Kinetic, Dynamic, and Pathway Studies of Glycerol Metabolism by *Klebsiella pneumoniae* in Anaerobic Continuous Culture: IV. Enzymes and Fluxes of Pyruvate Metabolism

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Abstract: The activities of pyruvate kinase (PK), pyruvate: formate-lyase (PFL), pyruvate dehydrogenase (PDH), and citrate synthase (CS) involved in the anaerobic glycerol conversion by *Klebsiella pneumoniae* were studied in continuous culture under conditions of steady states and sustained oscillations. Both the in vitro and in vivo activities of PK, PFL, and PDH are strongly affected by the substrate concentration and its uptake rate, as is the in vitro activity of CS. The flux from phosphoenolpyruvate to pyruvate is found to be mainly regulated on a genetic level by the synthesis rate of PK, particularly at low substrate concentration and low growth rate. In contrast, the conversion of pyruvate to acetyl-CoA is mainly regulated on a metabolic level by the in vivo activities of PFL and PDH. The ratio of in vitro to in vivo activities is in the range of 1 to 1.5 for PK, 5 to 17 for PFL and 5 to 80 for PDH under the experimental conditions. The regulation of in vivo activity and synthesis of these enzymes is sensitive to fluctuations of culture conditions, leading to oscillations of both the in vitro and in vivo activities. In particular, PFL is strongly affected during oscillations; its average in vitro activity is only about half of its corresponding steady-state value under similar environmental conditions. The average in vitro activities of PDH and PK under oscillations are close to their corresponding steady-state values. In contrast to all other enzymes measured for the glycerol metabolism by *K. pneumoniae* PFL and PDH are more effectively in vivo utilized under oscillations than under steady state, underlining the peculiar role of pyruvate metabolism in the dynamic responses of the culture. *Biotechnol Bioeng* **60**: 617–626, 1998.

Keywords: *Klebsiella pneumoniae;* glycerol; pyruvate kinase; pyruvate:formate-lyase; pyruvate dehydrogenase; in vitro and in vivo activities; dynamics

INTRODUCTION

Pyruvate metabolism is a key step in the central energy and anabolic metabolisms of most microorganisms. It supplies the cells with the intermediate acetyl-CoA. Under aerobic conditions acetyl-CoA is usually channeled into the tricarboxylic acid (TCA) cycle for the generation of energy in the form of $NADH_2$ and ATP and building blocks of biosynthesis such as amino acids and fatty acids. Under anaerobic conditions acetyl-CoA is mainly converted into fermentation products such as ethanol, acetic acid and lactic acid by many bacteria. The formation of these products supplies the cells also with energy or reducing power, which are needed for biosynthesis and/or formation of other products such as 1,3-propanediol in a number of microorganisms anaerobically grown on glycerol (Streekstra et al., 1987; Zeng et al., 1993; Zeng et al.,1997). The channeling of pyruvate into the different routes strongly depends on the metabolic pathways of the microorganism and the cultivation conditions.

The diversity of pyruvate metabolism gives cells flexibility to regulate the energy and intermediate fluxes under varied environmental conditions. However, it also often presents problems when using microorganisms for industrial production of bioproducts. The formation of acetic acid in *Escherichia coli* is, for example, a well-known phenomenon which hinders the achievement of a high cell density in conventional cultivation processes and can cause instability or incompatibility in cells overexpressing foreign proteins. In other processes such as microaerobic cultures (Zeng and Deckwer, 1996), lactic acid production (Cocaign-Bousquet et al., 1996; de Vos, 1996) and lysin production (Vallino and Stephanopoulos, 1994) the conversion of pyruvate to acetyl-CoA and TCA cycle intermediates can impair the formation of the desired products. On the other hand, the lack of pyruvate flux into the TCA cycle is the main reason for the production of lactate in animal cell culture (Neerman and Wagner, 1996). In the past, we have studied the kinetics and pathways for the bioconversion of glycerol to 1,3-propanediol (PD) by *Klebsiella pneumoniae* (Menzel et al., 1996, 1997a, b; Zeng et al., 1993, 1996,1997). The microbial production of PD is of industrial interest and is being

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commercially exploited (Deckwer, 1995; Potera, 1997; Stinson, 1995). It was shown that pyruvate metabolism is an important step affecting the yield and final concentration of PD (Zeng et al., 1993). Furthermore, unusual dynamic behavior such as sustained oscillation and hysteresis has been observed for the glycerol conversion by *K. pneumoniae* (Menzel et al., 1996), which appeared to be strongly associated with the pyruvate metabolism (Zeng et al., 1996). Thus, a more detailed and quantitative understanding of pyruvate metabolism is of general interest for process optimization and control and in the long term, may help in manipulating this central metabolic step by genetic means.

In bacteria, pyruvate is catabolized by one of the following enzymes: pyruvate dehydrogenase (PDH), pyruvate formate-lyase (PFL), and pyruvate:ferredoxin (flavodoxin) oxidoreductase (PFO) (Karlson, 1988; Knappe, 1990). Pyruvate dehydrogenase is usually the enzyme involved in aerobic pyruvate decarboxylation, while PFL and PFO are involved in anaerobic pyruvate cleavage. Pyruvate:ferredoxin (flavodoxin) oxidoreductase is mainly restricted to *Clostridia* and other strict anaerobes. For the anaerobic glycerol metabolism in *K. pneumoniae*, Zeng et al. (1993,1996) postulated that not only PFL, but other enzyme system(s) must be simultaneously involved. This appeared to be the case particularly for culture undergoing sustained oscillations. It was argued that the reason for the occurrence of oscillation and hysteresis lies in an unstable regulation of pyruvate metabolism of different enzymes triggered by substrate excess and drastic change(s) of environmental conditions. Recently, Menzel et al. (1997b) provided enzymatic evidence for the involvement of PDH in this anaerobic culture. However, information on the regulation of the in vitro and in vivo activities of these two enzymes (PFL and PDH), and particularly their dynamic responses under unsteady-state conditions (oscillation and after perturbation) is still lacking. Furthermore, no enzyme investigations into the upstream of pyruvate formation (i.e., from phosphoenolpyruvate) and its channeling into the TCA cycle have been reported for the anaerobic glycerol metabolism. This kind of information is needed for a more complete understanding of the pyruvate metabolism and the associated events in the other route of glycerol conversion leading to 1,3-propanediol. The latter is reported in an article by Ahrens et al. (1998).

In this article, the activities of enzymes associated with the pyruvate metabolism in *K. pneumoniae*, i.e. pyruvate kinase (PK), PFL, PDH, and citrate synthase (CS) are studied in continuous cultures under both steady-state and oscillation conditions. Intracellular concentrations of pyridine nucleotides (NAD and $NADH_2$) were measured to gain information on the regulation of enzyme activities. By comparing the in vitro and in vivo activities of the enzymes studied in this work and those in Ahrens et al. (1998) for glycerol dissimilation and 1,3-PD formation, it is intended to obtain quantitative and more complete information on the regulation of the metabolic pathways and fluxes and to identify possible limiting step(s) in the conversion of glycerol to 1,3-PD.

