Systems Microbiology

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Dedicated to our deceased colleagues, Jaroslav Stark and Emmanuelle Caron, pioneers in systems biology.

Preface

Systems biology aims to study the dynamic interactions of more than one component in a biological system in order to understand and predict the behaviour of the system as a whole. Typical approaches involve an iterative cycle of 'dry lab' modelling and 'wet lab' verification.

Bringing order to biological data that inherently have noise due to a multitude of variables has previously been considered too challenging. However, systems biology is now a rapidly expanding discipline fuelled by the 'omics' era that is coupled to several new technological advances that have increased the precision of data obtainable. This has provided the bit parts of complex living cells. New challenges arise to put these levels of information together including finding a common language of the difference omics data sets (e.g. genomics, transcriptomics, proteomics, metabolomics).

The sheer complexity of biological systems means that systems biology is a fledgling science. However, a focus on simple single cell organisms such as bacteria aids tractability and means that systems microbiology is a rapidly maturing science.

This book will include case studies on single microbial species (e.g. bacteria and archaea), systems analysis of microbial phenomena (e.g. chemotaxis and phagocytosis) and this is complemented with theoretical approaches and mathematical modelling.

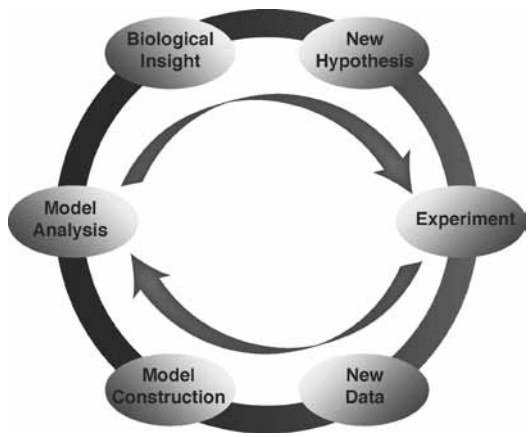

Figure 0.1 Systems biology illustrating the iterative cycle of 'dry lab' modelling and 'wet lab' verification. Concept and design by Jaroslav Stark.

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Sulfolobus Systems Biology: Cool Hot Design for Metabolic Pathways

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Theresa Kouril, Alexey Kolodkin, Melanie Zaparty, Ralf Steuer, Peter Ruoff, Hans V. Westerhoff, Jacky Snoep, Bettina Siebers and the SulfoSYS consortium

Abstract<Please check heading levels throughout>

Life at high temperature challenges the stability of macromolecules and cellular components, but also the stability of metabolites, which has received little attention. For the cell, the thermal instability of metabolites means it has to deal with the loss of free energy and carbon, or in more extremes, it might result in the accumulation of dead-end compounds. In order to elucidate the requirements and principles of metabolism at high temperature, we used a comparative blueprint modelling approach of the lower part of the glycolysis cycle. The conversion of glyceraldehyde 3-phosphate to pyruvate from the thermoacidophilic Crenarchaeon *Sulfolobus solfataricus* P2 (optimal growth temperature 80°C) was modelled based on the available blueprint model of the eukaryotic model organism *Saccharomyces cerevisiae* (optimal growth temperature of 30°C). In *S. solfataricus* only one reaction is different, namely glyceraldehyde-3-phosphate is directly converted into 3-phosphoglycerate by the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, omitting the extremely heat-instable 1,3-bisphosphoglycerate. By taking the temperature-dependent non-enzymatic (spontaneous) degradation of 1,3-bisphosphoglycerate in account, modelling reveals that a hot lifestyle requires a cool design.

Archaea

Archaea are fundamental components of biogeochemical cycles on earth and some are dominating in extreme environments (De Long 1998a, 2001; Chaban *et al.*, 2006). The assignment of Archaea as the third domain of life, in addition to Bacteria and Eukaryotes, based on the universal small subunit rRNA, was established in 1977 (Woese and Fox, 1977). Archaea are sometimes seen as a mosaic of bacterial and eukaryote life-forms, but they also exhibit numerous unique features. Bacterial-like features such as being unicellular, the lack of a nuclear membrane and organelles, the presence of circular DNA molecule, plasmids and the organization of genes in operon structures, are in contrast with mechanisms involved in information processing (e.g. transcription, translation), which represent less complex versions of the respective eukaryote equivalents.

However, Archaea differ in many aspects of their cell composition and molecular biology from Bacteria as well as from Eukaryotes. For example, instead of peptidoglycan (murein) in bacterial cell walls, in some methanogenic species pseudomurein is present (Konig *et al.*, 1982, 1983), while most other Archaea, consist of a paracrystalline surface layer (S-layer; König *et al.*, 2007) or harbour no cell wall at all (e.g. Thermoplasmatales; Itoh *et al.*, 2007).

In addition, Archaea are unique with respect to their metabolism, which is characterized by unusual, modified pathways. Beside the occurrence of special pathways like methanogenesis (for review see Thauer *et al.*, 2008), also some common pathways, like the oxidative pentose phosphate pathway found in Bacteria and Eukaryotes are absent from Archaea. Pentoses are either generated via the non-oxidative pentose phosphate pathway and/or the reversed ribulose monophosphate pathway (Orita *et al.*, 2005). Furthermore, the archaeal pathways are characterized by many, new and unusual enzymes, which substitute for classical bacterial and eukaryote counterparts (Siebers and Schönheit, 2005; Van der Oost and Siebers, 2007).

Life at high temperature

Although Archaea are ubiquitous and thrive in diverse habitats (De Long 1998b; De Long and Pace 2001) most Archaea that have been cultivated so far, are extremophiles, organisms that do not only tolerate, but require harsh environments for growth, e.g. low or high temperature, low or high pH, high salt concentrations or often combinations thereof.

