°C are in agreement with the requirements of this hyperthermophilic organism. The properties of the glucokinase were compared to those of less thermoactive gluco- and hexokinases [Kengen *et al*., 1995].

The glucokinase in *P. furiosus* was the first ADP-dependent kinase that was ever described, but apparently *P. furiosus* is not the only organism in which this type of kinase is present. ADP-dependent glucokinases have also been described from the Archaea *Thermococcus litoralis* [Koga *et al*., 2000] and *Archaeoglobus fulgidus* [Labes & Schönheit, 2003], although in another Archaeon, *Aeropyrum pernix*, an ATP-dependent glucokinase is present [Hansen *et al*., 2002b]. Thus, ATP-dependent kinases are not completely absent in Archaea, as was also implied by the presence of an ATP-dependent galactokinase in *P. furiosus* [Verhees *et al*., 2002]. Besides biochemical studies on the ADP-dependent glucokinases, also crystallographic studies have been done. The crystal structures of the ADP-dependent glucokinases from *T. litoralis* [Ito *et al*., 2001], *Pyrococcus horikoshii* [Tsuge *et al*., 2002], and *P. furiosus* [Ito *et al*., 2003] that have recently been determined, appeared to be similar. The enzymes consisted of large α/β-domains and small domains and showed structural similarity with an ATP-dependent ribokinase family. It was suggested that a large conformational change takes place during the enzymatic reaction [Tsuge *et al*., 2002], [Ito *et al*., 2003]. In the *P. furiosus* glucokinase, it was shown that glucose binds in a groove between the large and the small domain [Ito *et al*., 2003].

In **Chapter 3**, the characterisation of the ADP-dependent phosphofructokinase is described. The gene encoding this enzyme was identified in the genome of *P. furiosus* when the N-terminal amino acid sequence of the glucokinase was used to detect the corresponding gene. It turned out that the amino acid sequences of both kinases were 21.1% identical but not homologous to those of other sugar kinases. The gene encoding the ADP-dependent phosphofructokinase was cloned and expressed in *E. coli* and the recombinant phosphofructokinase was purified and characterised. The oxygen-stable protein had a homotetrameric structure and showed a specific activity of 88 U/mg at 50°C and a pH optimum of 6.5. As phosphoryl group donor, ADP could be replaced by GDP, ATP, and GTP to a limited extent. The K_m values for fructose-6-phosphate and ADP were 2.3 and 0.11 mM, respectively. The phosphofructokinase did not catalyse the reverse reaction, nor was it regulated by any of the known allosteric modulators of ATP-dependent phosphofructokinases. ATP and AMP were identified as competitive inhibitors of the phosphofructokinase, raising the K_m for ADP to 0.34 and 0.41 mM, respectively [Tuininga *et al*., 1999].

Amino acid sequence comparison of the phosphofructokinase of *P. furiosus* with those of proteins present in the GenBank data base showed homology with a hypothetical protein from *Methanococcus jannaschii*, which

turned out to be an ADP-dependent phosphofructokinase. The corresponding gene was cloned and expressed in *E. coli* and the protein was purified and characterised, as described in **Chapter 4**. The enzyme had an extremely low K_m for fructose-6-phosphate (9.6 µM), and it accepted both ADP and acetyl-phosphate as phosphoryl group donor. Further genome analysis revealed the presence of ADP-dependent phosphofructokinases in glycogen-producing members of the *Methanococcales* and *Methanosarcinales*, including both mesophilic and thermophilic representatives. Furthermore, uncharacterised homologs of this unusual kinase appeared to be present in several Eukarya [Verhees *et al*., 2001b]. Recently, the ADP-dependent phosphofructokinase of *M. jannaschii* was described to be bifunctional, showing phosphofructokinase as well as glucokinase activity [Sakuraba *et al*., 2002].

Although *P. furiosus* was the first organism in which an ADP-dependent phosphofructokinase was found, similar enzymes have since then been described in other Archaea as well, *i.e. Thermococcus zilligii*, that is closely related to *P. furiosus* [Ronimus *et al*., 1999a], [Ronimus *et al*., 2001a], and *Archaeoglobus fulgidus* [Labes & Schönheit, 2001]. Recently, from preliminary analysis of the crystallised ADP-dependent phosphofructokinase from *T. litoralis*, it was concluded that the overall structure of the enzyme resembles that of ADP-dependent glucokinases [Jeong *et al*., 2003]. However, in some other Archaea, ATP-dependent phosphofructokinases were found, *i.e.* in *Desulfurococcus amylolyticus* [Hansen & Schönheit, 2000] and *Aeropyrum pernix* [Hansen & Schönheit, 2001] as well as in the hyperthermophilic Bacterium *Thermotoga maritima* [Ding *et al*., 2001], [Hansen *et al*., 2002a]. A third type of phosphofructokinases was found in the Archaeon *Thermoproteus tenax* [Siebers *et al*., 1998] and in the hyperthermophilic Bacterium *Dictyoglomus thermophilum* [Ding *et al*., 1999]. This enzyme, that is pyrophosphate (PP_i)-dependent, is also found in some Bacteria and Eukarya and is distantly related to ATP-dependent phosphofructokinases.

