Metabolic Engineering 23 (2014) 9–21

Contents lists available at ScienceDirect

Metabolic Engineering

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

Metabolic control at the cytosol–mitochondria interface in different growth phases of CHO cells

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ARTICLE INFO

Article history:
Received 13 September 2013
Received in revised form
19 January 2014
Accepted 3 February 2014
Available online 10 February 2014

Keywords:
Mammalian cell culture
CHO
Compartmentation
Enzyme activities
Metabolic flux analysis
Mitochondria
Metabolic switches

ABSTRACT

Metabolism at the cytosol–mitochondria interface and its regulation is of major importance particularly for efficient production of biopharmaceuticals in Chinese hamster ovary (CHO) cells but also in many diseases. We used a novel systems-oriented approach combining dynamic metabolic flux analysis and determination of compartmental enzyme activities to obtain systems level information with functional, spatial and temporal resolution. Integrating these multiple levels of information, we were able to investigate the interaction of glycolysis and TCA cycle and its metabolic control. We characterized metabolic phases in CHO batch cultivation and assessed metabolic efficiency extending the concept of metabolic ratios. Comparing *in situ* enzyme activities including their compartmental localization with *in vivo* metabolic fluxes, we were able to identify limiting steps in glycolysis and TCA cycle. Our data point to a significant contribution of substrate channeling to glycolytic regulation. We show how glycolytic channeling heavily affects the availability of pyruvate for the mitochondria. Finally, we show that the activities of transaminases and anaplerotic enzymes are tailored to permit a balanced supply of pyruvate and oxaloacetate to the TCA cycle in the respective metabolic states. We demonstrate that knowledge about metabolic control can be gained by correlating *in vivo* metabolic flux dynamics with time and space resolved *in situ* enzyme activities.

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1. Introduction

Although the knowledge of metabolic pathways is very broad, methods for analyzing metabolic control mechanisms in mammalian cells at a systems level are still very limited. A huge number of valuable studies exploring various individual aspects of mammalian metabolism and of its regulation can be found in the literature. Investigated cells range from different mammalian cell lines, *e.g.* Chinese hamster ovary (CHO) cells, Baby hamster kidney (BHK) cells, hybridoma cells, or myeloma cells, to primary cells or tissues. Usually, a single aspect, *e.g.* the metabolic flux distribution in a specific growth phase, is analyzed and sometimes different conditions, such as nutrient availability, genetic background, or different biological systems are compared. Such studies are well suited for a qualitative description of differences between distinct conditions. However, detailed quantitative understanding of mammalian metabolism and its control requires systems-oriented combined studies, *e.g.* of *in vivo* activity and its regulation.

Metabolic compartmentation is a major characteristic in all eukaryotes and represents an essential means of metabolic regulation (Wahrheit et al., 2011). The interaction of cytosolic glycolysis and mitochondrial TCA cycle is central for cellular energy generation and metabolic efficiency. It often represents a bottleneck in production processes (Niklas et al., 2012a, 2012b, 2011b) and is at least partially impaired in a wide range of diseases including diabetes, cancer and neurodegenerative disorders (Fernyhough et al., 2010; Ferreira et al., 2010; Haas, 2010; Rosenstock et al., 2010; Schrauwen and Hesselink, 2008; Stacpoole, 2012). Despite this central role in metabolism, many aspects of this metabolic hub are still unknown and remain the target of current studies. Although the existence of a specific mitochondrial pyruvate transporter has been demonstrated in the 1970s, the coding gene was only very recently identified (Bricker et al., 2012; Herzig et al., 2012). Increasing our knowledge in metabolic compartmentation and in particular in understanding the interconnection of glycolysis and TCA cycle at the cytosol–mitochondria interface will substantially advance the optimization of producer cell lines and biotechnological processes as well as the development of novel therapeutic approaches.