Challenging habitats for prokaryotic growth are high-temperature environments, which comprise terrestrial and marine hot springs (e.g. geyser, black smokers, respectively). (Hyper)thermophiles grow in the range of 60°C up to 113°C (Stetter, 1999). One striking example is *Pyrolobus fumarii* with an optimal growth temperature of 106°C (growth range 90–113°C, pH 4.0–6.5; Blöchl *et al.*, 1997). Life at high temperature requires efficient adaptation strategies and in the past years a major focus has been the structural and mechanistic adaptation of cell components and macromolecules such as DNA, proteins and membranes (for review see Stetter 1999, Daniel and Cowan, 2000; Kawashima *et al.*, 2000). (Hyper) thermophilic organisms have evolved different strategies to adapt to environmental changes and beside intrinsic factors, which are determined within the macromolecules itself, extrinsic factors like the accumulation of compatible solutes, play a major role. Compatible solutes, such as amino acids (proline, glutamate, glycine), sugars (sucrose, trehalose), polyols and their derivates (Empadinhas *et al.*, 2006), can be accumulated up to high intracellular concentrations without interfering with metabolism. They protect against various stresses, such as desiccation, dehydration and osmotic, oxidative or temperature stress. One well-known example, detected in all three domains of life, is the disaccharide trehalose, which serves for example as osmolyte in order to balance changes in osmotic pressure and stabilizes proteins and membranes at high and low temperatures (for review see Elbein *et al.*, 2003). However, whereas the stability of macromolecules has been well addressed in the past, only minor attention has been paid to the effect of temperature on metabolites.

The model organism *Sulfolobus solfataricus* (strain P2, DSM1617; Zillig *et al.*, 1980) is an acidophilic thermophile, which belongs to the order of Sulfolobales within the Crenarchaeota. *S. solfataricus* was isolated from Solfatara at the Pisciarelli fumarole field in Naples, Italy and grows optimally at 80°C (60–92°C) and pH 3 (pH 2–4). It qualifies as an archaeal model organism for systems biology analyses, because it is genetically tractable and a well-characterized archaeal species (Worthington *et al.* 2003; Albers *et al.* 2006; Deng *et al.* 2009; Wagner *et al.* 2009). Furthermore, it is very easy to cultivate under defined conditions and grows heterotrophically with many different carbohydrates, such as monosaccharides (e.g. arabinose, glucose, galactose), disaccharides (e.g. maltose, sucrose) and polysaccharides (e.g. starch), as well as with amino acids or peptides (Grogan, 1989). Therefore, it qualifies for studying archaeal metabolic pathways. In addition, comprehensive microbiological,

biochemical and functional genomics data have been assembled over the past 25 years and effective standard operating procedures have been established recently (Zaparty *et al.*, 2010).

Archaeal carbohydrate metabolism

Polysaccharides are a major source of carbon in the three domains of life. Their utilization generally involves extracellular hydrolysis, uptake of oligosaccharides by specific transporters and their intracellular hydrolysis to generate hexoses, like glucose, galactose, mannose and fructose. Subsequently, these monosaccharides are oxidized via well-conserved central metabolic pathways.

The archaeal carbohydrate metabolic network (CMN) is as complex as that of Bacteria and primitive Eukaryotes, but is characterized by many novel enzymes and biochemical pathways. Even the basic pathways of sugar degradation, like the Embden–Meyerhof–Parnas (EMP) or the Entner–Doudoroff (ED) pathway have undergone slight modifications in these organisms compared with the 'classic' pathways in Bacteria and Eukaryotes (Selig *et al.*, 1997; Ronimus *et al.*, 2002; Verhees *et al.*, 2003; Siebers and Schönheit, 2005; Van der Oost and Siebers, 2007). Many of the archaeal enzymes lack similarity with their bacterial and eukaryote counterparts, or are members of different, 'new' enzyme families (e.g. ADP/ATP-dependent hexo(gluco)kinases, phosphoglucose isomerase; Hansen *et al.*, 2003; Siebers and Schönheit, 2005). Interestingly, despite the utilization of new enzymes most of the intermediates known so far (e.g. in the EMP pathway) are conserved, highlighting the major impact of thermodynamic constraints on metabolic reactions.

However, so far only very limited information is available for utilization and growth on alternative carbon sources in Archaea (e.g. pentoses, sugar acids, aldehydes) and therefore, reconstruction of the archaeal carbon metabolic network (CMN) is incomplete and regulatory mechanisms such as nutritional adaptation or carbon homeostasis, as well as energetics are still far from being understood.

Metabolic network reconstruction

A prerequisite for systems biology is a complete network reconstruction in order to identify all players of the investigated network. For the *Sulfolobus* Systems Biology (SulfoSYS; www.sulfosys.com) project, the central carbohydrate metabolism (including the EMP and branched ED pathway, trehalose and glycogen metabolism) of *S. solfataricus* P2 has been reconstructed and the effect of temperature change on the metabolic network has been investigated (Albers *et al.*, 2009; Zaparty *et al.*, 2010).

The established *S. solfataricus* CMN blueprint model was used recently for pathway reconstruction within about 30 thermoacidophilic Archaea, distributed among the archaeal kingdoms of Crenarchaeota and Euryarchaeota, with available genome sequence information (Auernik *et al.*, 2008; Zaparty and Siebers, 2011).

Previous studies revealed that *S. solfataricus* uses an unusual branched ED pathway for the catabolism of glucose and galactose to pyruvate (Lamble *et al.*, 2005; Snijders *et al.*, 2006). Because of the absence of a phosphofructokinase (PFK) homologue in *S. solfataricus*, it is suggested that the EMP pathway is utilized only in the gluconeogenic direction. The promiscuous ED pathway consists of a semi-phosphorylative (sp) and non-phosphorylative (np) branch (Fig. 8.1). Here, phosphorylation occurs via 2-keto-3-deoxygluconate kinase