MATERIALS AND METHODS

Strain, Culture Conditions, Sampling, Preparation of Cell Free Extract, and Product Analysis

Klebsiella pneumoniae DSMZ 2026 obtained from the German Collection of Microorganism and Cell Culture (DSMZ, Braunschweig, Germany) was used in this study. Culture medium composition, cultivation conditions, methods for the determination of fermentation products, and preparation of cell free extract were reported previously (Ahrens et al. 1998; Menzel et al. 1996; Menzel et al. 1997b). The sampling methods, inactivation of metabolism and sample treatment are summarized in Figure 1. The special fast sampling device used (Pec Tec, Mühlacker, Germany; Theobald et al., 1997) ensured taking anaerobic samples within 0.2 s residence time in the sampling device for enzyme assays and the determination of NAD and $NADH_2$.

Enzyme Assays

The pyruvate dehydrogenase (EC 1.2.4.1) activity was measured aerobically according to Hinman and Blass (1981). The reaction mixture contained 2.5 m*M* NAD, 0.2 m*M* thia-

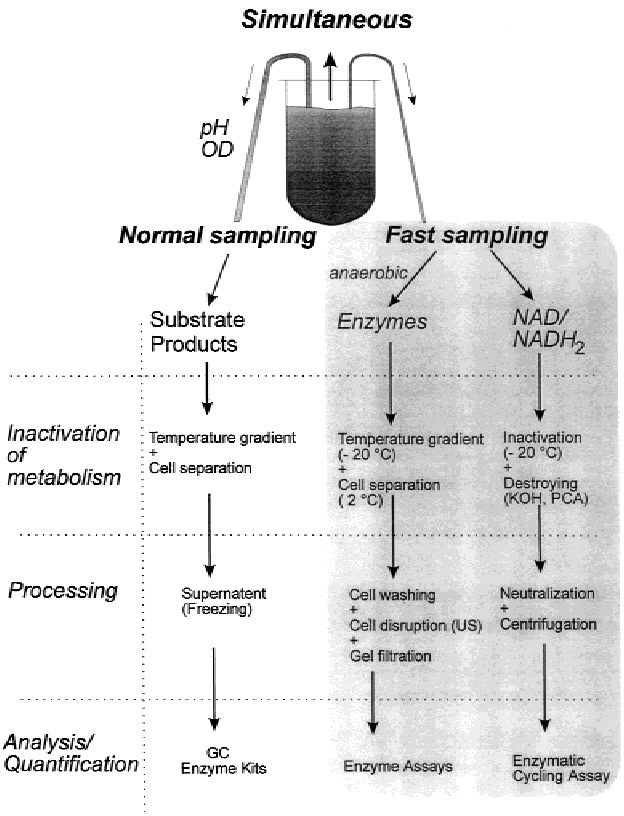

Figure 1. Sample preparation procedure for analysis and enzyme assays.

mine pyrophosphate, 0.1 *mM* coenzyme A, 0.3 m*M* DTT, 5 m*M* pyruvate, 1 m*M* magnesium chloride, desalted cell extract, 1 mg/mL bovine serum albumine, 0.6 m*M* p-iodonitrotetrazolium violet (INT), and lipoamide dehydrogenase (0.1 mg/mL) in 0.05*M* potassium phosphate buffer solution (pH 7.8). The assay was initiated by the addition of pyruvate, and the absorption was measured spectrophotometerally at 500 nm and 30°C. Units of activity were calculated using an experimentally determined extinction coefficient of INT of 15.01 $L \cdot mmol^{-1} \cdot cm^{-1}$.

Pyruvate formate-lyase (EC 2.3.1.54) activity was measured according to Knappe et al. (1974) under strictly anaerobic conditions. The assay contained in a volume of 1 mL:10 m*M* DTT, 0.1 m*M* $Fe(NH_4)2(SO_4)_2$, 5 m*M* L-malate, 1 m*M* NAD, 10 m*M* sodium pyruvate, 0.055 m*M* CoA, 20 μg/mL malate dehydrogenase (1200 u/mg) and 25 μg/mL citrate synthase (120 u/mg) in 0.1 *M* Tris-HCl buffer (pH 7.8). The reaction was started by addition of the desalted cell extract. $NADH_2$ formed was measured spectrophotometrically at 340 nm.

Pyruvate kinase (EC 2.7.1.40) activity was analyzed according to Oszaki and Shiio (1969) with the following assay composition: 3.3 m*M* $MnSO_4$, 0.15 m*M* $NADH_2$, 1 m*M* ADP, 27 u lactate dehydrogenase and the desalted cell extract. The reaction was started by addition of 2 m*M* phosphoenolpyruvate and the decrease of $NADH_2$ was followed by 340 nm.

One unit is defined as the amount of enzyme required to reduce 1 μmol of substrate (e.g., INT for PDH) per minute under the conditions specified. Specific enzyme activity is expressed as units per mg of protein.

Nucleotide Extraction and Assay

The special sampling device mentioned above was used for sampling directly into a tube containing the precooled extractive solution [OH or perchloric acid (PCA)], leading to a desactivation of the metabolism within 0.2–0.5 s (Theobald et al., 1997). $NADH_2$ was extracted with 2 *M* KOH, NAD with 35% PCA. The acid extraction (pH 1.0) destroyed the reduced forms and the alkaline extraction (pH 12.3), the oxidized forms of pyridine nucleotides (Lilius et al., 1979). For a better extraction the samples were immediately mixed for 5 s and left on ice for at least 10 min. After neutralization up to a pH of 7.2–7.4 (NAD) or 7.5–8.0 ($NADH_2$) using 2 *M* KOH or 0.5 N HCl, respectively, the samples were immediately assayed.

For the determination of NAD and $NADH_2$ the spectrophotometric ''enzymatic cycling'' assay of Bernowsky and Swan (1973) was applied. The assay mixture contained 2000 μL buffer (0.15 *M* glycylglycine/nicotinic acid buffer pH 7.4), 400 μL phenanzinium ethylsulfate (PES) (4 mg/mL), thiazolyl-blue (MTT) (5 mg/mL), 70 μL ethanol (p.A. grade), and 20 μL alcohol dehydrogenase (300 U/mL) according to Theobald et al. (1997). The reaction was started by addition of 50 μL neutralized sample. Absorption was spectrophotometrically followed for 15 min at 570 nm.

Calculations

The calculation of specific growth rate, product formation rates, and substrate uptake rates at steady states and during oscillations was previously described (Zeng et al., 1996). The in vitro activities of the enzymes on the basis of biomass were calculated as follows:

$$\text{Enzyme}_{\text{in vitro}} = \text{Enzyme}_{\text{spec}} \times Y_{\text{protein/biomass}} \quad (1)$$

where $\text{Enzyme}_{\text{spec}}$ is the specific enzyme activity based on protein (mol h^{-1} g protein^{-1}) and $Y_{\text{protein/biomass}}$ is the protein content of the crude cell extract related to the original biomass (g g^{-1}).

The in vivo enzyme activities ($\text{Enzyme}_{\text{in vivo}}$) during steady-state condition in $mmol \cdot g^{-1} \cdot h^{-1}$ can be calculated according to the pathway as follows:

$$PFL_{in\ vivo} = q_{For} + q_{H_2} \quad (2)$$

$$PDH_{in\ vivo} = q_{EtOH} + q_{HAc} - q_{For} - q_{H_2} \quad (3)$$

$$PK_{in\ vivo} = q_{glyc} - q_{PD} - q_{Suc} \quad (4)$$

where q_{glyc} is the specific substrate uptake rate and q_{For}, q_{H_2}, q_{EtOH}, q_{Hac}, q_{PD}, and q_{Suc} are the specific formation rates of formate, hydrogen, ethanol, acetate, 1,3-propanediol, and succinic acid in $mmol \cdot g^{-1} \cdot h^{-1}$.