Based on their biochemical properties and phylogeny, the phosphofructokinases can be classified in three families. Family A contains ATP- and PP_i-dependent enzymes from higher Eukarya, Bacteria and plants, whereas the only archaeal member of this family appears to be the PP_i-dependent phosphofructokinase from *Thermoproteus tenax*. While the ATP-dependent enzymes are usually major control points in the glycolytic pathway and therefore allosterically regulated by a number of effectors, the PP_i-dependent enzymes catalyse a reversible reaction and can be divided in two groups. Type I PP_i-phosphofructokinases are not allosterically regulated, but type II enzymes are activated by fructose-2,6-bisphosphate. All family B

phosphofructokinases are ATP-dependent, including the enzyme from the Archaeon *Aeropyrum pernix*. The enzymes show a broader substrate specificity than the family A ATP-dependent kinases. Family C consists of the ADP-dependent phosphofructokinases from Archaea, that are all non-allosteric enzymes [Ronimus & Morgan, 2001].

Another bioenergetically important step in the modified Embden-Meyerhof pathway of *P. furiosus* is the conversion of phosphoenolpyruvate (PEP) into pyruvate. In the classical Embden-Meyerhof pathway this step is catalysed by pyruvate kinase and yields 1 ATP molecule that is formed from ADP. *P. furiosus* possesses a pyruvate kinase, but recently it was suggested that in *P. furiosus* the reversible PEP synthase could be active in glycolytic direction, thereby producing ATP from AMP in one step [Sakuraba *et al.*, 1999]. Because of the large bioenergetic consequences of this suggested modification, the two enzymes were purified and characterised (**Chapter 5**). The PEP synthase appeared to be an unusual 2000-kDa multimeric protein, whereas the pyruvate kinase showed a normal tetrameric structure. Opposite to values from literature, the determined $\Delta G^{0\prime}$ value of the PEP synthase reaction was negative in glycolytic direction, indicating that in *P. furiosus* this reaction is thermodynamically feasible. Kinetic studies of both enzymes revealed allostericity of the pyruvate kinase for its substrate ADP. Furthermore, the affinity of the pyruvate kinase for ADP was decreased in the presence of AMP, which implies that in case of a high intracellular AMP level, the PEP synthase is favoured over the pyruvate kinase, which would increase the ATP yield of the pathway.

A hypothesis was presented in which the modified Embden-Meyerhof pathway of *P. furiosus* is regulated at the level of PEP by the integrated activities of pyruvate kinase and PEP synthase in glycolytic direction. The relative activities of the enzymes were suggested to be dependent on the energy level of the cell. The catabolic role of the PEP synthase allows the conservation of an extra ATP in the pathway, but it also recycles the AMP that is formed by the two ADP-dependent sugar kinases that are active in the first part of the pathway. However, another possibility for extra energy conservation in *P. furiosus* was recently described. A membrane-bound hydrogenase might serve as a proton pump, thereby creating a transmembrane proton gradient that can be used by an ATPase to produce ATP [Sapra *et al.*, 2003].

To get more insight into the bioenergetics of the glycolytic pathway of *P. furiosus*, chemostat culture experiments were done with cellobiose and pyruvate as the limiting carbon sources, as described in **Chapter 6**. Although

some technical problems were encountered with regard to the biomass determination, the growth yield data obtained from the chemostat cultures were highly comparable to those obtained in batch cultures of *P. furiosus* on cellobiose, maltose and pyruvate. This confirmed the previous suggestion that an additional ATP-conserving site might be present in the glycolysis [Kengen & Stams, 1994b]. Maximal growth yields and maintenance coefficients were calculated, which were somewhat different from literature values. Furthermore, an experiment using cysteine-free growth medium indicated that alanine, which is one of the end products of the fermentative metabolism, could also be produced directly from cysteine, possibly enzymatically by a cysteine desulfurase. The adenylate energy charge was determined in steady-state culture and although the ADP concentration appeared to be lower than in an *E. coli* culture, the energy charge of both organisms was highly comparable.

In conclusion, this research project has provided us with more information on the enzymology and bioenergetics of the modified Embden-Meyerhof pathway in the hyperthermophilic Archaeon *Pyrococcus furiosus*. The bioenergetically important enzymes were purified and characterised and the corresponding genes were identified. These genes and enzymes were compared to those in other hyperthermophilic Archaea, as well as in mesophilic organisms. The bioenergetic studies that were done in chemostat cultures confirmed the previous suggestion that extra ATP could be formed in the glycolysis of *P. furiosus*. As a possible site for this extra ATP production, the activity of the reversible PEP synthase in glycolytic direction was suggested, since this reaction appeared to be thermodynamically feasible at 80°C. The extra energy-yielding step could, however, also be found in the activity of a membrane-bound hydrogenase. Thus, with the combination of the pyruvate kinase, the PEP synthase and the membrane-bound hydrogenase, *P. furiosus* has an energy-generating system that can adjust the amount of energy gained from the sugar metabolism according to its energetic requirements under different conditions.