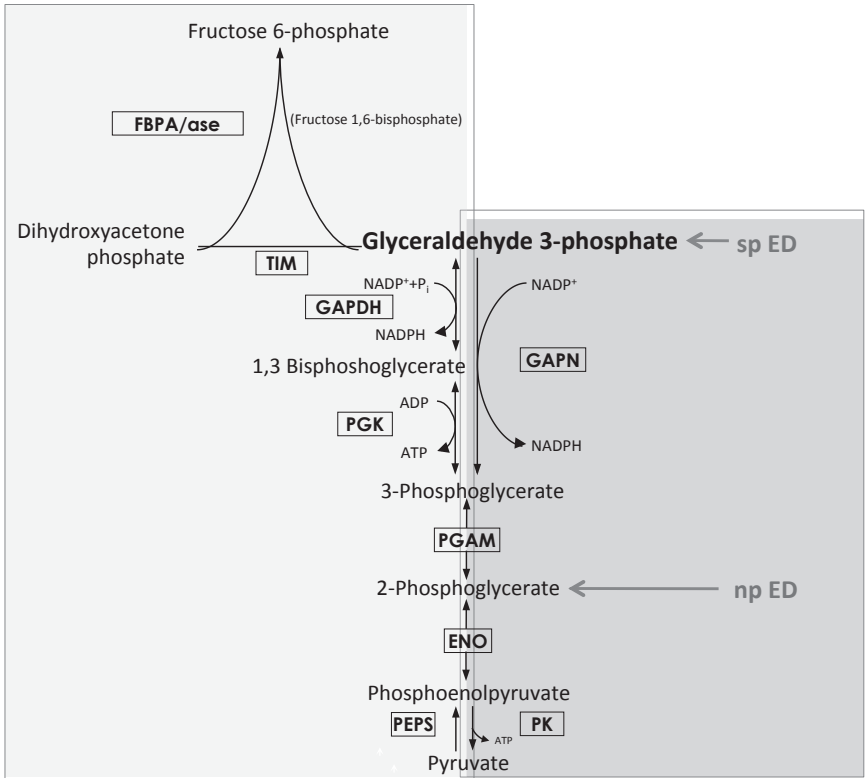

Figure 8.1 GAP conversion in *S. solfataricus*. Boxed in dark grey: reactions of the branched ED pathway; boxed in light grey: reactions of the anabolic EMP pathway. FBPA/ase, fructose-1,6-bisphosphate aldolase/phosphatase; TIM, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PEPS, phosphoenolpyruvate synthetase.

(KDGK) or glycerate kinase (GK), respectively (De Rosa *et al.*, 1984; Ahmed *et al.*, 2004; Lamble *et al.*, 2003, 2005).

The common part of the branched ED pathway in *S. solfataricus* involves the initial oxidation of glucose/galactose to gluconate/galactonate by glucose dehydrogenase (GDH; Lamble *et al.*, 2003; Haferkamp *et al.*, 2011) and the dehydration by gluconate/galactonate dehydratase (GAD; Lamble *et al.*, 2004; Lee and Kim, 2005) forming 2-keto-3-deoxygluconate/2-keto-3-deoxygalactonate (KDG/KDGal), the characteristic intermediates of this pathway. At the beginning of the sp ED branch KDG/KDGal is phosporylated to 2-keto-3-desoxy-6-phosphogluconate/2-keto-3- deoxy-6-phosphogalactonate (KDPG/KDPGal) via KDGK. KDPG/KDPGal as well as KDG/KDGal are cleaved by the bifunctional KD(P)G aldolase, yielding glyceraldehyde 3-phosphate (GAP) and pyruvate in the sp branch and glyceraldehydes (GA) and pyruvate in the np route, respectively (Buchanan *et al.*, 1999; Lamble *et al.*, 2003, 2005; Ahmed *et al.*, 2005). GAP is further metabolized either via the glyceraldehyde-3-phosphate dehydrogenase (GAPDH)/phosphoglycerate kinase (PGK) couple (Hess *et al.*, 1995; Jones *et al.*, 1995; Russo *et al.*, 1995) or via the non-phosphorylating GAPDH

(GAPN; Ettema *et al.*, 2008) ending in the lower common shunt of the EMP pathway, yielding a second molecule of pyruvate (Fig. 8.1).

In the np branch, GA is further oxidized to glycerate via glyceraldehyde dehydrogenase (GADH) or glyceraldehyde-oxidoreductase (GAOR), the latter has been characterized from *S. acidocaldarius* (aldehyde ferrodoxin oxidoreductase (AOR), Kardinahl *et al.*, 1999). Glycerate is then transformed to 2-phosphoglycerate (2PG) via glycerate kinase (GK) and channelled into the EMP pathway forming a second molecule of pyruvate (Zaparty and Siebers, 2011).

From GAP to pyruvate: the common shunt of the EMP pathway

A general feature of all (hyper)thermophilic Archaea analysed so far, is that they lack the characteristic bacterial and eukaryote regulation points at the beginning and end of the glycolytic EMP pathway. The archaeal ATP-dependent hexokinase, ADP-dependent glucokinase, ATP-, ADP-, and PP_i-dependent phosphofructokinases all lack allosteric properties, thus omitting the central control points of the classical EMP pathway as found in Bacteria and Eukaryotes. Also the archaeal pyruvate kinases (PK) exhibit reduced, if at all, regulatory potential (Siebers und Schönheit, 2005).

In *S. solfataricus* the upper part of the EMP pathway is only used for gluconeogenesis and the key metabolite at the branch point between gluconeogenic EMP and the lower glycolytic sp ED shunt of *S. solfataricus* is GAP (Fig. 8.1). In the anabolic direction GAP is converted to dihydroxyacetone phosphate (DHAP) by triosephosphate isomerase (TIM) and further metabolized to fructose 6-phosphate via an unusual, bifunctional fructose *1*,6-bisphosphate (FBP) aldolase/phosphatase (FBPA/ase, T. Kouril *et al.*, unpublished; Fig. 8.1). The FBPA/ase catalyses the direct conversion of DHAP and GAP to fructose 6-phosphate, as recently described for the hyperthermophilic Archaeon *Ignicoccus hospitalis* (Say *et al.*, 2010). This bifunctional enzyme seems to be restricted to Archaea and deep branching, mostly thermophilic and autothrophic Bacteria (Say *et al.*, 2010). However, bioinformatic analyses revealed, in addition, the presence of the archaeal-type class I FBP aldolase (Siebers *et al.*, 2001; Lorentzen *et al.*, 2003, 2005) homologue in *S. solfataricus*, which might catalyse reversible FBP formation. However, its role still has to be elucidated.

For the catabolic conversion of GAP, two different enzyme activities are known in *Sulfolobus*: (i) the classical GAPDH/PGK enzyme couple (Fabry *et al.*, 1987; Zwickel *et al.*, 1990; Jones *et al.*, 1995; Hess *et al.*, 1995; Russo *et al.*, 1995) and (ii) the GAPN (Brunner *et al.*, 1998, 2001; Ettema *et al.*, 2008), which catalyses the unidirectional oxidation of GAP to 3-phosphoglycerate (3PG; Fig. 8.1). In other Archaea, a third enzyme, the GAP oxidoreductase (GAP-OR), which catalyses the same reaction as GAPN, but uses ferredoxin instead of pyridine nucleotides as a co-substrate (Mukund *et al.*, 1995; Ettema *et al.*, 2008), has been identified. The formed 3PG is further converted to 2-phosphoglycerate (2PG), phosphoenolpyruvate (PEP) via phosphoglycerate mutase (PGAM) and enolase (ENO). Finally, a second molecule of pyruvate is formed via the pyruvate kinase (PK) reaction (Fig. 8.1).