CHO cells have been used as a mammalian model system for more than 50 years (Jayapal et al., 2007; Puck et al., 1958) and have therefore been termed as 'the mammalian equivalent of *Escherichia coli*' (Puck, 1985). Furthermore, CHO cells are of substantial significance

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http://dx.doi.org/10.1016/j.ymben.2014.02.001
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for the biopharmaceutical industry serving as the predominant cultivation system for the production of therapeutic proteins (Wurm, 2004).

In our study, we provide a comprehensive view on CHO central metabolism and its regulation. We used a unique combination of dynamic metabolic flux analysis (MFA) to obtain the time-resolved *in vivo* flux distribution and determination of compartmental *in situ* enzyme activities in different growth phases. This provides systems level information with functional, spatial and temporal resolution. In the first part of our study, we performed dynamic MFA to capture the dynamics of growth and metabolism upon changing environmental conditions. The cellular adaptation to changing environmental conditions during a batch cultivation leads to metabolic shifts resulting in different growth phases with distinct metabolic states (Niklas et al., 2011c). We identified and characterized different metabolic phases addressing the following questions: (a) what distinguishes the distinct growth phases, (b) which metabolic characteristics determine the metabolic state of each phase, (c) what triggers the phase shifts, and (d) how do the cells respond to these metabolic shifts? Three distinct metabolic states were distinguished, namely overflow metabolism, balanced metabolism and maintenance metabolism.

In order to assess and predict metabolic efficiency at different metabolic states, we expand the concept of metabolic flux ratios. The lactate/glucose ratio is an often-used parameter to characterize the metabolism of mammalian cells (Ahn and Antoniewicz, 2011; Niklas et al., 2012a). We propose the TCA cycle/glycolysis ratio as additional indicator of metabolic efficiency. Furthermore, we analyze the dynamics of these metabolic flux ratios to monitor metabolic shifts.

The first two growth phases are further investigated in the second part by determining specific enzyme activities in distinct growth phases. Applying selective permeabilization techniques in combination with conventional enzyme assays, we were not only able to quantify total enzyme activity but even more important their compartmental distribution (Niklas et al., 2011a).

The last part of our study focused on analyzing the metabolic shift from phase I, characterized by inefficient overflow metabolism, to an efficient and balanced metabolism in phase II by integrating the different data sets. The correlation of *in situ* enzyme activities indicating possible maximum enzyme rates with *in vivo* metabolic rates representing the actual activities represents a powerful approach to gain insight into metabolic control. *In situ* enzyme activities and *in vivo* fluxome integrate information of all upstream functional levels (genome, transcriptome, and proteome), including regulatory events (Sauer, 2006). Additional important information about enzyme localization was obtained from compartmental enzyme activity assays (Niklas et al., 2011a). We integrated information about *in situ* and *in vivo* metabolic activities (functional resolution), metabolic state (time resolution), and compartmental localization of activities (spatial resolution). This allowed us to draw conclusions about metabolic efficiency, rate-limiting steps, glycolytic channeling, and metabolic interactions at the cytosol–mitochondria interface. We demonstrate that metabolic control in the central metabolism and eventually potential targets for process optimization and biomedical research can be identified by correlating *in vivo* metabolic flux dynamics with respective time and space resolved *in situ* enzyme activities.

2. Material and methods

2.1. Cell culture

The CHO K1 cells were cultivated in protein free and chemically defined TC-42 medium (TeutoCell, Bielefeld, Germany) supplemented with 4 mM glutamine in 250 ml baffled shake flasks in a shaking incubator (2 in. orbit, 185 rpm, 37 °C, 5% CO_2 supply). Two parallel cultivations of 100 ml volume were inoculated with 2×10^5 cells/ml. Cell density, cell viability and average cell diameter were determined using an automated cell counter (Invitrogen, Darmstadt, Germany).

2.2. Analytics

Quantification of glucose, organic acids and proteinogenic amino acids in supernatants were performed by HPLC as described previously (Strigun et al., 2011).

