Journal of Biotechnology 157 (2012) 113–123

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Fermentative glycolysis with purified *Escherichia coli* enzymes for *in vitro* ATP production and evaluating an engineered enzyme

Bradley J. Stevenson[a,*], Jian-Wei Liu[a], Philip W. Kuchel[b], David L. Ollis[a]

[a] *Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia*
[b] *Singapore Bioimaging Consortium, Biomedical Sciences Institutes, 11 Biopolis Way, #02-02 Helios, Singapore 138667, Singapore*

ARTICLE INFO

Article history:
Received 3 May 2011
Received in revised form
13 September 2011
Accepted 16 September 2011
Available online 22 September 2011

Keywords:
Adenosine triphosphate
Glucose
Fermentation
Cell-free
Embden–Meyerhof pathway
^{31}P NMR spectroscopy

ABSTRACT

Each of the twelve enzymes for glycolytic fermentation, eleven from *Escherichia coli* and one from *Saccharomyces cerevisiae*, have been over-expressed in *E. coli* and purified with His-tags. Simple assays have been developed for each enzyme and they have been assembled for fermentation of glucose to ethanol. Phosphorus-31 NMR revealed that this *in vitro* reaction accumulates fructose 1,6-bisphosphate while recycling the cofactors NAD^+ and ATP. This reaction represents a defined ATP-regeneration system that can be tailored to suit *in vitro* biochemical reactions such as cell-free protein synthesis. The enzyme from *S. cerevisiae*, pyruvate decarboxylase 1 (Pdc1; EC 4.1.1.1), was identified as one of the major 'flux controlling' enzymes for the reaction and was replaced with an evolved version of Pdc1 that has over 20-fold greater activity under glycolysis reaction conditions. This substitution was only beneficial when the ratio of glycolytic enzymes was adjusted to suit greater Pdc1 activity.

1. Introduction

Fermentative glycolysis (Fig. 1) provides simple and efficient biochemistry for regenerating ATP while maintaining constant pH and phosphate concentration. ATP regeneration with homeostasis is essential for applications such as cell-free protein synthesis (CFPS). In this research, we reconstituted fermentative glycolysis *in vitro* from purified enzymes to recycle ATP and NAD^+ while accumulating the high-energy metabolite fructose 1,6-bisphosphate (FBP).

Several approaches are available for ATP regeneration and it is one of the most limiting aspects of CFPS. Pyruvate kinase (Pyk; EC 2.7.1.40) is often used, but the energy source, phospho-enol-pyruvate (PEP) can make up 50% of the cost of the reaction mixture (Kim et al., 2007a). Other sources of high energy phosphate, such as creatine phosphate, FBP (Kim et al., 2007a), or polyphosphate (Resnick and Zehender, 2000) can be used, but like PEP, they release phosphate that can sequester Mg^{2+} ions and inhibit protein translation (Kim and Kim, 2009). Glycolysis has been applied to CFPS by using the enzymes present in *Escherichia coli* extracts to regenerate ATP without accumulating phosphate. This approach also uses cheaper substrates such as glucose or glucose 6-phosphate (Calhoun and Swartz, 2005; Kim et al., 2007b), or maltodextrin (Wang and Zhang, 2009).

The contemporary approaches to ATP regeneration result in acidification of the reaction mixture. In order to maintain a suitable pH for protein translation, ATP regeneration usually requires a strongly-buffered solution separated from the protein-synthesis solution by a semi-permeable membrane or density gradient. An ATP regeneration method that does not produce acidic products would greatly simplify CFPS reaction preparation. To this end we revisited the reconstitution of fermentative glycolysis. This pathway uses a readily available energy source, glucose, recycles phosphate, and produces neutral products so that the pH can remain stable.