Due to its allosteric regulation, it is predicted that GAPN is the first and only regulation point of the sp ED pathway identified to date (Brunnner *et al.*, 1998, 2001; Lorentzen *et al.*, 2004; Ahmed *et al.*, 2005; Ettema *et al.*, 2008). The enzyme is efficiently activated by glucose 1-phosphate (G1P), a glycogen metabolism intermediate. Bioinformatics analyses revealed that GAPN seems to be present only in Archaea with an optimal growth temperature above

75°C, with the only exception of *Halobacterium* sp. NRC-1, a halophilic Archaeon with an optimal growth temperature of 37°C (Ettema *et al.*, 2008). In the catabolic direction, the use of GAPN instead of the GAPDH/PGK couple omits the extremely thermolabile intermediate 1,3-bisphosphoglycerate (1,3-BPG; half-life of 96 seconds at 60°C; Schramm *et al.*, 2001), therefore, a function of GAPN in metabolic thermoadaptation has been proposed previously (Ettema *et al.*, 2008). Detailed biochemical and transcriptional analyses in *Pyrococcus furiosus* and *Thermoproteus tenax* revealed a catabolic role for the GAPN and GAPOR, and an anabolic role for the GAPDH/PGK couple (Schäfer and Schönheit, 1993; Brunner *et al.*, 1998, 2001; Van der Oost *et al.*, 1998; Schut *et al.*, 2003; Lorentzen *et al.*, 2004).

However, the regulation at GAP level in *S. solfataricus* and other Archaea is still unclear and requires further biochemical investigations. The net ATP yield of the sp ED pathway is 1 mol ATP per mol of glucose, if the classical GAPDH/PGK couple is used, whereas no net energy is gained (0 mol ATP/mol glucose), if the GAPN or the np branch are used in glycolysis.

Blueprint modelling: glycolysis of *S. solfataricus* and *S. cerevisiae* are instantiations of the same master theme

Different biological organisms might be quite similar in a very important underlying mechanism of their function. First, building blocks (biomolecules) are basically the same. If L-amino acids and D-sugars had been chosen to be synthesized by the first ancestor organism, perhaps randomly, the evolution of successors would continue to work with those forms. If, at a certain stage, autotrophs have centred their metabolism on glucose, heterotrophs would be constrained to arrange their metabolism in a compatible way, to have compatible building blocks. We may also expect that major pathways, major biochemical reaction networks would remain similar through all organisms. First of all, the number of biologically affordable reactions is limited. We do not expect that any organism might ever synthesize glucose from water and CO_2 directly, in a single reaction; neither, would we expect that glucose would be oxidized to CO_2 and water directly. On the contrary, we would anticipate to find a thermodynamically reasonable, stepwise process compatible with life, which neither delivers nor consumes too much of Gibbs free energy. Since already emerged; most probably, the core scheme of this stepwise process would be inherited further and with the evolution of a biosphere as a whole; single organisms (species) would be constrained to stick to this consensus mechanism.

This seems to be also true for Archaea, although they use modifications of the classical EMP and ED pathway, most metabolites and catalysed reactions are similar, only the catalysts involved change. Similarity of biochemical pathways in various organisms justify a blueprint modelling approach: a blueprint (core) model may be built and then parameterized for a pathway of a particular organism, by varying only several parameters (assuming that the same kinetic type is used), such as the level of enzyme expression.

Here, we focus on a blueprint modelling of the lower part of glycolysis, the conversion of GAP to pyruvate. First, we project the reaction network diagram of this part of the glycolytic pathway in *S. solfataricus* over the reaction network of *S. cerevisiae*, an organism from the eukaryote kingdom, with an optimal growth at 30°C. The majority of the reactions overlap (Fig. 8.2) and only one reaction is different. As discussed above, apart from the classical GAPDH/PGK enzyme couple (reactions 1 and 2, Fig. 8.2), *S. solfataricus* employs

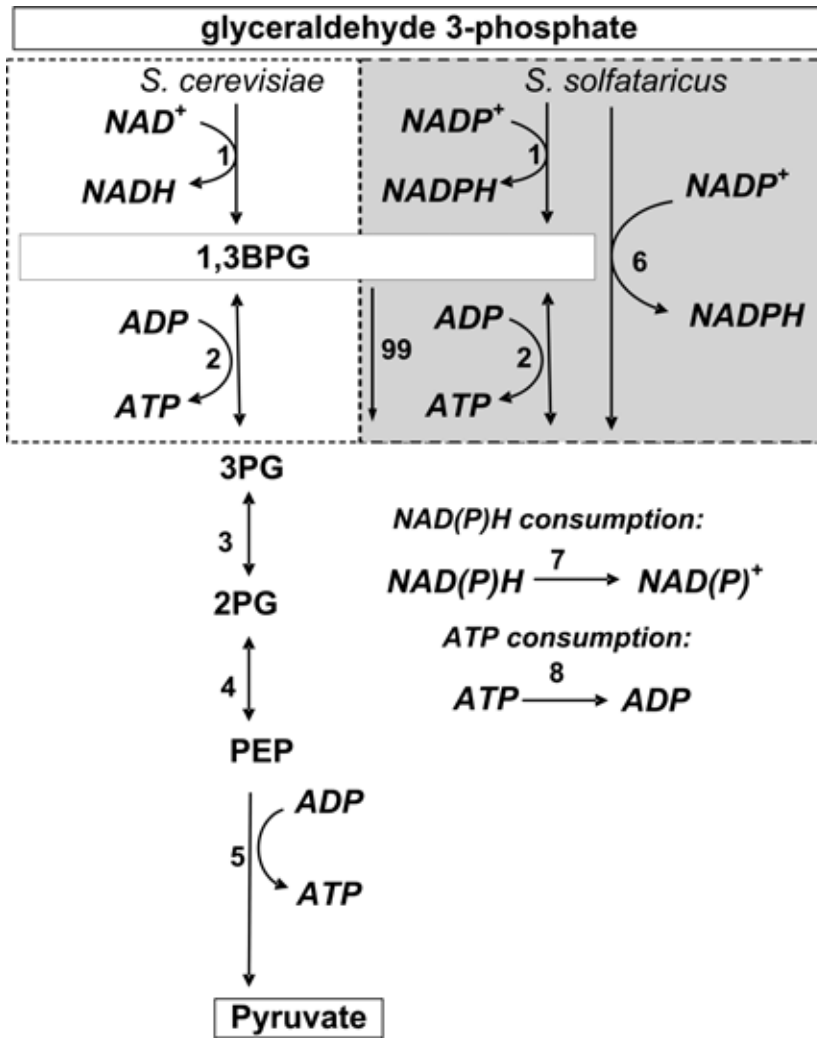

Figure 8.2 Blueprint scheme of the lower part of glycolysis (conversion of glyceraldehyde 3-phosphate to pyruvate) for *S. cerevisiae* and for *S. solfataricus*. GAP, glyceraldehyde-3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate.

