Metabolic characteristics of recombinant Chinese hamster ovary cells expressing glutamine synthetase in presence and absence of glutamine

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Abstract To elucidate the metabolic characteristics of recombinant CHO cells expressing glutamine synthetase (GS) in the medium with or without glutamine, the concentrations of extra- and intracellular metabolites and the activities of key metabolic enzymes involved in glutamine metabolism pathway were determined. In the absence of glutamine, glutamate was utilized for glutamine synthesis, while the production of ammonia was greatly decreased. In addition, the expression of recombinant protein was increased by 18%. Interestingly, the intracellular glutamine maintained almost constant, independent of the presence of glutamine or not. Activities of glutamate-oxaloacetate aminotransferase (GOT), glutamate-pyruvate aminotransferase (GPT), and glutamate dehydrogenase (GDH) increased in the absence of glutamine. On the other hand, intracellular isocitrate and the activities of its downstream isocitrate dehydrogenase in the TCA cycle increased also. In combination with these two factors, a 8-fold increase in the intracellular α -ketoglutarate was observed in the culture of CHO-GS cells in the medium without glutamine.

Keywords Animal cell culture · Cell metabolism · Glutamine · Glutamine synthetase · Recombinant CHO cell

Abbreviations

CHO-GS	Recombinant Chinese hamster ovary cell bearing the exogenous glutamine synthetase gene
GS	Glutamine synthetase
GOT	Glutamate-oxaloacetate aminotransferase
GPT	Glutamate-pyruvate aminotransferase
GDH	Glutamate dehydrogenase
PAG	Phosphate-activated glutaminase
C_X	Viable cell density
α -KG	α -Ketoglutarate
Fum	Fumarate
Cit	Citrate
Icit	Isocitrate
Mal	Malate
MSX	Methionine sulfoximine
Q_P	Specific production rate of metabolite
Q_S	Specific consumption rate of substrate
$Y_{P/S}$	Apparent yield coefficient of product to substrate

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Subscripts

0	Seeding time
t	Harvest time

Introduction

Glutamine is very important in *in vitro* cultures of mammalian cells, not only as an important energy source in catabolism, but also as the donor of carbon and nitrogen in anabolism. In comparison to other amino acids, more glutamine is needed and consumed quickly in cell culture, which usually results in the ammonia accumulation in medium. Ammonia, one of the major negative factors, can inhibit cell growth, diminish productivity and affect glycosylation pattern of recombinant proteins (Martinelle and Häggström 1993; Andersen and Goochee 1995). Therefore, it is very important to decrease ammonia production during cell culture.

Substitution of less-ammoniagenic substrates for glutamine can decrease the formation of ammonia in cell culture. However, some cell lines, such as BHK and Vero cells, usually take a longer period of time to adapt to and grow rather slowly in such a non-ammoniagenic medium (Hassell and Butler 1990). There is an increasing interest in the use of recombinant DNA techniques to manipulate cell metabolism. One well-established strategy is to introducing GS gene into a cell line, such as NS0 and CHO cells, to enable the cells to grow independently of the presence of glutamine in the medium (Dempsey et al. 2003; Wurm 2004). CHO cells have a low level of endogenous glutamine synthetase activity, which usually can maintain cell growth in the absence of glutamine. However, the growth will not be as good as in the presence of glutamine. Thus, co-expressed glutamine synthetase can be used as a selectable marker to identify successful transfectant in the glutamine-free medium, and the desired foreign gene can be amplified with methionine sulfoximine (MSX), an inhibitor of glutamine synthetase (Cockett et al. 1990). Under this condition, ammonia production could be greatly decreased.

The process optimization of cell culture is subject to the knowledge on the metabolic behavior of cells. However, less information is available on the intracellular metabolic characteristics of CHO-GS cells. The aim of this study is to elucidate the metabolism of recombinant CHO-GS cells in the absence and presence of

glutamine on the basis of the analysis of intracellular metabolites and key enzymes involved in the glutaminolysis pathway.

Materials and methods

Cell line and culture

A stable recombinant Chinese hamster ovary cell clone (CHO-GS), co-expressing glutamine synthetase and a recombinant protein, was used in this work. The CHO-GS cells were adapted to suspension growth and then routinely maintained in a home-made serum-free medium, composed of 1:1 mixture of glutamine-free DMEM/F12 supplemented with amino acids, fatty acids, vitamins, trace elements and some other additives. The media containing either 2.5 mmol l⁻¹ glutamate (glutamate-based medium) or 2.5 mmol l⁻¹ glutamine (glutamine-based medium) were used to investigate the cell metabolism in the absence and presence of glutamine. All chemicals were from Sigma, USA.