For comparing the in vitro and in vivo activities of enzymes the in vitro activities measured by the enzyme assays at 30°C have to be extrapolated to the fermentation temperature of 37°C ($= \text{Enzyme}_{\text{in vitro}}$) using a correction factor. This factor was 1.48 for PDH, 1.22 for PFL, and 1.45 for PK according to calibration curves prepared for the individual enzymes (data not shown).

RESULTS AND DISCUSSION

In Vitro and In Vivo Enzyme Activities Under Steady-State Conditions

Anaerobic continuous cultivations of *K. pneumoniae* were carried out at varied dilution rates (D) between 0.10 and 0.40 h^{-1}. For both substrate limitation and substrate excess conditions, steady states were obtained using the cultivation strategy described by Menzel et al. (1996) to avoid oscillations of the culture. The dependence of substrate consumption and product formation on the supply of substrate has been described in detail by Menzel et al. (1996) and Zeng et al. (1996). The data were used here to calculate the in vivo activities of the enzymes according to Eqs. (2)–(4). Furthermore, the in vitro activities of pyruvate kinase, pyruvate dehydrogenase, pyruvate:formate-lyase, and citrate synthase which channel acetyl-CoA into the TCA cycle were determined by enzyme assays.

The specific activity of PK (PK_{spec}) as functions of the glycerol concentration in the culture (C_{Glyc}^{Rct}) and the specific glycerol uptake rate (q_{Glyc}) at different dilution rates is illustrated in Figures 2A and B. A similar trend was obtained for the in vitro activity of PK based on biomass, because the

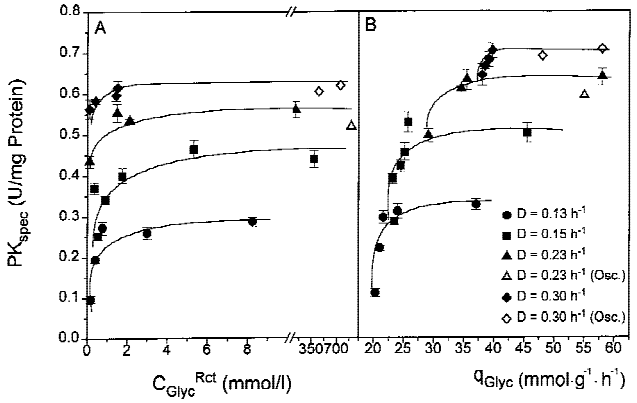

Figure 2. Specific activity of pyruvate kinase (PK) as functions of glycerol concentration in the reactor (C^{Rct}_{Glyc}) (**A**) and specific glycerol uptake rate (q_{Glyc}) (**B**) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

protein content of cells didn't change significantly under the experimental conditions (data not shown). No obvious or systematic dependence of $Y_{protein/biomass}$ on growth rate or substrate concentration could be ascertained. For each dilution rate, PK_{spec} showed a kind of saturation curve. It rapidly increased at low glycerol concentration (< 2 mmol/L) but leveled off at higher glycerol concentrations. The maximum enzyme level correlated with the dilution rate, indicating a growth rate coupled synthesis of the enzyme. As shown in Figure 2B, PK_{spec} also correlated well with the specific substrate uptake rate. The ratio of in vitro to in vivo activities of PK ($PK_{in\ vitro/in\ vivo}$) at different dilution rates is plotted against C^{Rct}_{Glyc} in Figure 3. It is interesting to note that at $D = 0.15\ h^{-1}$ $PK_{in\ vitro/in\ vivo}$ was close to 1.0 irrespective of C^{Rct}_{Glyc}, which is the minimum ratio of in vitro to in vivo activities that implies a complete utilization of the enzyme capacity. The $PK_{in\ vitro/in\ vivo}$ values calculated to be slightly lower than 1.0 for some of the steady states in Figure 3 might be due to an incomplete cell disruption and/or loss of enzyme activity during the preparation of cell extracts or an overestimation of the in vivo activity [Eq.(4)]. In general, it may be stated that at low dilution rates ($\leqslant 0.15\ h^{-1}$) the catalysis capacity of PK synthesized was optimally utilized, and the flux of phosphoenolpyruvate (PEP) to pyruvate was limited by the total amount of active enzyme. At higher dilution rates ($\geqslant 0.23\ h^{-1}$) $PK_{in\ vitro/in\ vivo}$ was close to 1 at very low glycerol concentrations, but increased to values of about 1.5–1.6 as the glycerol concentration increased. These results indicate that at high growth rates and high glycerol concentrations the activity of PK was either limited by its substrate PEP and coenzyme ADP or inhibited by factors such as intracellular pyruvate and ATP. The flux of PEP to pyruvate was not only regulated by the enzyme synthesis but also governed to certain extent by metabolic effects above a growth rate of $0.15\ h^{-1}$.

Menzel et al. (1997b) recently showed that in the anaerobic pyruvate metabolism of *K. pneumoniae* both PFL and PDH are simultaneously involved. The specific activities of these two enzymes measured at different dilution rates are shown in Figures 4 and 5 as functions of C^{Rct}_{Glyc} and q_{Glyc}, respectively. The specific activity of PFL turned out to be almost exclusively a function of C^{Rct}_{Glyc}, irrespective of the growth rate. It rapidly increased at low glycerol concentration and reached a saturation value of about 5.2 u/mg protein at a glycerol concentration above 5 mmol/L. The specific activity of PDH was also generally a saturation function of C^{Rct}_{Glyc}. However, a significant effect of growth rate was observed. It is interesting that the effect of growth rate seems to be merely restricted to growth rates $\leqslant 0.15\ h^{-1}$. At growth rates $> 0.15\ h^{-1}$ the specific activity of PDH followed a similar trend as PFL, reaching a saturation value at a glycerol concentration above 5 mmol/L. The saturation value of PDH (about 0.5 U/mg protein) was about 10% of that of PFL, indicating the dominant role of PFL under these conditions. Significantly higher values of specific PDH activity were obtained at $D < 0.15\ h^{-1}$, suggesting an increased contribution of PDH under these conditions.

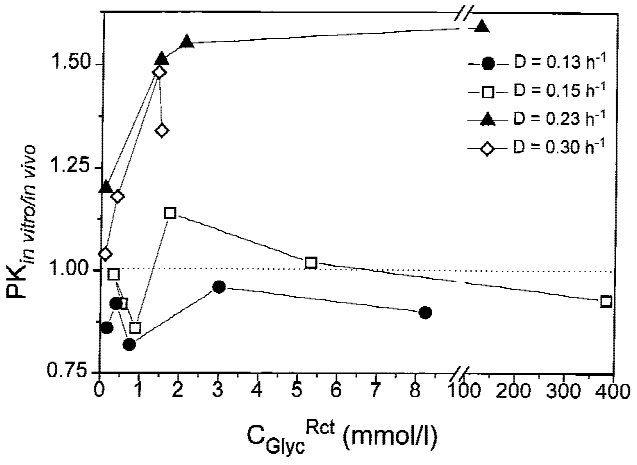

Figure 3. Ratio of the in vitro to in vivo activities of PK as a function of glycerol concentration in culture at different dilution rates.