the GAPN, which catalyses the unidirectional oxidation of GAP to 3PG (reaction 6, Fig. 8.2; Ettema *et al.*, 2008), and the organism uses $NADP^+$ instead of NAD^+ as cofactor for these reactions. *S. cerevisiae* lacks GAPN and is unable to convert GAP into 3PG directly (Fig. 8.2). Consequently, *S. cerevisiae* glycolysis is, in fact, the glycolysis of *S. solfataricus*, but without reaction 6. This allows the building of a mathematical model of glycolysis in *S. solfataricus* by adding the extra reaction into the model of *S. cerevisiae* and re-parameterizing the kinetics of the other reactions.

Another factor that has to be considered is the temperature dependent non-enzymatic (spontaneous) degradation of 1,3-BPG (reaction 99, Fig. 8.2). For *S. cerevisiae*, this reaction

may be neglected, but for *S. solfataricus* (living at high temperature) this has to be taken into account.

Building a mathematical model of glycolysis in *S. solfataricus*

The blueprint approach allows us to start with a well-known model of *S. cerevisiae* glycolysis, built in the Amsterdam group (Teusink *et al.*, 2000) and available at the JWS Online site (http://jjj.mib.ac.uk; Olivier and Snoep, 2004). Since we were interested only in the lower part of glycolysis (conversion of GAP into pyruvate), the corresponding part has been copied from the Teusink model. Initial concentrations of boundary metabolites (GAP and pyruvate) have been fixed (equal to steady state concentration in the Teusink model). The rate constant of oxidation of NADH (NADPH for *S. solfataricus*) has been assumed constant. The GAPN as well as the GAPDH of *S. solfataricus* have been shown to possess dual co-substrate specificity; however, $NADP^+$ has been shown to be the preferred co-substrate (Littlechild *et al.*, 2001; Ettema *et al.*, 2008). The rate of ATP consumption has been modelled with mass action kinetics. The ratio of the steady state ATP consumption flux and the GAP consumption flux indicate the efficiency of ATP production. Parameters for reactions 1, 5 and 6 have been experimentally measured within the SulfoSYS project (www.sulfosys.com). The specific activities (V_m values) were determined in cell-free extracts of *S. solfataricus* cells grown at 80°C [see SOP_SSO_080904 (fermenter set-up and fermentation); SOP_SSO_080905 (cell harvest); SOP_SSO_080913d (preparation of *S. solfataricus* crude extracts) in Zaparty *et al.* (2010)]. Purified, recombinant proteins were used to obtain Km values, performing enzyme assays according to standard protocols (Zaparty *et al.*, 2010; GAPN (reaction 6, Fig. 8.2) Ettema *et al.*, 2008; GAPDH (reaction 1, Fig. 8.2) Russo *et al.*, 1995; PK (reaction 5, Fig. 8.2) Schramm *et al.*, 2000). Parameters for reaction 2, 3 and 4 have been kept identical to the ones in the *S. cerevisiae* model (Teusink *et al.*, 2000). All rate equations, mass balances and parameters are shown in Table 8.1 and the model is available via JWS Online (e.g. http://jjj.mib.ac.uk, search e.g. for author Kouril), and can be exported in SBML format.

As shown in Fig. 8.2, when directly converting GAP into 3PG, *S. solfataricus* produces less ATP molecules per molecule of glucose than *S. cerevisiae*. This seems paradoxical, because the main function of glycolysis is exactly the synthesis of ATP. Why does *S. solfataricus* possess reaction 6? Would *S. solfataricus* not produce more ATP without this reaction?

The presence of GAPN might be understandable, if reactions 1 and 2 would be limiting for the total flux of glucose through the glycolytic pathway. However, if reactions 1 and 2 are sufficient for *S. cerevisiae*, living at low temperatures, the conversion of GAP into 3PG at high temperature should be even simpler due to the spontaneous degradation of 1,3-BPG. The non-enzymatic degradation of 1,3BPG would help to increase the total flux by stimulating reaction 1 (consuming its product) and by duplicating reaction 2. Another explanation for the existence of GAPN may be found, if there would be a high flux through reaction 99. Then the total mass flow would pass mostly through reactions 1 and 99 and there would be very little synthesis of ATP from GAP. Consequently, redirecting of the total flux from reactions 1 and 2 into reaction 6 would have almost no effect on the net outcome of ATP. Then, instead of producing GAPDH, *S. solfataricus* might produce GAPN. This maybe the reason why *Sulfolobus* possesses GAPN – simply because reaction 6 does not give any substantial disadvantages. However, it is not considered yet that 1,3-BPG might spontaneously degrade to compounds different from 3PG (e.g. 1PG) or other 'grey' metabolites. There are no studies

about the decay products available so far. These 'grey' metabolites may simply escape from further steps of glycolysis and thus, may be lost from the point of ATP production (carbon loss). Therefore, it may be important to assure that the flux goes through GAPN. Then, *S. solfataricus* faces the dilemma of either to lose ATP, due to employing GAPN instead of PGK, or may be to lose metabolites from the glycolytic pathway. Is this the complete picture for the trade-off between those two processes?

Futile ATP cycling: inspirations from the model

The mathematical model for *S. solfataricus* allowed us to compare the *S. solfataricus* and *S. cerevisiae* GAP to pyruvate conversion as instantiations of the same blueprint master-scheme. To be able to compare the *Sulfolobus* and *Saccharomyces* systems we need to express the efficiency of the lower branch of glycolysis in terms of ATP produced per GAP consumed. In this way the lower efficiency of *Sulfolobus* due to its use of the ED pathway does not interfere with the estimations of the efficiency of the second half of glycolysis.