The cultures were carried out in 120 cm² T-flasks with 30 ml medium at an initial viable cell density of 3.5×10^5 cells ml⁻¹. All T-flasks were put in a CO₂ incubator equilibrated with 5% CO₂ at 37°C. At 96 h post-inoculation, supernatant and cells were separately collected by centrifugation for the analysis of extra- and intracellular metabolites.

Assays

Viable cells were counted using the trypan blue exclusion method with a hemacytometer. Each sample was counted three times, and an average cell density was calculated. The average diameter of an individual viable cell was taken as 14 µm, which gave a single cell volume of 1.44×10^{-9} ml (Schmid and Keller 1992). Glucose, lactate, and ammonia were measured with respective assay kits (Inst. Biol. Prod., Shanghai, China). The concentrations of extra- and intracellular amino acids were determined by a reverse HPLC method (Fiorina et al. 1989).

To analyze the organic acids in TCA cycle, about 1×10^8 viable cells were harvested and

washed by cold 0.45% sodium chloride twice. Subsequent removal of supernatant was followed by addition of 1 ml of 0.3 mol l⁻¹ perchloric acid as extractant. The reaction mixture was kept on ice for 10 min and then centrifuged at 12,000g for 15 min. The supernatant was neutralized by 0.3 mol l⁻¹ KOH. After centrifugation at 12,000g for 15 min again, supernatant was collected and frozen at -80°C till analysis by an ion-exchange chromatography (Lu et al. 2003).

In enzyme activity assays, 6–8 × 10⁷ cells were harvested and pelleted by centrifugation at 600g for 8 min. After decanting the supernatant, the pellet was washed twice in cold phosphate buffered saline (PBS) and then gently resuspended in 1 ml extraction buffer (4 mmol l⁻¹ MgCl₂, 2 mmol l⁻¹ DTT, 0.2 mmol l⁻¹ PBS, pH 7.5) with a pipette. Each sample was sonicated for 2 min (output power 360 w, pulse work time 0.6 s, intermittent time 0.4 s) on ice and centrifuged at 12,000g for 15 min at 4°C. Then, the supernatant was collected and frozen at -80°C. All enzyme activities were determined with a spectrophotometer. Glutaminase (E.C. 3.5.1.2) was measured according to Meister (1985). Glutamate dehydrogenase (E.C. 1.4.1.2), NAD-dependent isocitrate dehydrogenase (E.C. 1.1.1.41), NADP-dependent isocitrate dehydrogenase (E.C. 1.1.1.42), and citrate synthase (E.C. 4.1.3.7) were assayed based on the methods described by Bergmeyer (1983a, b). Glutamine synthetase (E.C. 6.3.1.2), glutamate-oxaloacetate aminotransferase (E.C. 2.6.1.1), and glutamate-pyruvate aminotransferase (E.C. 2.6.1.2) were determined using their corresponding assay kits (Jiancheng Biotechnology Co., Nanjing, China).

All experiments were performed in triplicate in this work.

Calculations

The apparent yield coefficients of product to substrate ($Y_{P/S}$, mol mol⁻¹), the specific consumption rates of substrate (Q_S , mmol cell⁻¹ d⁻¹), and the specific production rates of metabolite (Q_P , mmol cell⁻¹ d⁻¹) were calculated using the following equations:

$$Y_{P/S,t} = (C_{P,t} - C_{P,0}) / (C_{S,0} - C_{S,t})$$

$$Q_{S,t} = 2 \times 10^{-3} (C_{S,0} - C_{S,t}) / [(C_{X,0} + C_{X,t})(t/24)]$$

$$Q_{P,t} = 2 \times 10^{-3} (C_{P,t} - C_{P,0}) / [(C_{X,0} + C_{X,t})(t/24)]$$

where, C_X , C_S , and C_P represent viable cell density (cells ml⁻¹), substrate concentration (mmol l⁻¹), and product concentration (mmol l⁻¹), and subscripts 0 and t indicate the seeding and harvest times (h), respectively.