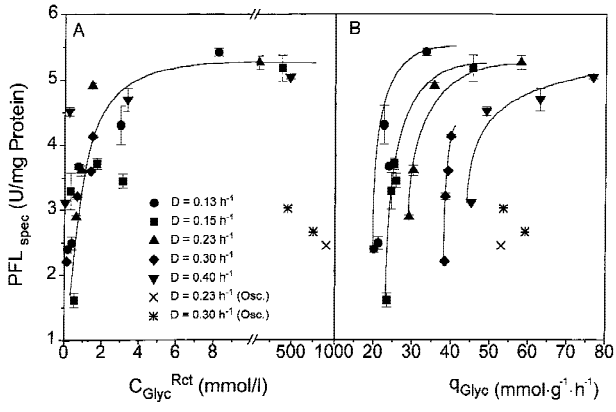

Figure 4. Specific activity of pyruvate formate lyase (PFL) as functions of glycerol concentration in the reactor (C^{Rct}_{Glyc}) (**A**) and specific glycerol uptake rate (q_{Glyc}) (**B**) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

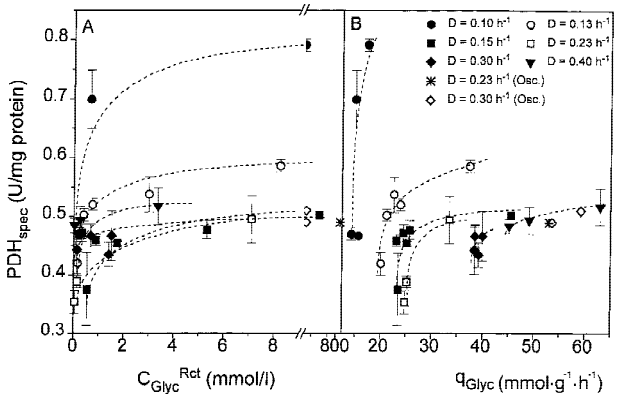

Figure 5. Specific activity of pyruvate dehydrogenase (PDH) as functions of glycerol concentration in the reactor (C_{Glyc}^{Rct}) (**A**) and specific glycerol uptake rate (q_{Glyc}) (**B**) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

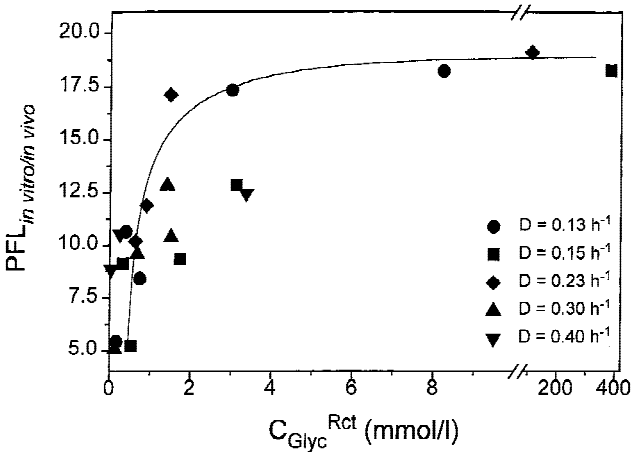

Figure 6. Ratio of in vitro to in vivo activities of PFL as a function of glycerol concentration in culture at different dilution rates.

The different behavior of the culture with respect to the synthesis of PDH at growth rates higher or lower than the boundary growth rate (about 0.15 h^{-1}) is clearer when plotting PDH_{Spec} against q_{Glyc} (Fig. 5B). A plotting of PFL_{Spec} against q_{Glyc} (Fig. 4B) confirmed that the saturation value of PFL_{Spec} achieved was independent of the growth rate. However, it suggests a dependency of PFL_{Spec} on μ in the unsaturated range of PFL_{Spec}. This seemingly contradicting observation in comparison with Figure 4A is due to the fact that q_{Glyc} is a function of both growth rate and substrate concentration (Zeng and Deckwer, 1995). Under substrate-limiting conditions, it is almost only a linear function of μ. Under conditions of large substrate excess the effect of C_{Glyc}^{Rct} becomes dominant, with q_{Glyc} increasing over a relatively wide range of C_{Glyc}^{Rct}. With these two aspects in mind, the different appearances of Figures 4A and B can be understood. Because q_{Glyc} is a variable reflecting the physiological state (uptake rate or flux of substrate) of cells, its use can sometimes give more information (Ahrens et al. 1998). For instance, in addition to what was mentioned above, Figures 4B and 5B revealed that both PDH_{Spec} and PFL_{Spec} are very sensitive to the uptake rate of substrate in their unsaturated ranges. At an identical substrate uptake rate, both PDH_{Spec} and PFL_{Spec} drastically increased at decreasing growth rates. In contrast, the specific activity of the preceding enzyme PK increased with increasing growth rate (Fig. 2B).

To understand the different behavior of the enzymes a comparison of their in vitro and in vivo activities is helpful. Figures 6 and 7 compare the in vivo and in vitro activities of PFL and PDH as functions of C_{Glyc}^{Rct}. Under substrate limitation, the in vivo activity of PDH was very low while $PFL_{in\ vivo}$ linearly increased with the substrate concentration (Menzel et al., 1997a). Obviously, the whole flux of pyruvate to acetyl-CoA was mainly catalyzed by PFL under substrate limitation. Under substrate excess, however, $PDH_{in\ vivo}$ increased and $PFL_{in\ vivo}$ decreased. Both reached a stationary level at high glycerol concentrations (> 700 mmol/L) (Menzel et al., 1997b). For both enzymes, their in vitro activities were significantly higher than the in vivo activities under substrate excess: the ratio of in vitro to in vivo activities being higher than 7 for PDH and about 20 for PFL. These results indicate a decisive regulation of enzyme activity on a metabolic level. The relatively high ratios of in vitro to in vivo activities for these enzymes seem to be partly due to a low intracellular concentration of pyruvate imposed by the low specific activity of PK (Fig.2) and a limitation of PEP flux to pyruvate (Fig.3). The increase of $PFL_{in\ vitro/in\ vivo}$ with C_{Glyc}^{Rct} may indicate a decreased intracellular concentration of pyruvate (Fig.6). The change of $PDH_{in\ vitro/in\ vivo}$ is somewhat irregular, probably due to the fact that PDH is an enzyme complex with multiple subunits, and a number of other factors such as NAD and $NADH_2$, that in addition to pyruvate are involved in the activity regulation of this enzyme complex.