In *S. cerevisiae* at low temperature, neither reaction 99 nor reaction 6 are present, then two molecules of ATP will be produced per molecule of GAP converted to pyruvate. We expected a decrease in efficiency (ATP/GAP) after the addition of reaction 99 (spontaneous degradation of 1,3BPG to 3PG), since this could lead to 1,3BPG consumption uncoupled from ATP synthesis. We expected that at low temperature the effect of spontaneous 1,3BPG degradation would not be very severe, the flux through reaction 99 would be low and the ATP/GAP flux ratio would be somewhere between one and two. We also expected that at high temperature, the total pool of 1,3BPG would be degraded spontaneously, the flux through reaction 2 would approach zero and, consequently, the net ATP/GAP flux ratio would become equal to one. Model simulations confirmed these predictions for the case of low temperature. The ATP/GAP flux ratio was found to be between 1 and 2 (Table 8.2); but, at high temperature the ATP/GAP flux ratio dropped even further to values below 1. This phenomenon might be explained by futile cycling of ATP. The PGK reaction is simulated as a reversible reaction and the increased conversion of 1,3BPG due to degradation at high temperatures can lead to a change in 3PG/1,3BPG ratios and thereby increasing the reverse rate of PGK. The latter is coupled with the consumption of ATP and could be observed in the model as the negative flux value through reaction 2. The activity of the futile cycle reduced the ATP/GAP flux ratio below 1 and this indicates that removal of PGK could actually lead to a higher ATP/GAP flux ratio, although this would remove a potential ATP production site and limit the ratio to 1. Indeed, when reactions 1 and 2 in the *S. solfataricus* model were removed, the ATP/GAP flux ratio did not drop below 1, showing that this design was more advantageous.

Conclusions and future perspective

In the course of the SulfoSYS project (Albers *et al.*, 2009) a blueprint modelling approach with input of real kinetic data allowed us to address the design of a partial glycolysis model, in other words conversion of GAP to pyruvate for the thermoacidophilic archaeon *S. solfataricus*.

Metabolite instability is one important challenge for organisms growing at high temperature. Especially as some triosephosphates are heat-labile compounds, which need to be quickly converted to more heat-stabile carbohydrates in order to prevent carbon and energy

Table 8.1 Model description <throughout confirm that the units mM/min (i.e. mol/l/min) are correct, not mmol/min>

Reactions		Parameters
v_1	GAPDH: GAP oxidation to 1,3-BPG (coupled with $NADP^+$ reduction) $$\frac{\frac{V_{mfGAPDH}\cdot GAP(t)\cdot NADP(t)}{K_{mGAPDHGAP}\cdot K_{mGAPDHNADP}}-\frac{V_{mbGAPDH}\cdot BPG(t)\cdot NADPH(t)}{K_{mGAPDHBPG}\cdot K_{mGAPDHNADPH}}}{\left(1+\frac{GAP(t)}{K_{mGAPDHGAP}}+\frac{BPG(t)}{K_{mGAPDHNADP}}\right)\cdot\left(1+\frac{NADP(t)}{K_{mGAPDHBPG}}+\frac{NADPH(t)}{K_{mGAPDHNADPH}}\right)}$$ (mM/min)	$K_{mGAPDHGAP}$ = 4.6 mM $K_{mGAPDHNADP}$ = 0. 31 mM $K_{mGAPDHBPG}$ = 92.4 mM $K_{mGAPDHNADPH}$=0.025 mM *$V_{mfGAPDH}$ = $V_{mbGAPDH}$ = 0.066 U/mg = 66 mM/min (SulfoSys data)
v_2	PGK (reversible): when in direction from BPG to 3PG generates ATP (Prb): V_{mPGK}*(K_{eqPGK}*BPG*(SUMAXP-(SUMAXP^2–2*SUMAXP*Prb+8*K_{eqAK}*SUMAXP*Prb+Prb^2–4*K_{eqAK}*Prb^2)^0.5)/(1–4*K_{eqAK})-(-SUMAXP+Prb-4*K_{eqAK}*Prb+(SUMAXP^2–2*SUMAXP*Prb+8*K_{eqAK}*SUMAXP*Prb+Prb^2–4*K_{eqAK}*Prb^2)^0.5)*3PG/(2–8*K_{eqAK}))/($K_{mPGKATP}$*$K_{mPGK3PG}$*(1+(SUMAXP-(SUMAXP^2–2*SUMAXP*Prb+8*K_{eqAK}*SUMAXP*Prb+Prb^2–4*K_{eqAK}*Prb^2)^0.5)/((1–4*K_{eqAK})*$K_{mPGKADP}$)+(-SUMAXP+Prb-4*K_{eqAK}*Prb+(SUMAXP^2–2*SUMAXP*Prb+8*K_{eqAK}*SUMAXP*Prb+Prb^2–4*K_{eqAK}*Prb^2)^0.5)/((2–8*K_{eqAK})*$K_{mPGKATP}$))*(1+BPG/$K_{mPGKBPG}$+3PG/$K_{mPGK3PG}$)) (mM/min)	K_{eqAK} = 0.450 K_{eqPGK} = 3200 $K_{mPGKADP}$ = 0.2 mM $K_{mPGKATP}$ = 0.3 mM $K_{mPGKBPG}$ = 0.003 mM $K_{mPGK3PG}$ = 0.53 mM V_{mPGK} = 1306.45 mM/min SUMAXP = 4.1 mM As for yeast (Teusink *et al.*, 2000)
v_3	PGAM (reversible): 3PG to 2PG $$\frac{\frac{V_{mPGAM}}{K_{mPGAM3PG}}\cdot(3PG(t)-\frac{2PG(t)}{K_{eqPGM}})}{1+\frac{3PG(t)}{K_{mPGAM3PG}}+\frac{2PG(t)}{K_{mPGAM2PG}}}$$ (mM/min)	V_{mPGAM} = 2525.81 M/min K_{eqPGAM} = 0.19 $K_{mPGAM3PG}$ = 1.2 mM $K_{mPGAM2PG}$ = 0.08 mM As for yeast (Teusink *et al.*, 2000)
v_4	ENO (reversible): 2PG to PEP $$\frac{\frac{V_{mENO}}{K_{mENO2PG}}\cdot(2PG-\frac{PEP}{K_{eqENO}})}{1+\frac{2PG}{K_{mENO2PG}}+\frac{PEP}{K_{mENOPEP}}}$$ (mM/min)	V_{mENO}=365.8 mM/min $K_{mENO2PG}$ = 0.04 mM K_{eqENO} = 6.7 $K_{mENOPEP}$ = 0.5 mM As for yeast (Teusink *et al.*, 2000)
v_5	PK (dephosphorylation of PEP to PYR, coupled with ATP (Prb) synthesis): V_{mPYK}/($K_{mPYKPEP}$*$K_{mPYKADP}$)*(PEP*(SUMAXP-(Prb^2–4*K_{eqAK}*Prb^2–2*Prb*SUMAXP+8*K_{eqAK}*Prb*SUMAXP+SUMAXP^2)^0.5)/(1–4*K_{eqAK})-PYR*((Prb-4*K_{eqAK}*Prb-SUMAXP+(Prb^2–4*K_{eqAK}*Prb^2–2*Prb*SUMAXP+8*K_{eqAK}*Prb*SUMAXP+SUMAXP^2)^0.5)/(2–8*K_{eqAK}))/K_{eqPYK})/((1+PEP/$K_{mPYKPEP}$+PYR/$K_{mPYKPYR}$)*(1 +(Prb-4*K_{eqAK}*Prb-SUMAXP+(Prb^2–4*K_{eqAK}*Prb^2–2*Prb*SUMAXP+8*K_{eqAK}*Prb*SUMAXP+SUMAXP^2)^0.5)/(2–8*K_{eqAK})/$K_{mPYKATP}$+(SUMAXP-(Prb^2–4*K_{eqAK}*Prb^2–2*Prb*SUMAXP+8*K_{eqAK}*Prb*SUMAXP+SUMAXP^2)^0.5)/(1–4*K_{eqAK})/$K_{mPYKADP}$)) (mM/min)	V_{mPYK}* = 0.04 U/mg = 40 mM/min (measured for 70°C, for 80°C its value should be a bit higher) $K_{mPYKPEP}$ =0.26 mM $K_{mPYKADP}$ =0.113 mM (SulfoSys data) K_{eqAK} = 0.450 K_{eqPYK} = 6500 $K_{mPYKATP}$ =1.5 mM $K_{mPYKPYR}$=21 mM SUMAXP = 4.1 mM As for yeast (Teusink *et al.*, 2000)