Cell-free fermentative glycolysis, provided by yeast lysate, has been used successfully to regenerate ATP for glutamine (Wakisaka et al., 1998) or 2′-deoxynucleoside synthesis (Horinouchi et al.,

Abbreviations: 13BPG, 1,3-bisphosphoglycerate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; Adh, alcohol dehydrogenase; CFPS, cell-free protein synthesis; DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; Eno, enolase; F6P, fructose 6-phosphate; Fba, fructose-1,6-bisphosphate aldolase; FBP, fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Glk, glucokinase; Gpi, glucose-6-phosphate isomerase; PEP, phospho-enol-pyruvate; Pdc, pyruvate decarboxylase; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); Pfk, 6-phosphofructokinase; Pgk, phosphoglycerate kinase; Pgm, phosphoglycerate mutase; Pi, inorganic phosphate; Pyk, pyruvate kinase; TEP, triethylphosphate; Tpi, triose-phosphate isomerase; TPP, thiamine pyrophosphate.

* Corresponding author. Present address: CSIRO Ecosystem Sciences, GPO Box 1700, Canberra, ACT 2601, Australia. Tel.: +61 2 62464464.
E-mail address: Bradley.Stevenson@csiro.au (B.J. Stevenson).

0168-1656/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2011.09.019

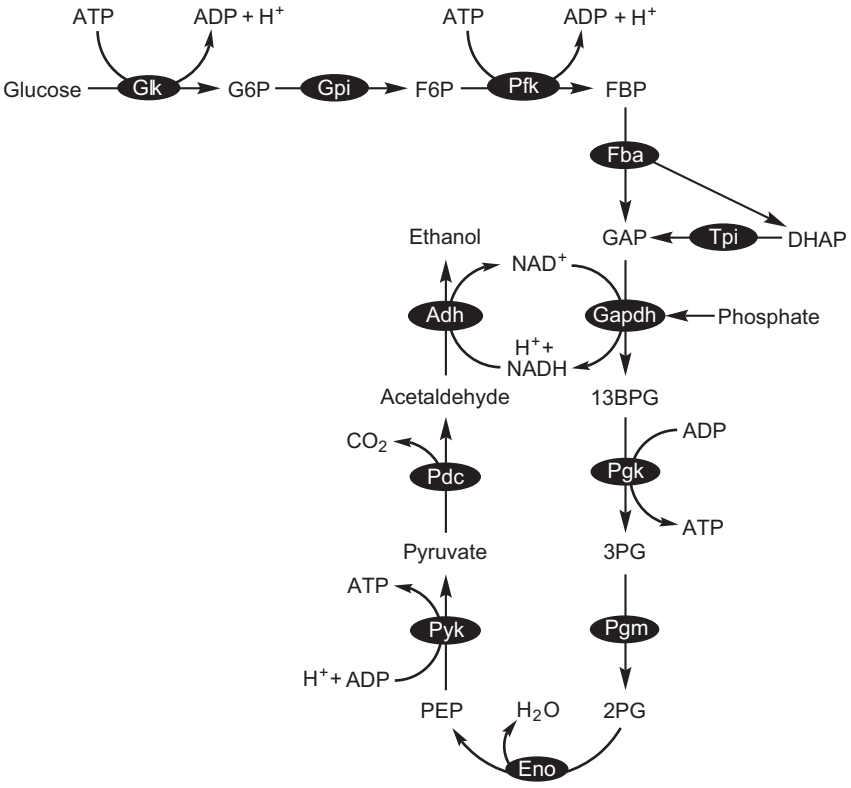

Fig. 1. Fermentative glycolytic pathway. Unidirectional arrows have been used to indicate the direction of net flux through the metabolic pathway. However, only the kinases (Glk, Pfk, Pyk) and Pdc reactions are effectively unidirectional, the others are strictly bidirectional. Abbreviations are listed on the title page.

2005). Although yeast lysate offers an economical source of enzymes for fermentative glycolysis, it includes enzymes that may produce futile cycles and side reactions that reduce the efficiency of ATP regeneration. For this reason we have examined the feasibility reconstituting fermentative glycolysis from individually purified enzymes.