8

Samenvatting

Enzymologie en bio-energetica van de glycolytische route van *Pyrococcus furiosus*

Dit proefschrift behandelt het onderzoek dat ik de afgelopen jaren gedaan heb naar het suikermetabolisme van het hyperthermofiele micro-organisme *Pyrococcus furiosus*. In dit hoofdstuk worden enkele veelgebruikte termen uit het proefschrift uitgelegd en wordt een samenvatting gegeven van de behaalde resultaten.

Hyperthermofiele micro-organismen

Micro-organismen zijn, zoals de naam al aangeeft, microscopisch kleine beestjes, die met het blote oog niet te zien zijn. Deze organismen komen overal op aarde voor: in het menselijk lichaam, in de oceanen, in heet-waterbronnen, in de lucht, enzovoort. Voor micro-organismen die onder extreme omstandigheden leven, zoals bij een erg hoge of erg lage temperatuur, in heel zout water of onder hoge druk, wordt de term "extremofielen" gebruikt. Onder deze extremofielen vallen ook de thermofielen ("warmteminnenden"), die bij temperaturen boven de 60°C groeien en de hyperthermofielen ("hitteminnenden"), die zelfs boven de 80°C het best gedijen. *Pyrococcus furiosus*, het organisme waarom het in dit proefschrift gaat, is zo'n hyperthermofiel. Zijn optimum groeitemperatuur is 100°C, dus hij groeit het snelst in kokend water.

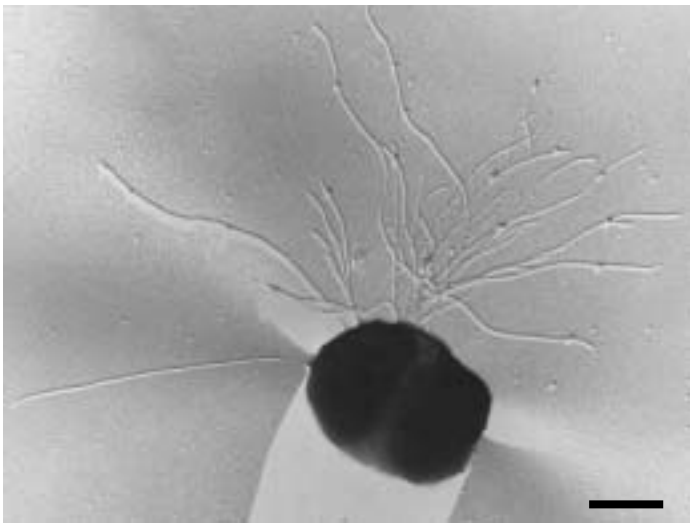
In theorieën over het ontstaan van leven op aarde wordt over het algemeen aangenomen dat de aarde uit een hete oersoep bestond toen het eerste leven zich ontwikkelde. Het lijkt dan ook logisch dat de eerste levende wezens op aarde hyperthermofielen waren. Dit is een van de redenen waarom *Pyrococcus* en zijn soortgenoten interessante beestjes zijn. Door te kijken naar verschillen tussen deze oude hyperthermofielen en jongere organismen, zoals planten en zoogdieren, kunnen we meer inzicht krijgen in de evolutie. Bovendien kan de vergelijking van hyperthermofielen met "normale" bacteriën informatie opleveren over de speciale overlevings-mechanismen die de hyperthermofielen hebben ontwikkeld om onder die extreme omstandigheden te kunnen leven. Tot slot zijn hyperthermofielen voor sommige industriële processen interessant, omdat ze bestand zijn tegen hoge temperaturen en vaak ook tegen andere extreme omstandigheden die in de industrie gebruikt worden.