A channeling of acetyl-CoA into citric acid cycle intermediates via citrate synthase will reduce the acetyl-CoA

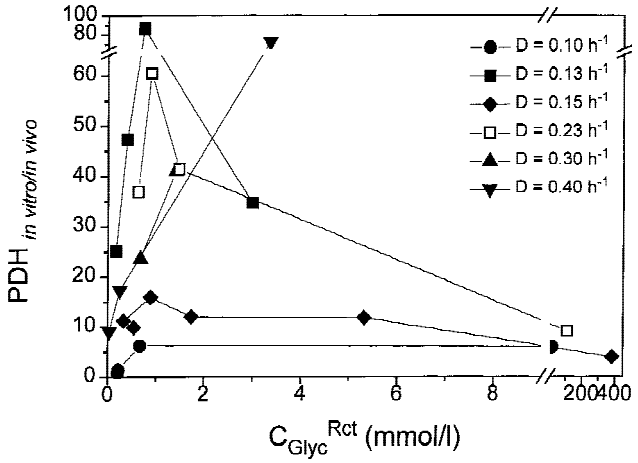

Figure 7. Ratio of in vitro to in vivo activities of PDH as a function of glycerol concentration in culture at different dilution rates.

amount available for the formation of ethanol and acetic acid and therefore affect the $PDH_{in\ vivo}$ value calculated by Eq. (2). To estimate the extent of flux of acetyl-CoA into citric acid cycle intermediates the specific activity of CS (CS_{spec}) was measured. The results of CS assays for different dilution rates are shown in Figure 8. CS_{spec} seemed to be a strict function of C^{Rct}_{Glyc} (Fig.8A). Independent of the growth rate, it decreased from about 0.11 U/mg protein to a level as low as 0.03–0.04 U/mg protein at high values of C^{Rct}_{Glyc} (substrate excess). As pointed out above for PFL (Fig. 4), the apparent independence of CS_{spec} on the growth rate might be falsified by the relationship between C^{Rct}_{Glyc} and q_{Glyc}. As revealed in Figure 8B, CS_{spec} increased with D at a given uptake rate of substrate, reflecting the fact that under anaerobiosis the intermediates of the TCA cycle are mainly required for biomass synthesis. The in vivo activity of CS was not estimated in this work. Assuming a complete in vivo utilization of CS the acetyl-CoA flux catalyzed by CS would be in a range of 20% of the sum of the fluxes of ethanol and acetic acid under substrate limitation (C^{Rct}_{Glyc} < 0.5 mmol/L) and about 4% of the sum of ethanol and acetic acid fluxes under glycerol excess (C^{Rct}_{Glyc} > 4 mmol/L). Because most steady states in this work had a C^{Rct}_{Glyc} value significantly higher that 0.5 mmol/L it can be concluded that the acetyl-CoA flux to citrate had no significant influence on the calculation of in vivo activity of PDH.

It is worth mentioning that the culture showed distinctively different dynamic behavior with respect to perturbations of culture conditions at different dilution rates (Menzel et al., 1996). At pH 7 and dilution rates above 0.15 h^{-1} *K. pneumoniae* is very sensitive to an increase in substrate concentration, and responds by sustained oscillations of growth and product formation upon a certain level of perturbations of the substrate concentration. However, no oscillations were observed for cells grown at dilution rates below 0.15 h^{-1}, even after a relatively drastic change of the substrate concentration. The results shown in Figures 3 and 5 appear to give some clues for this different dynamic behavior of the culture. As discussed above, distinct regulation patterns were found for the in vitro to in vivo ratio of PK and for the specific activity of PDH around the growth rate of 0.15 h^{-1}. It seems that both an increased synthesis rate of PDH and a decreased regulation of PK on a metabolic level increase the stability of the culture. It may be assumed that the regulation of enzyme activity on a metabolic level is more sensitive to substrate fluctuations than the regulation of enzyme synthesis on a genetic level. Therefore, due to the different nature of enzyme regulations at different growth rates cells can display different dynamic behavior. Among the enzymes studied in this work and in Ahrens et al. (1998), PK and PDH are the only two enzymes that showed such distinct activity regulation mechanisms around the critical growth rate (0.15 h^{-1}) for the occurrence of oscillations, suggesting important roles of these enzymes in determining the dynamic behavior of *K. pneumoniae*. The unique irreversible step of glycolysis catalyzed by PK in this fermentation process may be of particular importance in this regard. The other irreversible glycolysis reactions catalyzed by hexokinase and phosphofructokinase are not involved in the glycerol conversion, because glycerol enters the glycolysis only at the level of dihydroxyacetonephosphate (DHAP).

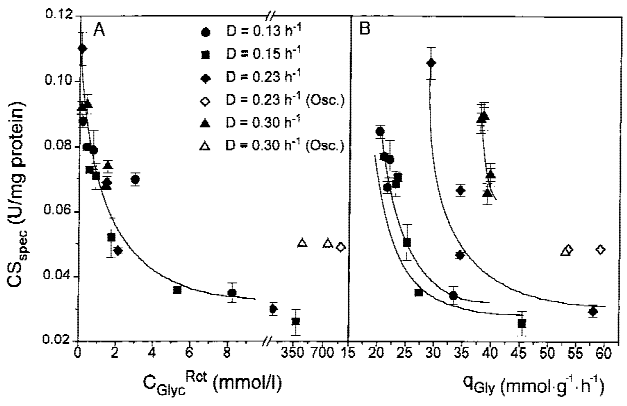

Figure 8. Specific activity of citrate synthetase (CS) as functions of glycerol concentration in the reactor (C^{Rct}_{Glyc}) (**A**) and specific glycerol uptake rate (q_{Glyc}) (**B**) at different dilution rates. Also shown are the mean values of oscillation cycles at the corresponding dilution rate and average glycerol concentration or glycerol uptake rate.

Intracellular NAD and $NADH_2$ Concentrations Under Steady States

The intracellular concentrations of NAD and $NADH_2$ and their ratio are involved in the regulation of in vivo activity of several enzymes in the anaerobic glycerol metabolism. The activities of PDH, glycerol dehydronase (GDH), and 1,3-propanediol oxidoreductase (PDOR) are known, for example, to be enhanced by NAD, but inhibited by $NADH_2$. To obtain information in this regard, the intracellular concentrations of NAD and $NADH_2$ were measured for two dilution rates at varied glycerol concentration. The results are summarized in Figure 9. Both the $NADH_2$ and NAD concentrations appeared to be independent of the growth rate. They decreased with C^{Rct}_{Glyc} at low concentrations and

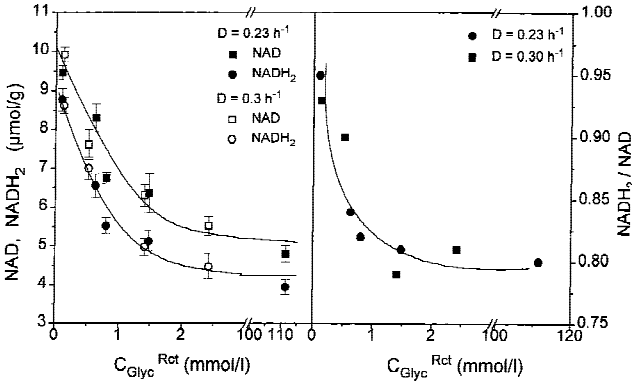

Figure 9. (**A**) Intracellular concentrations of NAD and $NADH_2$, and (**B**) ratio of $NADH_2$/NAD as functions of glycerol concentration in the reactor (C^{Rct}_{Glyc}).

became relatively constant at $C^{Rct}_{Glyc} > 3$ mmol/L, being about 5 μmol/g DW for NAD and 4 μmol/g DW for $NADH_2$. Under the experimental conditions the concentration of NAD in the cells was always higher than that of $NADH_2$, resulting in a $NADH_2$/NAD ratio lower than 1.0 (Fig. 9B). The $NADH_2$/NAD ratio also declined with increasing C^{Rct}_{Glyc} and reached a constant value of 0.8 at $C^{Rct}_{Glyc} > 1.5$ mmol/l. Similarly as for the citrate synthetase (Fig. 8B) it can be shown that the concentrations of NAD and $NADH_2$ and the $NADH_2$/NAD ratio all declined with q_{Glyc}. From these results, it appears that the variation of the in vitro to in vivo ratio as shown in Figure 7 for PDH is not mainly caused by the pyridine nucleotides. In fact, several other effectors including the intracellular concentrations of PEP, pyruvate, acetyl-CoA, ADP and TCA cycle intermediates are involved in regulating the activity of PDH (Karlson, 1988; Knappe, 1990). In contrast, for the other two enzymes GDH and PDOR the decrease of their in vitro to in vivo ratios with C^{Rct}_{Glyc} (Ahrens et al., 1998) correlates with the decrease of $NADH_2$/NAD, indicating a possible role of NAD and $NADH_2$ in the regulation of enzyme activity.

