^{13}C NMR Flux Ratio Analysis of *Escherichia coli* Central Carbon Metabolism in Microaerobic Bioprocesses

Jocelyne Fiaux,[§] Charlotte I. J. Andersson,[||] Niklas Holmberg,[⊥,†] Leif Bülow,[||] Pauli T. Kallio,[⊥] Thomas Szyperski,[‡,§] James E. Bailey,[⊥] and Kurt Wüthrich*[,§]

Institut für Molekularbiologie und Biophysik and Institut für Biotechnologie Eidgenössische Technische Hochschule-Hönggerberg CH-8093 Zürich, Switzerland
Department of Pure and Applied Biochemistry Center for Chemistry and Chemical Engineering Lund University, P.O. Box 124 S-221 00 LUND, Sweden

Received October 30, 1998

Active pathways and catalytic capacities in microorganisms are often highly sensitive to the cell environment. For example, oxygen supply can have qualitative effects on biocatalytic configuration and rates.[2−4] This paper addresses the behavior of wild-type *E. coli* MG1655 cells carrying a high copy number plasmid under microaerobic conditions ($[O_2] < 0.02$ mmol/L), a situation that is encountered frequently in nature as well as in the biotechnology industry.[2,5] Only a few stable isotope labeling experiments have so far been pursued to investigate anaerobic and/or microaerobic metabolism.[6−13] In this paper, information on active metabolic topology and carbon pathway flux ratios was obtained from the aforementioned *E. coli* strain under microaerobic conditions,[14] which is a typical biocatalyst configuration, using biosynthetic fractional ^{13}C-labeling of proteinogenic amino acids with a mixture of 10% [$^{13}C_6$]-glucose/90% unlabeled glucose as the sole carbon source and 2D [^{13}C,^{1}H]-correlation NMR spectroscopy for analysis of the resulting products.[13,14] Analysis of the breakdown of the six-carbon skeleton of glucose from the ^{13}C fine structures allows both determination of ratios of metabolic fluxes and an efficient analysis of the bioreaction network topology.[13,15−17]

Most ^{13}C-labeling patterns which were observed for the microaerobic *E. coli* MG1655 system (Figure 1; Table 1; Table S1 in the Supporting Information) are similar to those previously reported for *E. coli* strain W3110 grown in a batch culture under strictly anaerobic conditions,[13,18] indicating that the topology of active central metabolic pathways and the flux ratios are essentially the same under these microaerobic growth conditions[14] as under strictly anaerobic conditions (Figure 2). Thus, comparison of pyruvate and phosphoenolpyruvate ^{13}C-labeling patterns[13] reveals that 71% of the C1−C2 bonds in pyruvate have been reversibly cleaved at least once by pyruvate formate-lyase (Figure 1, a and b; Table 1).[19] Consistent with this finding, formate is detected as the major byproduct in the culture medium.[14] Since pyruvate formate-lyase is strongly suppressed by oxygen,[19,20] its activity indicates that the intracellular concentration of free oxygen must be close to zero. The NMR data further show that oxaloacetate is synthesized solely via anaplerotic carboxylation of phosphoenolpyruvate (Figure 1c). The flux from 2-oxoglutarate to oxaloacetate is zero, showing that the tricarboxylic acid cycle is interrupted.[13] This is typical for anaerobiosis, because strong repression

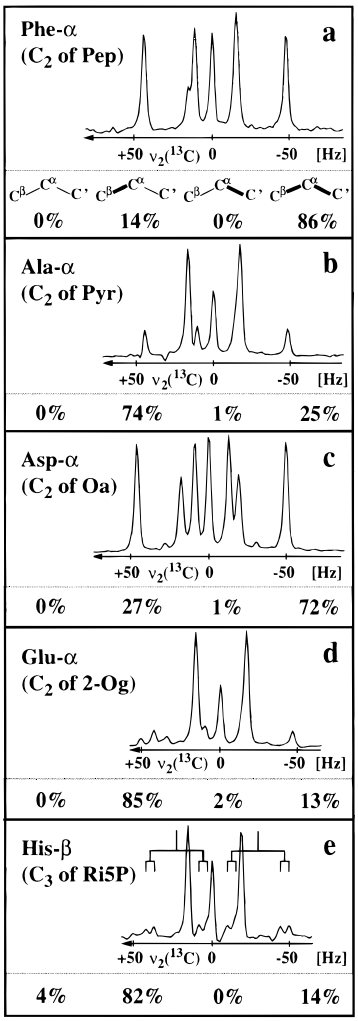

Figure 1. ^{13}C scalar coupling fine structures: (a) phenylalanine C$^{\alpha}$, (b) alanine C$^{\alpha}$, (c) aspartate C$^{\alpha}$, (d) glutamate C$^{\alpha}$, (e) histidine C$^{\beta}$. The fractionally ^{13}C-labeled amino acids[13,18] were obtained from wild-type *E. coli* cells grown as described in ref 14. Panels (a) to (e) derive from C_2 of phosphoenolpyruvate (Pep), pyruvate (Pyr), oxaloacetate (Oa), 2-oxoglutarate (2-Og), and C_3 of ribose-5-phosphate (Ri5P), respectively. In panel (e), the doublet of doublets arising from $^{13}C^{\gamma}-^{13}C^{\beta}-^{13}C^{\alpha}$ is further split by the two-bond scalar coupling $^{2}J_{C^{\beta}C^{\delta}}$, which documents the presence of intact C_5-fragments arising from the oxidative branch of the pentose phosphate pathway.[13,16,17] The 2D [^{13}C,^{1}H]-COSY spectrum was recorded on a Bruker DRX 500 spectrometer.[14] In each panel, the relative abundances (in %) of the different carbon fragments shown in panel (a) are indicated, where the preserved bonds from the source molecules are depicted in bold. These were calculated as described previously from the analysis of the ^{13}C fine structure.[13]