Reactions		Parameters
v_6	GAPN (irreversible): Oxidation of GAP to 3PG, coupled with $NADP^+$ reduction: $\frac{V_{mGAPN} \cdot GAP(t) \cdot NADP(t)}{K_{mGAPNGAP} \cdot NADP(t) + K_{mGAPNNADP} \cdot GAP(t) + GAP(t) \cdot NADP(t) + K_{mGAPNGAP} \cdot K_{mGAPNNADP}}$ (mM/min)	$^*V_{mGAPN}$ =0.06 U/mg= 60 mM/min $K_{mGAPNGAP}$ =1 mM $K_{mGAPNNADP}$ =0.18 mM (SulfoSys data)
v_7	Oxidation of NADPH+H^+ to $NADP^+$: $k_1 \cdot NADPH(t)$ (mM/min)	k_1 = 170/min (fitted)
v_8	Consumption of ATP (Prb): $k_8 \cdot Prb(t)$ (mM/min)	k_8 = 2.005 min^{-1} (fitted)
v_{99}	Spontaneous (non-enzymatic) degradation of BPG to 3PG: $k_{99} \cdot 3PG(t)$ (mM/min)	k_{99} (varied) variable 10^5 and 10^7
Balance equations		
dNADP/d*t*		$(v_7 - v_6 - v_1)$ (mM/min)
dNADPH/d*t*		$(v_6 + v_1 - v_7)$ (mM/min])
d3PG/d*t*		$(v_{99} + v_{6+} v_2 - v_3)$ [mM/min]
d2PG/d*t*		$(v_3 - v_4)$ (mM/min)
dPEP/d*t*		$(v_4 - v_5)$ (mM/min)
dBPG/d*t*		$(v_1 - v_2 - v_{99})$ (mM/min)
dPYR/d*t*		(v_5) (mM/min)
dTRIO/d*t*		$(-v_1 - v_6 - v_{99})$ (mM/min)
dPrb/d*t* (=ATP)		$(v_2 + v_5 - v_8)$ (mM/min)
Conserved moieties		
NADP(t) + NADPH(t)		1.58 (mM)
Initial conditions		
Prb(0)		6.3 (mM)
NADP(0)		1.54 (mM) [1.48 mM for design 4 (only GAPN)]
BGP(0)		0.0000522 (mM)
NADPH(0)		0.04 (mM) [0.1 mM for design 4 (only GAPN)]
3PG(0)		0.08 (mM) [1.28 mM for design 4 (only GAPN)]
PEP(0)		0.09 (mM) [1.37 mM for design 4 (only GAPN)]
2PG(0)		0.01 (mM) [0.23 mM for design 4 (only GAPN)]
PYR(0)		(fixed) 8.5 (mM)
GAP(0)		(fixed) 0.77 (mM)
SUMAXP		(fixed) 4.1 (mM)

GAP, glyceraldehyde 3-phosphate; BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; Prb, corresponds to 2ATP + ADP, SUMAXP, corresponds to ADP, ATP and AMP, fixed.
$^*V_m$ was measured in U/mg of crude (total) protein (μmol/min/mg) and converted to (mM/min) using the conversion factor 1 g of total protein corresponds to 1 ml of cell volume (parameter set in the realistic range, for yeast 1 g = 3.7 ml (Teusink *et al*, 2000), for mitochondria 1 g = 1 ml)

Table 8.2 Steady-state fluxes in (mM/min) for *S. cerevisiae* and *S. solfataricus*. Mass balances, rate equations and parameters of corresponding models are shown in Table 8.1

	S. cerevisiae instantiation		*S. solfataricus* instantiation	
	Low temperature	High temperature (moderate rate of reaction 99)	Very high temperature (very high rate of reaction 99)	
	Reaction 1 Reaction 2	Reaction 1 Reaction 2 plus reaction 99 ($kf_{99} = 10^5$)	Reaction 1 Reaction 2 plus reaction 99 ($kf_{99} = 10^7$)	Reaction 6 only
ATP flux (ATP consumption)	12.6	10.14	1.82	25.6
GAP flux reaction v3 (v1 + v6)	6.3	6.3	6.3	25.6
ATP/GAP Flux ratio	2	1.61	0.3	1
Flux through reaction 99	0	2.48	10.8	–
Flux through reaction 2	6.3	3.82	-4.5	–
Flux through reaction 6	–	–	–	25.6

loss (Ahmed *et al.*, 2004; Ettema *et al.*, 2008; Say *et al.*, 2010). However, the decay products of the spontaneous degradation of *1*,3BPG are still unknown and part of current studies. If other 'grey' metabolites are formed, they may escape from glycolysis.