Results and discussion

Cell growth and glycolysis

Glutamine plays a key role in cell metabolism as nitrogen donor and energy substrate. Its depletion may cause severe decrease of cell viability (Mercille and Massie 1994). However, in the culture of CHO-GS cells, because glutamine can be synthesized under the action of glutamine synthetase, the cell growth must not rely on the presence of glutamine in medium. Table 1 shows the growth and metabolism characteristics of CHO-GS cells in the glutamate- and glutamine-based media with initial 17.5 mmol l⁻¹ glucose. The maximum viable cell densities of 1.41 × 10⁶ cells ml⁻¹ and 1.12 × 10⁶ cells ml⁻¹ were obtained in glutamate- and glutamine-based cultures, respectively. Cell viability maintained as high as about 95% in both cultures. In glutamate-based culture, the specific consumption rate of glucose and the yield coefficient of lactate to glucose ($Y_{Lac/Glc}$) increased by 14% and 36%, respectively, indicating that more glucose was consumed via catabolism pathway to lactate, instead of via anabolism pathway to the precursors for cell growth. It differed from the culture of hybridoma cells in which no increase in lactate production was observed at extremely low glutamine level (Ljunggren and Häggström 1994).

Glutaminolysis

Table 2 showed the specific consumption rates of amino acids in both glutamate- and glutamine-based cultures. Minus means that the designated

Table 1 Growth and metabolism of CHO-GS cells at 96 h in glutamate- and glutamine-based cultures

Medium	C_x (10^6 cells ml^{-1})	Viability (%)	Q_{Glc} (10^{-9} mmol $\text{cell}^{-1} \text{d}^{-1}$)	$Y_{\text{Lac/Glc}}$ (mol mol^{-1})
Glutamate-based	1.41 ± 0.09	96.0 ± 1.27	3.2	1.70
Glutamine-based	1.12 ± 0.14	94.8 ± 1.19	2.8	1.25

amino acids were produced during the cultures. In the glutamine-based culture, glutamine was utilized quickly, accompanying with high specific production rates of alanine and glutamate, both are the catabolic products of glutamine. In the glutamate-based culture where glutamate was a major substrate instead of glutamine, glutamine became a produced amino acid. In this case, glutamate could either be utilized as a substrate for biosynthesis, or be further metabolized to α -ketoglutarate for entering into the TCA cycle. On the other hand, the specific consumption rates of most amino acids were increased in the glutamate-based culture over that in the glutamine-based culture. Among them, the specific consumption rate of asparagine increased by 36%. That might derive from some asparagine to be utilized for glutamine synthesis by transferring NH_4^+ to glutamate. Kurano et al. reported that asparagine could support CHO cell growth as a stable substitute for glutamine (Kurano et al. 1990).

In addition, intracellular amino acids were determined during the batch culture of

CHO-GS cells (Table 2). Interestingly, in both glutamine- and glutamate-based cultures, the intracellular glutamine maintained at the similar level, showing that the cells could absolutely get enough glutamine supply through the action of glutamine synthetase in the medium without glutamine. Generally, the uptake rate of glutamine was much higher than glutamate, so it is reasonable that the metabolic flux of glutaminolysis pathway in the glutamine-based culture is higher by 81% than that in the glutamate-based culture. Therefore, it is not surprised to observe that the intracellular concentrations of glutamate and alanine were increased by 80% and 40% in glutamine-based culture. The relatively high specific production rates for glycine and high intracellular concentration of glycine showed that glycine was also major productive amino acid in both cultures, and the accumulation might be related with the consumption of serine.

The apparent yield coefficients of alanine to glutamate ($Y_{\text{Ala/Glu}}$) and glutamine ($Y_{\text{Ala/Gln}}$) were determined, respectively, in glutamate- and glutamine-based cultures (Table 3). The $Y_{\text{Ala/Glu}}$ of $1.01 \text{ mmol mmol}^{-1}$ in glutamate-based culture was significantly higher than $Y_{\text{Ala/Gln}}$ of $0.37 \text{ mmol mmol}^{-1}$ in glutamine-based culture, indicating that the glutaminolysis pathway shifted to be prone to the alanine production. Christie and Butler also observed the higher $Y_{\text{Ala/Glu}}$ in the batch culture of BHK cells in glutamine-free medium (Christie and Butler 1999).

The accumulation of ammonia obviously decreased from 2.1 mmol l^{-1} in glutamine-based culture to 0.7 mmol l^{-1} in glutamate-based culture. Ammonia is one of the major toxic byproducts in cell culture. Therefore, its reduction in the glutamate-based culture will be benefit for achieving the improved cell growth and then protein expression in the mass cultivation of CHO-GS cells.