Pyrococcus furiosus

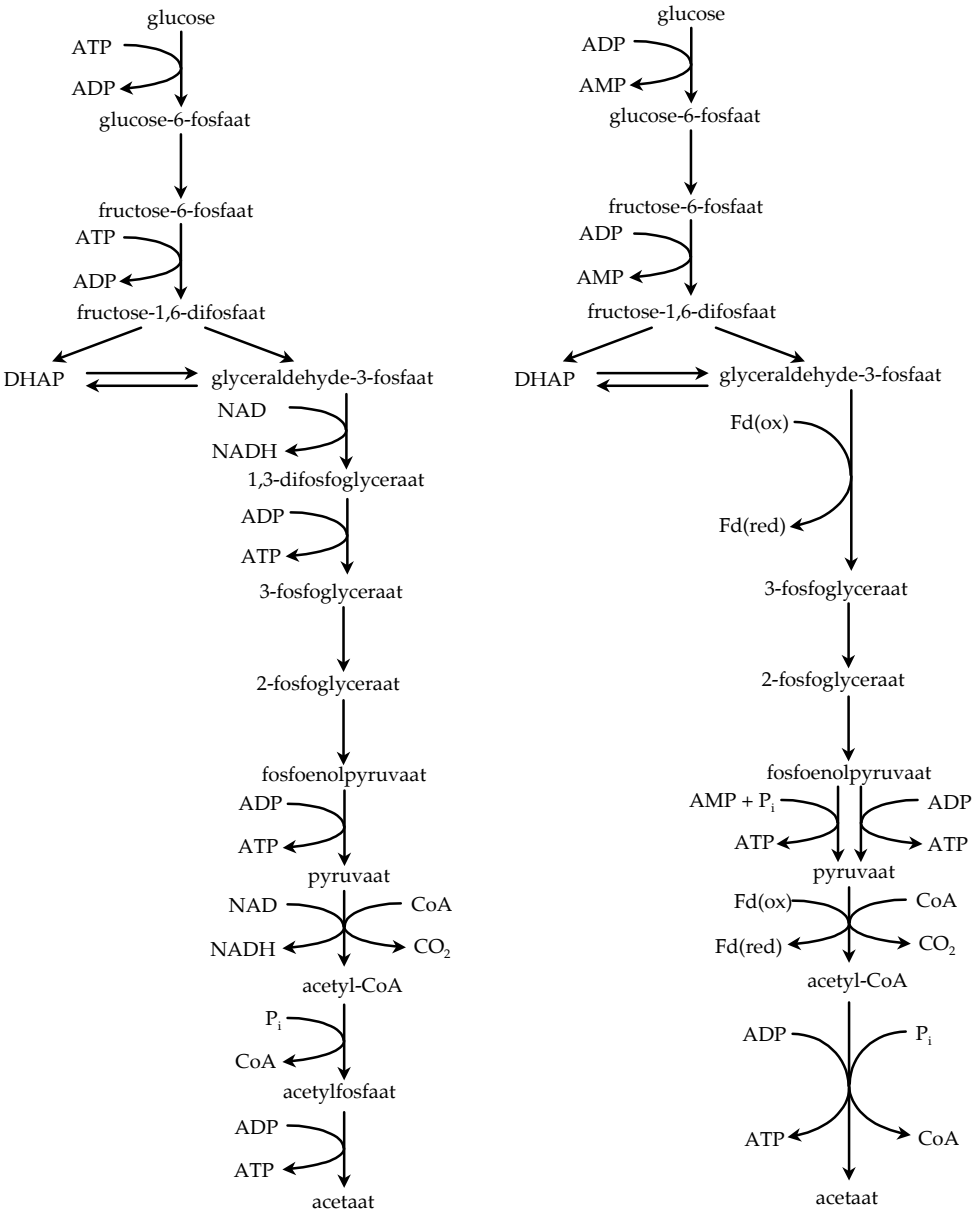
De naam *Pyrococcus furiosus* is afgeleid uit het Latijn en betekent "ziedende vuurbal". Ziedend omdat het beestje zo snel groeit, hij verdubbelt zich elke 37 minuten, en vuurbal omdat het een bolvormig organisme is dat in kokend water groeit (zie Figuur 1). *P. furiosus* is in 1986 ontdekt en opgekweekt uit vulkanisch verhit water in de zee bij Italië. Omdat het zo snel groeit en relatief makkelijk te kweken is, is *Pyrococcus* een veelbestudeerde hyperthermofiel geworden. Hij kan groeien op verschillende voedingsstoffen, zoals zetmeel (aardappelzetmeel vindt hij lekkerder dan maïszetmeel), moutsuiker (maltose) en melksuiker (lactose).

Suikermetabolisme van *P. furiosus*

Een van de onderwerpen die bestudeerd zijn in *Pyrococcus* is het suikermetabolisme, dat is de stofwisseling van de bacterie. Al snel bleek *Pyrococcus* een aantal variaties te vertonen op de gebruikelijke suikerafbraak, zoals die plaatsvindt in andere bacteriën als *E. coli* en *Salmonella* en ook in de mens. Een van de bekende suikerafbraakroutes heet "glycolyse" en staat getekend in Figuur 2, zowel de gevarieerde route van *Pyrococcus* als de klassieke route, bekend uit andere organismen. De route begint met suikers die door het organisme worden opgenomen uit hun omgeving, die daarna stapje voor stapje worden omgezet in kooldioxide (CO_2) en azijnzuur (acetaat). Elk stapje in deze keten wordt uitgevoerd door een specifiek enzym, dat is een eiwit dat een bepaalde reactie kan laten verlopen. In grote lijnen is de glycolyse van *Pyrococcus* gelijk aan de route die bekend is uit andere organismen, maar toch zijn er een paar belangrijke verschillen.