The intracellular concentrations of NAD and $NADH_2$ and the $NADH_2$/NAD ratio shown above for *K. pneumoniae* are much lower than those found for *Clostridium butyricum*. Reimann (1997) reported a NAD concentration as high as 9 μmol/g DW and a $NADH_2$ concentration of about 25 μmol/g DW for *C. butyricum* DSM 5431 anaerobically grown on glycerol under substrate excess. The $NADH_2$/NAD ratio was as high as 2.8. Both the pool of NAD+$NADH_2$ and the $NADH_2$/NAD ratio in this strain can be significantly reduced by an addition of DL-glyceraldehyde into the culture, leading to an increased consumption of glycerol and an increased formation of 1,3-PD (Petitdemange, personal communication). For two mutants of *C. butyricum* DSM 5431 that produce more 1,3-PD at high substrate concentrations than the wild-type strain, the concentrations of NAD and $NADH_2$ were found to be much lower (about 2–3 and 6–8 μmol/g, respectively) than those in the wild-type strain. Considering that *K. pneumoniae* generally has a higher productivity of 1,3-PD than *C. butyricum* (Zeng et al., 1993), these results seem to support the conjecture that a lower intracellular pool of NAD+$NADH_2$ and/or a lower $NADH_2$/NAD ratio is favorable for the formation of 1,3-PD. However, this conjecture seems to contradict the fact that $NADH_2$ is a cosubstrate for 1,3-PD formation, and NAD inhibits this reaction (Daniel et al., 1995). In fact, an evaluation of the experimental data of Petitdemange et al. (personal communication) and Reimann (1997) on the basis of specific formation rate of 1,3-PD (q_{PD}) by cells reveals that the *C. butyricum* cultures with addition of DL-glyceraldehyde had lower values of qPD than the uninfluenced normal culture. The *C. butyricum* mutants studied by Reimann (1997) also had slightly lower values of q_{PD} than the wild-type strain. The only consistent conclusion regarding the effect of pyridine nucleotides from our work and those with *C. butyricum* (Reimann,1997) seems to be that a lower intracellular pool of NAD+$NADH_2$ favors the synthesis of biomass, because the biomass concentration increased with a decreased pool of NAD+$NADH_2$ in all these cultures. This may be understood by considering the fact that the regeneration of $NADH_2$ constitutes a problem for cells when anaerobically grown on glycerol. It may also be one of the reasons why *K. pneumoniae* grows much more effectively on glycerol than *C. butyricum* strains. The effects of nucleotide concentrations on cell growth deserve more investigation.

In Vitro and In Vivo Enzyme Activities Under Sustained Oscillations

The activities of enzymes PK, CS, PFL, and PDH were investigated for cultures undergoing sustained oscillations at two dilution rates 0.23 and 0.30 h^{-1}. The consumption rate of substrate and formation rates of biomass and different products during the oscillations were exactly the same as those reported by Menzel et al. (1996) and Zeng et al. (1996). For the same cultures, Ahrens et al. (1998) measured the in vitro activities of glycerol dehydrogenase (GDH), glycerol dehydratase (GDHt), and 1,3-propanediol oxidoreductase (PDOR) and compared them with the corresponding in vivo activities. It was found that both the in vitro and in vivo activities of GDH, GDHt, and PDOR oscillated with nearly the same frequencies. Oscillations have also been found for the activities of PK, CS, PFL, and PDH as shown in Figure 10 for the oscillation cycle at $D = 0.23\ h^{-1}$. Similar results were obtained for the oscillations at $D = 0.30\ h^{-1}$. Again, the oscillation frequency was almost the same for the four enzymes, reaching their maxima and minima slightly in advance of the maxima and minima of CO2 evolution, growth rate (cf. Menzel et al., 1996) and those of GDH, GDHt, and PDOR (Ahrens et al., 1998). The amplitude of oscillations was different for the enzymes. PFL displayed the largest fluctuation of its in vitro activity, having a minimum of 1.65 and a maximum of 3.3 U/mg protein. This corresponded to 100% fluctuation of the enzyme concentration. Such a large fluctuation was also observed for CS, though the absolute in vitro activity of this enzyme was much lower. Similar, and even larger fluctuations were reported for GDH, GDHt, and PDOR in the same culture (Ahrens et al., 1998). In contrast, the fluctuations of PK and PDH in vitro activities were lower, being about 20– 40% of their absolute values. These results indicate that among the enzymes tested, PFL synthesis is most significantly affected during the oscillation. This becomes more obvious when comparing the average values of in vitro activities during an oscillation cycle with their corresponding steady-state values at the same dilution rate and average C^{Rct}_{Glyc} for the different enzymes. As found for the enzymes associated for the glycerol dissimilation and 1,3-PD formation (Ahrens et al., 1998) the average values of PKspec and PDH_{Spec} during oscillations were also nearly the same as those of the corresponding steady-state values (Figs. 2 and 5). However, the average value of PFL_{Spec} was only half of that under steady state (Fig. 4). On the other hand, the average value of

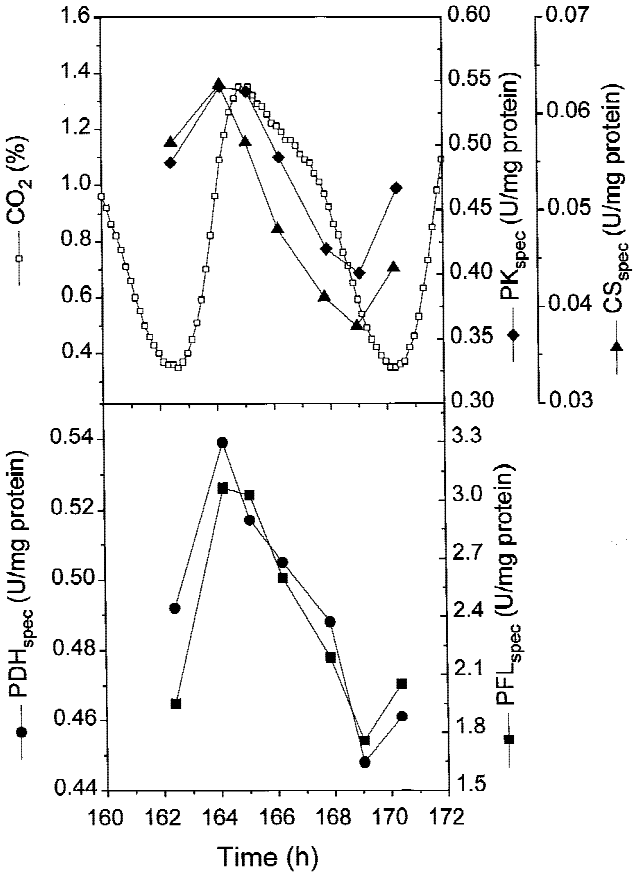

Figure 10. CO_2 concentration in the effluent gas and specific activities of enzymes PK, CS, PDH, and PFL during an oscillation cycle in a continuous culture with feed glycerol concentration = 99 g/l and D = 0.23 h^{-1}.