Table 2 The specific consumption rates of amino acid (Q_{AA}) and intracellular amino acid concentration ($C_{\text{AA, I}}$) in glutamate-based (–Gln) and glutamine-based (+Gln) cultures of CHO-GS cells

Amino acids	Q_{AA} (10^{-10} mmol $\text{cell}^{-1} \text{d}^{-1}$)		$C_{\text{AA, I}}$ ($\mu\text{mol l}^{-1}$)	
	–Gln	+Gln	–Gln	+Gln
Asp	–0.74	–0.81	40	37
Glu	3.7	–0.43	51	92
Asn	4.9	3.6	23	20
Ser	3.3	2.7	35	40
Gln	–0.84	24.7	34	39
His	–0.11	–0.30	11	10
Gly	–3.3	–3.2	111	187
Ala	–3.7	–9.1	77	108
Arg	1.1	1.1	75	84
Ile	0.58	0.71	24	27
Leu	1.2	1.5	44	48

Table 3 The apparent yield coefficients of alanine to glutamate ($Y_{\text{Ala/Glu}}$) and alanine to glutamine ($Y_{\text{Ala/Gln}}$) and the ammonia concentrations ($C_{\text{NH}_4^+}$) in glutamate- and glutamine-based cultures of CHO-GS cells

Medium	$Y_{\text{Ala/Glu}}$ (mol mol ⁻¹)	$Y_{\text{Ala/Gln}}$ (mol mol ⁻¹)	$C_{\text{NH}_4^+}$ (mmol l ⁻¹)
Glutamate-based	1.01	/	0.72
Glutamine-based	/	0.37	2.10

Enzymatic changes

In glutamine catabolism (Fig. 1), the first step of glutaminolysis pathway is the deamidation of glutamine to glutamate, which is catalyzed by glutaminase. Then, glutamate is converted into the TCA cycle intermediate, α -ketoglutarate. However, for CHO-GS cells cultured in the absence of glutamine, glutamine is synthesized from glutamate and ammonia. In this work, the enzyme activities of glutaminolysis pathway were determined in the glutamate- and glutamine-based cultures of CHO-GS cells.

In our experiments, intracellular GS activity was significantly up to 2.5-fold under glutamate-based condition (Fig. 2A), showing that GS activity is down regulated in the presence of glutamine in medium. The depletion or absence of glutamine has been considered as the major metabolic switch for an increase in GS activity (Milman et al. 1975). Eisenberg et al. (2000) also reported that the GS activity was inversely proportional to the level of glutamine, and was regulated by glutamine at the post-transcriptional level.

Some key enzymes in amino acid metabolism were determined (Fig. 2). The activities of GOT were significantly higher than those of glutamate-pyruvate aminotransferase (GPT) and glutamate dehydrogenase (GDH) in both glutamate- and glutamine-based cultures of CHO-GS cells. It indicated that glutamate metabolism mostly relies

Fig. 1 Scheme of the metabolic pathways of glutaminolysis and the enzymes involved in CHO cell culture. ① Phosphate-activated glutaminase (PAG), ② Glutamate-oxaloacetate aminotransferase (GOT), ③ Glutamate-pyruvate aminotransferase (GPT), ④ Glutamate dehydrogenase (GDH), ⑤ Glutamine synthetase (GS)

on the transamination route, rather than the deamidation route. Similar results were documented by flux analysis and enzyme activity measurement that the transamination route appeared to be the main pathway in myeloma, BHK, and CHO cells, in which glutamate-oxaloacetate aminotransferase (GOT) was the major enzyme involved (Vriezen and van Dijken 1998; Yallop et al. 2003).

Aspartate could be formed through either GOT transamination route or the deamidation of asparagine. In the glutamate-based culture, the specific consumption rate of asparagine increased by 36% (Table 2), while the activity of GOT was

Fig. 2 Activities of some key enzymes in glutamine metabolism route in glutamate-based (■) and glutamine-based (□) cultures of CHO-GS cells. (A) Glutamine synthetase (GS) and glutamate-oxaloacetate aminotransferase (GOT), (B) glutamate-pyruvate aminotransferase (GPT), glutamate dehydrogenase (GDH), and glutaminase (PAG)

increased by 45% in the glutamine-based culture (Fig. 2A), showing that more aspartate should have accumulated, however, the specific production rates of aspartate were almost the same in both cultures. This suggested that, in the glutamate-based medium, produced aspartate was partially utilized in the anabolism of cell growth.