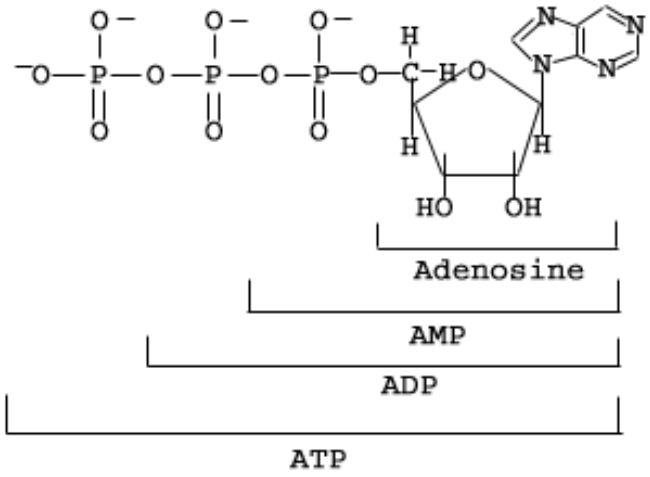


FIGUUR 1 Electronenmicroscopische foto van *Pyrococcus furiosus*. Op deze foto is goed te zien dat *Pyrococcus* een bosje flagellen heeft, waarmee het zich kan voortbewegen. Het streepje geeft een lengte aan van 1 µm, oftewel 1 duizendste mm.



FIGUUR 2 Klassieke glycolyse (links) en gevarieerde glycolyse van *Pyrococcus furiosus*. Zoek de 6 verschillen!

Zo wordt er in de eerste stappen van de route twee keer een energierijk molecuul geïnvesteerd om de suiker uiteindelijk zo efficiënt mogelijk af te kunnen breken. Normaal gesproken is dit molecuul ATP (een adenosine-molecuul met 3 gebonden fosfaatgroepen, zie Figuur 3) maar in *Pyrococcus* bleken de enzymen in de eerste stappen niet ATP maar ADP (hetzelfde molecuul maar dan met 2 gebonden fosfaatgroepen) te gebruiken. Dit was nog nooit eerder aangetoond voor soortgelijke enzymen uit welk ander organisme dan ook. De reden voor deze variatie was (en is nog steeds) niet helemaal duidelijk. Het investeren van ATP en ADP kost evenveel energie en beide moleculen zijn aanwezig in de cellen. Het zou zo kunnen zijn dat het bij



FIGUUR 3 Structuurformule van ATP, ADP en AMP.

hoge temperaturen een bepaald voordeel heeft om ADP in plaats van ATP te gebruiken, hoewel er ook hyperthermofielen bekend zijn die wel ATP gebruiken voor dezelfde stap. Het zou ook kunnen dat *Pyrococcus* een heel oud type enzym bevat, dat pas later in de evolutie overstapte op ATP.

Verderop in de route van *Pyrococcus* is een enzym actief dat een stap uitvoert die normaal gesproken in twee stapjes, door twee verschillende enzymen, wordt uitgevoerd. Het gaat hier om de omzetting van glyceraldehyde-3-fosfaat naar 3-fosfoglyceraat. Ook dit was nog nooit eerder vertoond. Deze variatie lijkt onlogisch, omdat de twee losse stapjes energie opleveren (in de vorm van ATP) voor het organisme, terwijl de variatie in *Pyrococcus* geen energie oplevert. Het is echter gebleken dat dit nieuwe enzym ervoor zorgt dat *Pyrococcus* een extra controlepunt heeft in de suikerafbraak, dat kan worden in- of uitgeschakeld als er meer of minder suiker aanwezig is in de omgeving of als er meer of minder energie nodig is in de cel. Bovendien lijkt het erop dat de variatie-stap toch gekoppeld is aan een energie-leverende stap, maar dan in een andere vorm, buiten de glycolyse.

Het onderzoek

Aan het begin van het onderzoek was er nog veel onduidelijk over de afzonderlijke stapjes van de glycolyse in *Pyrococcus* en over hoeveel energie de route precies oplevert en op welke plekken. Daarom is er gekeken naar de enzymen die de energetisch interessante stappen uitvoeren (de enzymologie) en naar de energie-opbrengst van de route in zijn geheel (de bio-energetica). De resultaten van dit onderzoek staan beschreven in dit proefschrift.

In **Hoofdstuk 1** is een inleiding gegeven op het onderwerp in het algemeen. Er wordt uitgelegd wat hyperthermofielen zijn, welke variaties op de bekende suikerafbraakroutes bekend zijn in de verschillende organismen en wat er bij het begin van het onderzoek bekend was over de glycolyse van *Pyrococcus*.

Hoofdstuk 2 gaat over het enzym dat het eerste stapje van de glycolyse uitvoert, namelijk het glucokinase. Het enzym is gezuiverd uit cellen van *Pyrococcus* en daarna in detail bestudeerd. Het enzym gebruikt ADP in plaats van ATP en kan bijna geen andere suikers omzetten dan glucose (druivensuiker). Met behulp van de aminozuurvolgorde van het enzym kon het bijbehorende gen gevonden worden op het chromosoom van *Pyrococcus*. Een gen is een stukje DNA dat de genetische code bevat voor een specifiek eiwit.