As a proof of principle, we aimed to demonstrate ATP and NAD^+ recycling with fermentative glycolysis in isolation. In this situation, the stoichiometry of glycolysis as defined by Scopes (1997) is:

$$\begin{aligned} & 2 \cdot \mathrm{ATP} + \mathrm{glucose} \rightarrow 2 \cdot \mathrm{ADP} + \mathrm{FBP} && \text{(Reaction 1)} \\ + \;& 2 \cdot \mathrm{ADP} + \mathrm{glucose} + 2 \cdot \mathrm{P_i} \rightarrow 2 \cdot \mathrm{ATP} + 2 \cdot \mathrm{ethanol} + 2 \cdot \mathrm{CO_2} && \text{(Reaction 2)} \\ = \;& 2 \cdot \mathrm{glucose} + 2 \cdot \mathrm{P_i} \rightarrow \mathrm{FBP} + 2 \cdot \mathrm{ethanol} + 2 \cdot \mathrm{CO_2} && \text{(Reaction 3)} \end{aligned}$$

Reaction 1 represents the net reaction of the enzymes glucokinase (Glk; EC 2.7.1.2), glucose-6-phosphate isomerase (Gpi; 5.3.1.9) and 6-phosphofructokinase (Pfk; EC 2.7.1.11). Reaction 2 represents the net reaction for the entire glycolytic pathway: where the net result is ADP phosphorylation for ATP regeneration. The sum of these two reactions gives the net reaction for fermentative glycolysis running in isolation (Reaction 3, the Harden–Young equation). Once all glucose molecules are phosphorylated to yield FBP, ADP is no longer produced and ATP regeneration by phosphoglycerate kinase (Pgk; EC 2.7.2.3) or Pyk cannot occur. At this point, only half of the glucose can be converted to ethanol and CO_2. The remaining carbohydrate is trapped as phosphorylated intermediates represented as FBP in reactions 1 and 3. FBP would exist in equilibrium with triose-phosphates: glyceraldehyde-3-phosphate (GAP), dihydroxyacetone phosphate (DHAP), and 1,3-bisphosphoglycerate (13BPG) (Fig. 1). This equilibrium of high-energy metabolites would then be available to regenerate ATP (Reaction 4).

$$\mathrm{FBP} + 2 \cdot \mathrm{P_i} + 4 \cdot \mathrm{ADP} \rightarrow 4 \cdot \mathrm{ATP} + 2 \cdot \mathrm{ethanol} + 2 \cdot \mathrm{CO_2} \quad \text{(Reaction 4)}$$

In this study, we mix eleven His-tagged *E. coli* enzymes with His-tagged pyruvate decarboxylase 1 (Pdc1; EC 4.1.1.1) from *Saccharomyces cerevisiae* to enable fermentative glycolysis to run in isolation while regenerating ATP and NAD^+. However, Pdc1 is highly regulated and not suited for this application since it is inhibited at above neutral pH and by phosphate. We therefore tested a version of Pdc1 selected for increased activity in these conditions by directed evolution (Stevenson et al., 2008). Overall, the methods presented here enabled reconstitution of fermentative glycolysis such that each enzyme, cofactor and substrate can be defined and tested.

2. Materials and methods

2.1. Enzymes

E. coli was used as a convenient source of all the enzyme genes, except for Pdc, which was obtained from *S. cerevisiae*. Pdc1 was selected over the other two isoenzymes, Pdc5 or Pdc6, since it was responsible for most of the activity in *S. cerevisiae* (Hohmann, 1991). Likewise, some of the other enzymes were present as isozymes in *E. coli*: Glk was selected as the only soluble enzyme for the first step in glycolysis (Fukuda et al., 1983); phosphofructokinase 1 (Pfk1; EC 2.7.1.11) was selected since, unlike Pfk2, it is not inhibited by ATP and FBP (Babul, 1978); of the two phosphoglycerate mutase (Pgm; EC 5.4.2.1) isozymes, dPgm was selected because it is smaller and more active than iPgm (Fraser et al., 1999); FBP-activated pyruvate kinase 1 (Pyk1; EC 2.7.1.40) was selected over AMP-activated Pyk2 (Fraenkel, 1987) since AMP would not be present in our system; and alcohol dehydrogenase P (AdhP; EC 1.1.1.1) was selected over other isoenzymes due to its high activity (Shafqat et al., 1999).

Each of the other six enzymes: Gpi, fructose-1,6-bisphosphate aldolase (Fba; EC 4.1.2.13), triose-phosphate isomerase (Tpi; EC 5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh; EC 1.2.1.12), phosphoglycerate kinase (Pgk; EC 2.7.2.3), and enolase (Eno; EC 4.2.1.11), are unique in *E. coli* and were selected for isolation.