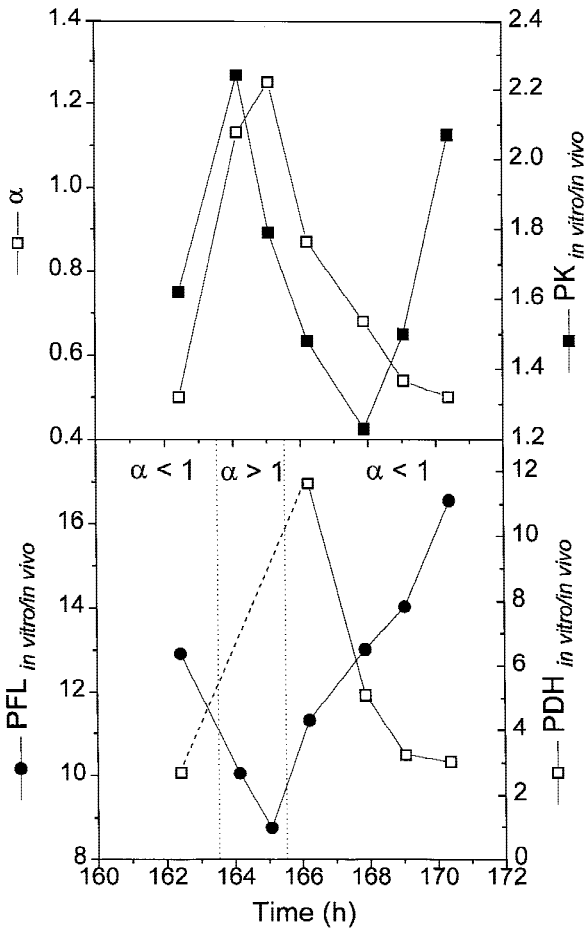

Figure 11. Ratios of in vitro to in vivo activities for enzymes PK, PFL and PDH during an oscillation cycle in the continuous culture of Figure 10. Also shown is the ratio of acetyl-CoA flux catalyzed by PFL to the total acetyl-CoA flux $\left(\alpha = \frac{q_{H_2} + q_{Formate}}{q_{Ethanol} + q_{Acetate}}\right)$.

CS_{spec} was higher under oscillations than under steady state (Fig. 8). These experimental data of enzyme measurement confirmed the findings from the analysis of metabolic fluxes (Zeng et al., 1996), which suggested that pyruvate metabolism is the main step affected under oscillation conditions. The specific formation rates of all the products originating from pyruvate metabolism (CO_2, H_2, formic acid, ethanol, acetic acid, lactic acid, and 2,3-butanediol) showed significant differences under conditions of oscillation and steady state. It is now clear that these differences are mainly caused by the alteration of PFL synthesis.

The simultaneous fluctuation of PDH and PFL at the same pace (Fig. 10) is not expected in the sense that the ratio of the acetyl-CoA flux catalyzed by PFL to the total acetyl-CoA flux (α) oscillated as well (Zeng et al., 1996). Thus, the in vivo activities and/or the ratios of in vitro to in vivo activities of these two enzymes must oscillate in different manners. Figure 11 depicts the α value and the ratios of in vitro to in vivo activities of PFL, PDH, and PK during an oscillation cycle. Indeed, the oscillations of $PFL_{in\ vitro/in\ vivo}$ and $PDH_{in\ vitro/in\ vivo}$ reached their maxima and minima at almost opposite time points. $PDH_{in\ vitro/in\ vivo}$ followed more closely the oscillation of $PK_{in\ vitro/in\ vivo}$. This means that with the increase of in vivo PK activity or the increase of PEP flux to pyruvate the in vivo activity of PDH also increases while the in vivo activity of PFL decreases. This is in accordance with the experimental observations under steady-state conditions (Figs. 6 and 7). A comparison of the average values of ratios of in vitro to in vivo activities as shown in Figure 11 with those of Figures 6 and 7 reveals that both PFL and PDH are more effectively utilized under oscillating conditions. In contrast, the average value of $PK_{in\ vitro/in\ vivo}$ is slightly higher under oscillation than under steady state, indicating a less effective utilization of the enzyme capacity. Such a reduced utilization was also found for GDH and DDOR during oscillations (Ahrens et al., 1998). These results seem to underline again, the peculiar role of pyruvate metabolism during the oscillations.

The increased utilization of both PFL and PDH may be a metabolic response of cells to overcome the metabolic burden imposed by a large perturbation of culture conditions. The same metabolic burden may also hinder the synthesis of PFL. The exact nature of the metabolic stress and the mechanisms of regulation on both metabolic and genetic levels are not clear. In fact, different regulation mechanisms

are involved in PFL and PDH apart from the activation by substrates and inhibition by endproducts. The in vivo activity of PFL is inhibited by high intracellular concentrations of dihydroxyacetone (DHA), dihydroxyacetone-phosphate (DHAP), and acetyl-CoA, while several TCA cycle intermediates, ATP and $NADH_2$ are inhibitors of PDH (Carlsson et al., 1985). In addition, PDH is activated by PEP and ADP. For *E. coli* and *Enterococcus faecilis* the most important factor reducing PDH activity is the $NADH_2$/NAD ratio (Hansen and Henning, 1966; Snoep et al. 1990). A $NADH_2$/NAD ratio of about 0.6 was found to inhibit the PDH activity in *E. faecilis* by 50%, and a ratio of 1.65 by 90% (Snoep et al., 1990). We measured the intracellular concentrations of NAD and $NADH_2$ during oscillations. A certain level of fluctuations was found for both, but no clear patterns of oscillations could be ascertained (not shown). This might be due to the fact that the regulation of intracellular nucleotides renders a response on a much shorter time scale, i.e., minutes rather than hours as observed for the enzyme concentrations. Nevertheless, results from seven different samples during an oscillation cycle indicated that the average concentrations of NAD (7.6 μmol/g) and $NADH_2$ (6.6 μmol/g) are obviously higher than those (about 4.8 and 3.9 μmol/g, respectively) at high glycerol concentrations under steady state (Fig. 9). More experimental data on the intracellular metabolites and cofactors are needed to improve our understanding of the regulation of in vitro and in vivo activities of the enzymes, particularly for the dynamic behavior under nonsteady-state conditions as shown above.