Het gen van het glucokinase bleek helemaal niet te lijken op genen van glucokinases uit andere organismen, maar wel op het gen voor het enzym dat de derde stap van de glycolyse uitvoert, namelijk het fosfofructokinase. Dit enzym wordt beschreven in **Hoofdstuk 3**. Ook dit enzym gebruikt ADP in plaats van ATP en bovendien bleek het niet gevoelig te zijn voor een aantal stofjes die normaal gesproken het enzym remmen of juist stimuleren en daarmee de snelheid van de glycolyse reguleren. Blijkbaar reguleert *Pyrococcus* de snelheid van de route dus op een andere manier. De beide ADP-afhankelijke kinases zijn erg aan elkaar verwant en blijken bij een nieuwe familie van suikerkinases te horen.

Tegelijk met het gen voor het fosfofructokinase in *Pyrococcus* werd ook het gen voor het fosfofructokinase in een hyperthermofiele methaanbacterie, *Methanococcus jannaschii*, gevonden. Dit was opmerkelijk, omdat methaanbacteriën normaal gesproken geen glycolyse hebben. Ze halen hun energie namelijk uit methaanvorming in plaats van uit suikerafbraak. Daarom werd ook dit enzym gezuiverd en in detail bestudeerd, wat beschreven staat in **Hoofdstuk 4**. Het enzym gebruikt evenals het enzym uit *Pyrococcus* ADP om de suiker mee te activeren. Waarschijnlijk wordt de stap in *Methanococcus* normaal gesproken in de omgekeerde richting uitgevoerd, in een suiker-opbouwende route, de gluconeogenese. Bovendien bleek *Methanococcus* niet de enige methaanbacterie te zijn waarin een soortgelijk enzym en gen voorkomt. In een heel aantal andere methaanbacteriën is hetzelfde enzym aangetoond, zowel in hyperthermofielen als in methaanbacteriën die bij lagere temperaturen groeien.

Een andere belangrijke stap in de glycolyse is een van de laatste stappen in de route, de omzetting van fosfo-enolpyruvaat (fosfo-enol-

pyrodruivenzuur) naar pyruvaat (pyrodruivenzuur). Normaal gesproken wordt deze stap uitgevoerd door het enzym pyruvaatkinase, waarbij ADP in ATP wordt omgezet. Deze stap levert dus energie op in de vorm van 1 fosfaatbinding. Een groep Japanse onderzoekers heeft een aantal jaren geleden gesuggereerd dat in *Pyrococcus* de stap zou worden uitgevoerd door het enzym fosfo-enolpyruvaatsynthase (afgekort PEP-synthase), waarbij AMP in ATP wordt omgezet, wat dus energie oplevert in de vorm van 2 fosfaatbindingen (zie Figuur 3). Dat het enzym aanwezig is in *Pyrococcus* was al bekend, maar normaal gesproken is het actief in de omgekeerde richting in de suikeropbouwende gluconeogenese. De stap is energetisch ongunstig in de glycolyserichting, wat het onwaarschijnlijk maakt dat *Pyrococcus* hier toch energie uit zou kunnen halen. Bovendien is in *Pyrococcus* ook een pyruvaatkinase aanwezig, wat vreemd is als het organisme door het gebruik van het andere enzym extra energie zou kunnen halen uit de suikerroute. Vanwege deze onduidelijkheden zijn de beide enzymen, pyruvaatkinase en PEP-synthase, gezuiverd en bestudeerd (**Hoofdstuk 5**). Heel verrassend bleek dat de energie van de omzetting van het PEP-synthase in *Pyrococcus* wel degelijk gunstig is in de glycolyserichting, zodat de suggestie van de Japanse onderzoekers waar zou kunnen zijn. Uit onze resultaten bleek dat beide enzymen actief zijn in de glycolyse, alnaargelang de gewenste hoeveelheid energie en de aanwezige hoeveelheid AMP in de cel. Blijkbaar heeft *Pyrococcus* hier een nieuwe manier van regulatie van de suikerafbraakroute gevonden.

Over de energie-opbrengst van de hele route bestond in het begin van het onderzoek onduidelijkheid. Daarom is dit bestudeerd en beschreven in **Hoofdstuk 6**. Eerdere proeven die de bio-energetica beschreven waren gedaan in flesjes. Hierin veranderen de omstandigheden voortdurend, doordat de suiker die in het begin wordt toegevoegd langzaam opraakt en de produkten van de route in het flesje ophopen. Een betere methode voor energetische studies is dan ook de "chemostaat" oftewel "continu-culture". In dit geval wordt *Pyrococcus* gekweekt in een fermentor, waaraan heel langzaam vers medium met suiker wordt toegevoegd en even langzaam het oude medium wordt verwijderd. Op deze manier kunnen de omstandigheden in de fermentor precies constant gehouden worden.

Helaas zijn er bij deze proeven wat technische problemen opgetreden. Zo werd het medium al troebel voor het begin van de proeven, waardoor de hoeveelheid cellen in de fermentor niet duidelijk vast te stellen was. Desondanks werd door deze experimenten bevestigd wat al eerder gesuggereerd was door onderzoekers op ons laboratorium, namelijk dat er

ergens in de route extra energie gewonnen moet worden. Waar dat precies moet gebeuren is uit de proeven niet duidelijk geworden, maar vlak voor het eind van het onderzoek publiceerden Amerikaanse onderzoekers een artikel waarin beschreven staat hoe *Pyrococcus* energie kan maken met behulp van produkten van de glycolyse die de cel uit getransporteerd worden. Dit zou een oplossing kunnen zijn.