In both organisms, the mesophile *S. cerevisiae* and the thermophile *S. solfataricus*, GAP is produced via two different glycolytic pathways. *S. cerevisiae* metabolizes GAP via the classical EMP pathway, whereas in *S. solfataricus* GAP is formed via the sp ED branch; no GAP is produced in the np ED branch of the *S. solfataricus* pathway. In the model, the main focus lies on the ATP production in terms of ATP produced per molecule of GAP converted into pyruvate.

S. cerevisiae forms two ATP per molecule of GAP at low temperature, which might drop to below one ATP per molecule GAP at high temperature, by taking the temperature induced non-enzymatic degradation of 1,3BPG into account (futile cycling leads to a negative flux value of ATP through PGK reaction). The circumvention of 1,3BPG by the use of GAPN, as in *S. solfataricus*, results in the formation one ATP per GAP molecule.

Consequently it may be advantageous to remove GAPDH/PGK and involve GAPN or GAP-OR enzymes in (hyper)thermophiles, which catalyse the direct oxidation of GAP to 3PG. This fits with the above-mentioned bioinformatics analyses, showing that GAPN seems to be mostly present in Archaea with an optimal growth temperature above 75°C (Ettema *et al.*, 2008).

We have shown here that the GAPDH/PGK enzyme pair may cause a futile cycling: at high temperatures 1,3BPG would degrade spontaneously and it can be re-synthesized by reversed PGK activity resulting in the consumption of ATP. Thus, to circumvent the futile cycle it is essential to remove the PGK even more so than adding GAPN (or GAP-OR). However, GAPN cannot substitute entirely for the GAPDH/PGK couple. Both GAPDH and PGK are essential for gluconeogenesis.

With our blueprint modelling approach we have shown that the GAPDH/PGK route could lead to a net ATP consumption at high temperatures due to futile cycling over the PGK reaction. It could be shown that in this case it was better to utilize GAPN (in absence of PGK). If the net flux over the PGK is in the forward direction, this will lead to an ATP/GAP flux ratio higher than 1, and then the GAPDH/PGK route is more efficient than the GAPN route. The relative fluxes over the GAPDH/PGK, GAPN and spontaneous degradation of 1,3BPG at high temperature is dependent on the kinetics for these reactions, which are currently under experimental investigation.

Indeed, in previous studies we observed a tremendous activation of GAPN by G1P (intermediate in glycogen metabolism). The activity of GAPN increased about four-fold on addition of G1P (0.01 mM; Ettema *et al.*, 2008), such an activation could lead to an increased flux over the GAPN route.

In summary, the 'hot' design of *S. solfataricus* does not seem to be a strange design wasting ATP compared with the 'cool' design of *S. cerevisiae* (Fig. 8.3), but rather representing a 'cool' adaptation strategy to high temperature. We present two possible mechanisms to explain why at high temperatures with an unstable 1,3BPG, the GAPN route may be advantageous over the GAPDH/PGK route for the conversion of GAP to 3PG. The first mechanism relates directly to the degradation of 1,3BPG which could lead to a carbon drain via 'grey' metabolites. The second mechanism relates to futile cycling leading to a net ATP drain via the PGK reaction.

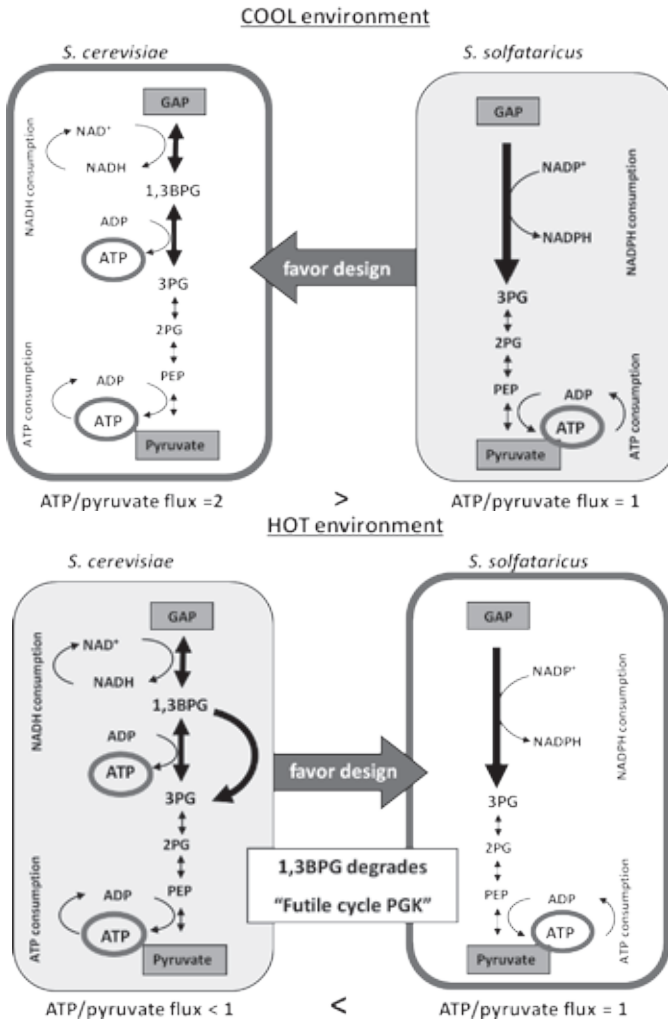

Figure 8.3 At low temperature, the conversion of GAP into 3PG via 1,3BPG is more preferable because two molecules of ATP may be generated per one molecule of GAP (ATP/GAP flux ratio = 2). At high temperature, ATP would be lost due to futile cycling: spontaneous degradation of 1,3BPG and re-synthesis of 1,3BPG from 3PG would consume ATP. Thus, at high temperature, it would be more preferable to omit 1,3BPG by the utilization of GAPN.

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