2.3. Metabolic flux analysis

Time resolved metabolic flux analysis was carried out using the method and metabolic network model described by Niklas et al. (2011c). The stoichiometric matrix of the metabolic network model is depicted in Table S1.

2.4. Enzyme assays

For determination of specific enzyme activities in different growth phases, cells from two different cultures, the first one being between 48 h and 96 h cultivation time (phase I) and the second one between 96 h and 144 h after seeding (phase II), were harvested in parallel. Enzyme assays were performed in 96-well plates as described in Niklas et al. (2011a) using selective permeabilization techniques for discrimination between cytosolic and mitochondrial activities. For each condition, complete permeabilization with Triton X-100, selective permeabilization with digitonin, and respective negative controls were performed with four technical replicates each. The change of absorbance at 340 nm was monitored over time for all NADH or NADPH dependent enzyme assays ($\varepsilon_{340\ nm} = 6.22\ M^{-1}\ cm^{-1}$). For the citrate synthase assay, absorbance of dithionitrobenzoic acid (DTNB) at 412 nm was measured ($\varepsilon_{412\ nm} = 14.15\ M^{-1}\ cm^{-1}$). In the applied 96-well format, layer thickness ($d = 0.746$ cm) was experimentally determined with NADH solutions at different concentrations using Lambert–Beer's law.

Assays for glucose-6-phosphate isomerase (PGI; EC:5.3.1.9), lactate dehydrogenase (LDH; EC:1.1.1.27), glucose-6-phosphate dehydrogenase (G6PDH; EC:1.1.1.49), fructose-1,6-bisphosphatase (FBPase; EC:3.1.3.11), NAD^+- and $NADP^+$-dependent isocitrate dehydrogenase (IDH_{NAD}; EC:1.1.1.41, and IDH_{NADP}; EC:1.1.1.42), malic enzyme (ME; EC:1.1.1.40), and glutamate dehydrogenase (GDH; EC:1.4.1.3) were carried out according to Niklas et al. (2011a). Assay solutions for other enzymes were as follow: Hexokinase (HK; EC:2.7.1.1): 1 mM $MgCl_2$, 6 mM ATP, 1 mM $NADP^+$, 10 mM glucose, 0.35 U glucose-6-phosphate dehydrogenase, and PBS. Phosphofructokinase (PFK; EC:2.7.1.11): 5 mM $MgCl_2$, 0.15 mM NADH, 0.05 mM ATP, 0.1 U aldolase, 0.6 U glycerol phosphate dehydrogenase, 9.4 U triose phosphate isomerase, 0.25 mM fructose-6-phosphate, and PBS. Pyruvatekinase (PK; EC:2.7.1.40): 15 mM $MgCl_2$, 0.25 mM NADH, 1 mM ADP, 1.2 U lactate dehydrogenase, 12 mM phosphoenolpyruvate, and PBS. After permeabilization the cell suspension was diluted 1:5 before used for this assay. Citrate synthase (CS; EC:2.3.3.1): 0.1 mM acetyl-Coenzyme A, 0.2 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.5 mM oxaloacetate, and PBS. Phosphoenolpyruvate carboxykinase (PEPCK; EC:4.1.1.32): 1 mM $MgCl_2$, 0.15 mM NADH, 1 mM phosphoenolpyruvate, 1.5 mM GDP, 5 mM $KHCO_3$, > 12 U malate dehydrogenase, and PBS. Alanine aminotransferase (ALAT; EC:2.6.1.2): 0.15 mM NADH, 0.02 mM pyridoxalphosphate, 200 mM alanine, 10 mM α-ketoglutarate, > 17 U lactate dehydrogenase, and PBS. Aspartate aminotransferase (ASAT; EC:2.6.1.1): 0.15 mM NADH, 0.02 mM pyridoxalphosphate, 50 mM aspartate, 10 mM α-ketoglutarate,

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