In **Hoofdstuk 7** worden de resultaten van het onderzoek samengevat en wordt een overzicht gegeven van wat er sinds de ontdekking van de ADP-afhankelijke kinases in *Pyrococcus* over dergelijke enzymen in andere organismen is ontdekt.

Tot slot

Door het onderzoek beschreven in dit proefschrift is meer bekend geworden over de verschillende enzymen die actief zijn in de suikerafbraakroute van *Pyrococcus furiosus*. De kinases die actief zijn in de eerste stappen gebruiken ADP in plaats van ATP en zijn erg aan elkaar verwant. Dergelijke enzymen blijken in meer organismen voor te komen. In een van de laatste stappen van de glycolyse zijn twee enzymen actief: het pyruvaatkinase en het PEP-synthase, waarvan het laatste dubbel zoveel energie oplevert. De proeven over de energie-opbrengst van de route hebben eerdere resultaten bevestigd dat er ergens in de route extra energie gevormd moet worden. Die extra energie zou dus kunnen komen uit het PEP-synthase, maar ook uit het door de Amerikanen beschreven transportsysteem. Met de combinatie van het pyruvaatkinase, het PEP-synthase en het transportsysteem heeft *Pyrococcus* dus een combinate van energie-opleverende systemen, waarmee de energie-opbrengst uit het suikermetabolisme aangepast kan worden aan de energiebehoefte van de cel onder verschillende omstandigheden.

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Curriculum vitae

Op zaterdag 19 juni 1971 werd Judith Estella Tuininga geboren in Roden. Na een aantal omzwervingen belandde het gezin - inmiddels uitgebreid met een zusje en een broertje - in Baflo, waar Judith de Openbare Lagere School Baflo doorliep. Vervolgens ging ze in Warffum naar Rijksscholengemeenschap 'Het Hogeland' en in de zomer van 1989 vertrok ze met haar atheneumdiploma op zak naar Wageningen om levensmiddelentechnologie te gaan studeren.

Bij het practicum Inleiding Levensmiddelenmicrobiologie sloeg de vonk over, waarna Judith besloot de specialisatie levensmiddelenmicrobiologie te kiezen. Ze deed een afstudeervak bij de sectie Levensmiddelenmicrobiologie van de vakgroep Levensmiddelentechnologie naar het ontstaan van nisine-resistente mutanten van *Listeria monocytogenes* en *Bacillus cereus*. Na dit afstudeervak vertrok ze voor ruim 6 maanden naar Kopenhagen, waar ze bij de Kongelige Veterinær- og Landbohøjskole in het kader van een Erasmus-stage meewerkte aan onderzoek naar startercultures voor de vleesindustrie. Na terugkomst deed Judith een tweede afstudeervak, nu bij de werkgroep MicFys van de vakgroep Microbiologie, waar ze de ADP-afhankelijke kinasen uit *Pyrococcus furiosus* onderzocht. Tijdens dit afstudeervak sloeg er weer een vonk over, nu voor het hyperthermofiele micro-organisme. In augustus 1995 studeerde ze af.

Na een jaar van vruchteloze pogingen om de arbeidsmarkt te betreden, begon Judith in oktober 1996 aan een na-doctoraal onderzoeksproject bij de werkgroep BacGen van de vakgroep Microbiologie. Hier leerde ze werken met moleculaire technieken en onderzocht ze een transcriptie-regulator uit *P. furiosus*.

In september 1997 begon ze als onderzoeker in opleiding aan het onderzoek naar de enzymologie en bio-energetica van de glycolyse van *P. furiosus*, waarin ze in eerste instantie het werk van haar afstudeervak kon voortzetten. Na een jaar werd de aanstelling omgezet van voltijds naar 4 dagen per week, waardoor het project met een jaar verlengd werd. De resultaten van het onderzoek staan beschreven in dit proefschrift. Sinds oktober 2003 is Judith werkzaam als docent microbiologie aan het ROC Leiden.

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Aftiteling

Nu de inhoudelijke onderdelen van mijn proefschrift op papier staan, is het tijd geworden om terug te blikken. Als ik de afgelopen jaren aan me voorbij laat glijden, is het alsof ik een film terugzie. En het is me een film geweest! Maar wel één met kenmerken uit heel verschillende genres, van *detective* en *love story* tot *comedy* en *film noir*, met gelukkig dit boekje en het feest als *happy end*! Zes jaar geleden heb ik de rol van regisseur toebedeeld gekregen en mijn naam prijkt dan ook op de omslag, maar het had onmogelijk tot een goed einde kunnen komen zonder de hulp van een heleboel anderen. Daarom op deze plaats de aftiteling.