In addition, the activities of GPT and GDH were higher in the glutamate-based culture (Fig. 2B) than in the glutamine-based culture. GPT catalyzes the formation of α -ketoglutarate and alanine from glutamate and pyruvate. The observed increase of the yield coefficient of alanine to glutamate in glutamate-based culture (Table 3) might be directly attributed to the increased activities of GPT. GDH activity was observed higher in the glutamate-based culture than in the glutamine-based culture (Fig. 2B). The deamidation metabolism via GDH pathway can produce more ATP than transamination metabolism. Martinelle et al. found by NMR method in hybridoma and myeloma cell cultures that the flux through GDH pathway increased when cells grew in glucose-limited and glutamine-limited conditions (Martinelle et al. 1998). It was evident that GDH pathway was very important for energy production under stress conditions.

Phosphate-activated glutaminase (PAG) catalyzes the deamidation of glutamine to glutamate and is the rate-limiting step for glutamine utilization (Fitzpatrick et al. 1993). In our experiment (Fig. 2B), the activity of PAG improved by 22% in glutamine-based culture, with the increase of glutamine utilization rate.

Intracellular organic acids

Intracellular organic acids of CHO-GS cells in glutamate- and glutamine-based cultures were determined by the application of ion-exchange chromatography with suppressed conductivity detector (Fig. 3). The peak of pyruvate did not appear in the chromatography in both cultures due to the extremely low presence of pyruvate inside cell. This indicated that pyruvate produced through glycolysis pathway was quickly converted to lactate or alanine, or fully oxidized in TCA cycle, independent of the presence of glutamine in medium. A similar observation was also

Fig. 3 Concentrations of organic acids in TCA cycle, such as citrate (Cit), isocitrate (Icit), fumarate (Fum), malate (Mal), and α -ketoglutarate (α -KG), in glutamate-based (■) and glutamine-based (□) cultures of CHO-GS cells

reported in the culture of hybridoma cells (Bonarius et al. 1996).

The concentration of isocitrate was distinctively high among all the acids of the TCA cycle in CHO-GS cells and increased by 18% in glutamate-based culture over that in the glutamine-based condition. It was contrary to tumor cells (Coleman and Lavietes 1981), hybridoma cells (Mancuso et al. 1994), and CHO-*dhfr*⁻ cells (Lu et al. 2005) in which isocitrate was hardly detectable. The level of citrate was significantly decreased by 71%, to meet a low activity of citrate synthase in glutamate-based medium (Table 4).

The production of α -ketoglutarate can be catalyzed through either isocitrate dehydrogenase pathway or glutaminolysis pathway. It was observed that there was an eight-fold increase of α -ketoglutarate concentration in the absence of

Table 4 Activities of citrate synthase, NAD-dependent isocitrate dehydrogenase, and NADP-dependent isocitrate dehydrogenase in glutamate- and glutamine-based cultures of CHO-GS cells

Enzyme	Activity ($\mu\text{mol min}^{-1}$ (mg protein) ⁻¹)	
	Glutamate-based	Glutamine-based
Citrate synthase	0.115 ± 0.011	0.135 ± 0.008
NAD-dependent isocitrate dehydrogenase	0.014 ± 0.002	0.011 ± 0.003
NADP-dependent isocitrate dehydrogenase	0.038 ± 0.004	0.029 ± 0.001

glutamine, accompanying with the increased activities of intracellular GOT, GPT, and GDH, which might transform glutamate to α -ketoglutarate through transamination or deamination pathways. In addition, isocitrate also could be converted to α -ketoglutarate by NAD- and NADP-dependent isocitrate dehydrogenase pathways. Combined increase of two enzyme activities by 27% and 31%, respectively, were also related to an increase in the level of α -ketoglutarate in the glutamate-based culture (Table 4).

Conclusions

The growth and metabolism of recombinant CHO-GS cells were characterized in the glutamate- and glutamine-based cultures. GS expression system enabled the cells to grow better on the glutamate-based medium, but without significant difference in cell viability. In the aspects of amino acid metabolism, the presence of glutamine in medium resulted in the high specific consumption rate of glutamine and the large ammonia production. On the contrary, in the glutamate-based culture, glutamine was slightly produced and ammonia accumulation was greatly decreased. There was no significant difference in the intracellular glutamine, showing that the CHO-GS cells could absolutely get enough glutamine supply in the medium without glutamine. On the other hand, an eight-fold increase of α -ketoglutarate concentration was observed in glutamate-based culture, accompanying with the activity increases of some intracellular enzymes.

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