CONCLUSION

The in vitro and in vivo activities of pyruvate kinase, pyruvate:formate-lyase, pyruvate dehydrogenase, and citrate synthase in *K. pneumoniae* grown on glycerol are strongly affected by the substrate concentration and its uptake rate respectively. The formation of pyruvate is mainly regulated on a genetic level by the synthesis of PK, particularly at low substrate concentration and low growth rate. In contrast, the conversion of pyruvate to acetyl-CoA is mainly regulated on a metabolic level by the in vivo activities of PFL and PDH. Distinctive regulation patterns of the in vitro and in vivo activities of PK and PDH were observed around the critical growth rate of 0.15 h^{-1} for the occurrence of oscillations, suggesting that the different stability of the culture at different dilution rates is due to the different regulation mechanisms of the activities of these enzymes. Both the metabolic and genetic networks for the enzymes studied are sensitive to fluctuations of culture conditions at high dilution rates, leading to oscillations of their in vitro and in vivo activities. In contrast to most enzymes involved in glycerol metabolism, PFL and PDH are more effectively utilized during oscillations than under steady-state conditions.

References

Ahrens, K., Menzel, K., Zeng, A.-P., Deckwer, W.-D. 1998. Kinetic, dynamic and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. Part III: Enzymes and fluxes of glycerol dissimilation and 1,3-propanediol formation. Biotechnol. Bioeng., in press.

Bernowsky, C., Swan, M. 1973. An improved cycling assay for nicotinamid adenine dinucleotid. Anal. Biochem. **53**: 452–458.

Carlsson, J., Kujala, U., Edlund, M.-J. 1985. Pyruvate dehydrogenase activity in *Streptococcus mutants.* Infect. Immun. **49**(3): 674–678.

Cocaign-Bousquet, M., Garrigues, C., Loubiere, P., Lindley, N. D. 1996. Physiology of pyruvate metabolism in *Lactococcus lactis.* Antonie van Leeuwenhoek **70**: 253–267.

Daniel, R., Boenigk, R., Gottschalk, G. 1995. Purification of 1,3-propanediol dehydrogenase from citrobacter freundii and cloning, sequencing, and overexpression of the corresponding gene in *Escherichia coli*. J. Bacteriol. **177**: 2151–2156.

Deckwer, W.-D. 1995. Microbial conversion of glycerol to 1,3-propanediol. FEMS **16**: 143–149.

de Vos, W. M., 1996. Metabolic engineering of sugar catabolism in lactic acid bacteria. Antonie van Leeuwenhoek **70**: 223–242.

Hansen, R. G., Henning, U., 1966. Regulation of pyruvate dehydrogenase activity in *Escherichia coli* K12. Biochim. Biophys. Acta **122**: 352–355.

Hinman, R. G., Blass, J. P. 1981. An NADH-linked spectrophotometric assay for pyruvate dehydrogenase complex in crude tissue homogenates. J. Biol. Chem. **256**: 6583–6586.

Karlson, P. 1988. Kurzes Lehrbuch der Biochemie. Georg Thieme Verlag, Stuttgart.

Knappe, J., Blaschkowski, H. P., Gröbner, P., Schmitt, T. 1974. Pyruvate-formate lyase of *Escherichia coli:* The acetyl-enzyme intermediate. Eur. J. Biochem. **50**: 253–263

Knappe, J. 1990. Anaerobic dissimilation of pyruvate, pp. 151–155. In: F.C Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*, Vol. 1. ASM, Washington, DC.

Lilius, E. M., Multanen, V.-M., Toivonen, V. 1979. Quantitative extraction and estimation of intracellular nicotinamid dinucleotids in *Escherichia coli.* Anal. Biochem. **99**: 22–27.

Menzel, K., Zeng, A.-P., Biebl, H., Deckwer, W.-D. 1996. Kinetic, dynamic and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. Part I: The phenomena and characterization of oscillation and hysteresis. Biotechnol. Bioeng. **52**: 549–560.

Menzel, K., Zeng, A.-P., Deckwer, W.-D. 1997a. High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae.* Enzyme Microbiol. Technol. **20**: 82–86.

Menzel, K., Zeng, A.-P., Deckwer, W.-D. 1997b. Enzymatic evidence of involvement of pyruvate dehydrogenase in anaerobic glycerol metabolism by *Klebsiella pneumoniae.* J. Biotechnol. **56**:135–142.

Neerman, J., Wagner, R. 1996. Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell Lines, insect and primary liver cells. J. Cell. Physiol. **166**: 152–169.

Ozaki, H., Shiio, I. 1969. Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum.* J. Biochem. **66**: 297–311.

Potera, C. 1997. Genencor & Dupont create ''green'' polyester. Genetic Eng. News **17**, 11.

Reimann, A. 1997. Produktion von 1,3-Propandiol aus Glycerin durch *Clostridium butyricum* DSM 5431 und produkttolerante Mutanten. Ph.D dissertation. Technical University of Braunschweig, Germany.

Snoep, J. L., Teixera, de Mattos M. J., Postma P. W., Neijssel, O. M. (1990) Involvement of pyruvate dehydrogenase in product formation in pyruvate-limited anaerobic chemostat cultures of *Enterococcus faecilis* NCTC 775. Arch. Microbiol. **154**: 50–55.

Stinson, S. C. 1995. Fine and intermediate chemicals makers emphasize new products and processes. Chem. Eng. News **17**: 10–14.

Streekstra, U., Teixera de Mattos, M. J., Neijssel, O. M., Tempest, D. W. 1987. Overflow metabolism during anaerobic growth of *Klebsiella aerogenes* NCTC 418 on glycerol and dihydroxyacetone in chemostat culture. Arch. Microbiol. **147**: 268–275.

Theobald, U., Mailinger, W., Baltes, M., Rizzi, M., Reuss, M. 1997. In vivo analysis of metabolic dynamics in *Saccharomyces cerevisiae:* I. Experimental observations. Biotechnol. Bioeng. **55**: 305–316.

Vallino, J. J., Stephanopoulos, G. 1994. Carbon flux distribution at the pyruvate branch point in *Corynebacterium glutamicum* during lysin overproduction. Biotechnol. Prog. **10**: 320–326.

Zeng, A.-P., Biebl, H., Schlieker, H., Deckwer, W.-D. 1993. Pathway analysis of glycerol fermentation by *Klebsiella pneumoniae:* Regulation of reducing equivalent balance and product formation. Enzyme Microb. Technol. **15**: 770–779.

Zeng, A.-P., Deckwer, W.-D. 1995. A kinetic model for substrate and energy consumption of microbial growth under substrate-sufficient conditions. Biotech. Prog. **11**: 71–79.

Zeng, A.-P., Menzel, K., Deckwer, W.-D. 1996. Kinetic, dynamic and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture. Part II: Analysis of metabolic fluxes and pathways under oscillation and steady-state conditions. Biotechnol. Bioeng. **52**: 561–571.

Zeng, A.-P., Deckwer, W.-D. 1996. Bioreaction techniques under low oxygen tension and oxygen limitation: from molecular level to pilot plant reactor. Chem. Eng. Sci. **51**: 2305–2314.

Zeng, A.-P., Biebl, H., Deckwer, W.-D. 1997. Microbial conversion of glycerol to 1,3-propanediol - Recent progress, Chapter 14, pp. 264–279. In: B. C. Saha and J. Woodward (eds.), Fuels and chemicals from biomass. ACS Symposium Series 666. American Chemical Society, Washington, DC.