Om te beginnen werd de hoofdrol in het hele verhaal met glans gespeeld door *Pyrococcus furiosus*. Hij liet zich steeds weer gewillig binnenstebuiten keren en ondanks alle grillen en "sterallures" bleef het een fantastisch beest om mee te werken!

Naast of achter mijn regisseursstoel zaten mijn drie begeleiders, die ervoor zorgden dat ik het scenario niet uit het oog verloor. In de eerste plaats wil ik mijn copromotor Servé bedanken voor alles dat ik van hem geleerd heb, en dat is heel veel! Hij liet mij tijdens een practicum kennismaken met *Pyrococcus* en heeft daarna jaren met me opgescheept gezeten. Ik kon altijd bij hem terecht om praktische tips te vragen, om te filosoferen over onverwachte resultaten of om gewoon een praatje te maken. Mijn tweede begeleider Fons promoveerde tijdens mijn OIO-periode van copromotor naar promotor en daar ben ik heel blij om. Hij had een heel andere - vaak verhelderende - kijk op het onderzoek en maakte net als Servé altijd tijd voor me. Zijn snelheid van manuscripten nakijken is en blijft ongekend! De derde begeleidersrol was voor Willem. In het begin van wat dichterbij, later meer op afstand, bleef zijn invloed op mijn onderzoek en het proefschrift toch altijd duidelijk.

In de openingsscène van de film werden Corné en ik aan elkaar uitgehuwelijkt, doordat onze projecten dicht met elkaar verweven waren. Hoewel het soms niet bepaald een sprookjeshuwelijk leek, kijk ik toch terug op een hele prettige samenwerking, waar ik veel van geleerd heb en waar mooie dingen uit

voortgekomen zijn! Cornés copromotor John wil ik bedanken voor het aanstekelijke enthousiasme waarmee hij zich met onze projecten bezighield.

Het Laboratorium voor Microbiologie fungeerde als filmset waar het allemaal gebeurde. Het was er een komen en gaan van mensen die voor korte of langere tijd allemaal aan een eigen (film)project werkten, of aan dat van een collega. Vooral de medewerkers en oud-medewerkers van de werkgroep MicFys (onder meer Arthur, Wim, Bo, Caroline, Miriam, Bernd, Anne-Meint, Heleen, Sander, Maurice, Wout, Melike, John, Nico, Fons, Ed, Frank, Sonja, Bram, Caroline, Jan, Peter & Peter) wil ik van harte bedanken voor het beantwoorden van mijn vragen, de praktische tips, de feedback en de gezelligheid tijdens pauzes, barbecues, kerstdiners, avondjes uit, congressen en natuurlijk het reisje naar Duitsland en Zwitserland!

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Voor stunts die ik op de vakgroep niet kon uitvoeren, heb ik hulp gezocht van buitenaf. George Ruijter van (toen nog) MGIM heeft geholpen bij een poging het PFK te zuiveren met behulp van hun kleurstofkolommetjes, Hans Wassink van Biochemie leverde gezuiverd POR uit *Pyrococcus* voor enzymassays, Gerrit van Koningsveld van Levensmiddelenchemie bediende de MALDI-TOF om het molecuulgewicht van het *Methanococcus*-PFK te achterhalen, Joop Groenewegen van Virologie bood me door de elektronenmicroscoop een kijkje op het PEP-synthase, Melanie Kuiper van Levensmiddelenmicrobiologie leerde me ATP-metingen doen en Peter Barneveld van Fysische en Kolloïdchemie beantwoordde mijn vragen over thermodynamica. Hoewel maar een deel van deze stunts in het proefschrift is terechtgekomen, ben ik hen allemaal dank verschuldigd voor het vrijmaken van hun tijd en hun apparatuur. Ook buiten de vakgroep, maar binnen het project, speelden de mensen uit Groningen hun rol. Sonja, Sonja, Arnold & Wil gaven met hun eigen onderzoek en hun feedback op dat van mij een breder kader aan mijn werk.

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Ook buiten de muren van de vakgroep waren er heel veel mensen geïnteresseerd in de voortgang van het project en gelukkig maar, want wat is een film zonder publiek? Bovendien vormden ze met elkaar een welkome bron van afleiding. Ik wil graag de meiden van de Kopervrouw (Geri, Mirjam, Dorine, Karina, Viona & Sylvie) bedanken voor de in- en ontspannende zondagmiddagen, de meiden van de mensagroep (Judith, Inge & Katinka) voor alle gezellige uitstapjes en bijkletsafspraken, de jongens van 6A (Tom, Marc, Igor, Sjoerd, Harm, Jan & Freek) voor een onverwachte hernieuwde kennismaking met het studentenleven, Kjille voor de morele steun tijdens de beginscènes en - samen met Rob - voor de uren op de dansvloer en alle andere vrienden (met name Bart, Douwe, Tycho, Bart, Willemijn, Erik & Kristof) voor hun belangstelling en het aanhoren van mijn oeverloos geklets over dat onbegrijpelijke beestje.

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Hierbij voor iedereen: "Jongens, bedankt!"

THE END

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