* To whom correspondence should be addressed.
[§] Institut für Molekularbiologie und Biophysik.
[||] Center for Chemistry and Chemical Engineering.
[⊥] Institut für Biotechnologie.
[†] Present address: Unilever Research Colworth, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK.
[‡] Present address: Department of Chemistry, State University of New York at Buffalo, 815 Natural Sciences Complex, Buffalo, NY 14260-3000.

(1) Abbreviations used: *E. coli*, *Escherichia coli*; NMR, nuclear magnetic resonance; 2D, two-dimensional; DO, dissolved oxygen concentration; A_{600}, optical density at 600 nm; DCW, dry cell weight; COSY, correlation spectroscopy.

(2) Konz, J. O.; King, J.; Cooney, C. L. *Biotechnol. Prog.* **1998**, *14*, 393−409.
(3) Spiro, S.; Guest, J. R. *Trends Biochem. Sci.* **1991**, *16*, 310−314.
(4) Iuchi, S.; Lin, E. C. C. *Cell* **1991**, *66*, 5−7.
(5) Bailey, J. E. *Chem. Eng. Sci.* **1995**, *50*, 4091−4108.
(6) Ogino, T.; Arata, Y.; Fujiwara, S.; Shoun, H.; Beppu, T. *Biochemistry* **1978**, *17*, 4742−4745.
(7) Ogino, T.; Arata, Y.; Fujiwara, S. *Biochemistry* **1980**, *19*, 3684−3691.
(8) den Hollander, J. A.; Urgubil, K.; Brown, T. R.; Bednar, M.; Redfield, C.; Shulman, R. G. *Biochemistry* **1986**, *25*, 203−211.
(9) Campbell-Burk, S. L.; den Hollander, J. A.; Alger, J. R.; Shulman, R. G. *Biochemistry* **1987**, *26*, 7493−7500.
(10) Grivet, J. P.; Durand, M.; Tholozan, J. L. *Biochimie* **1992**, *74*, 897−901.
(11) Santos, H.; Fareleira, P.; Xavier, A. V.; Chen, L.; Liu, M.-Y.; LeGall, J. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 551−557.
(12) Chalk, P. A.; Roberts, A. D.; Blows, W. M. *Microbiology* **1994**, *140*, 2085−2092.
(13) Szyperski, T. *Eur. J. Biochem.* **1995**, *232*, 433−448.

10.1021/ja983786y CCC: $18.00
Published on Web 01/29/1999

Table 1. Origin of Intermediates in the Central Carbon Metabolism of *E. coli* Cells under Microaerobic Conditions[a]

metabolites	fraction of total pool[b] [%]
Pep originating from pentoses (upper bound)	46 ± 5
E4P originating from fructose (lower bound)	31 ± 4
Ri5P originating from G3P and S7P (TK reaction)	85 ± 2
Ri5P originating from E4P (TK + TA reactions)	9 ± 2
Pyr reversibly converted to AcCoA and formate at least once	71 ± 3
AcCoA originating from pyruvate	96 ± 3
Oa originating from Pep	97−100
Oa reversibly converted to fumarate at least once	25 ± 7
2Og originating from Oa and AcCoA	95−100
Ic originating from glyoxylate and succinate	0−4
Gly originating from CO_2 and C_1 unit	0−3
Ser originating from Gly and C_1 unit	35 ± 2

[a] The data have been obtained as described previously.[13,16] TK and TA denote transketolase and transaldolase, respectively, and Ic designates isocitrate. The abbreviations for the other metabolites are given in the legend of Figure 2. [b] The numbers describe the fraction of the total pool of the metabolite indicated in bold that originates from the specified pathway, or has undergone the specified reversible interconversion reaction. Some of these flux ratios also appear in Figure 2.

of the 2-oxoglutarate dehydrogenase complex leads to dissection of the tricarboxylic acid cycle into a reductive and an oxidative branch, which provide respectively succinate and 2-oxoglutarate for biosynthetic demands.[3,13,19,21] About 25% of the oxaloacetate molecules have at least once been reversibly interconverted to the symmetric metabolites fumarate or succinate (Figure 1c; Table 1), which demonstrates that fumarate generation for synthesis of succinyl-CoA does not take place in an irreversible fashion.

A key finding with respect to the pentose phosphate pathway is the detection of intact C_5-fragments in ribose-5-phosphate (Figure 1e),[16,17] which shows that the oxidative branch of the pentose phosphate pathway participates in the generation of NADPH to satisfy biosynthetic requirements. In conjunction with the lower and upper bounds that could be derived for erythrose 4-phosphate synthesis from fructose and for phosphoenolpyruvate synthesis

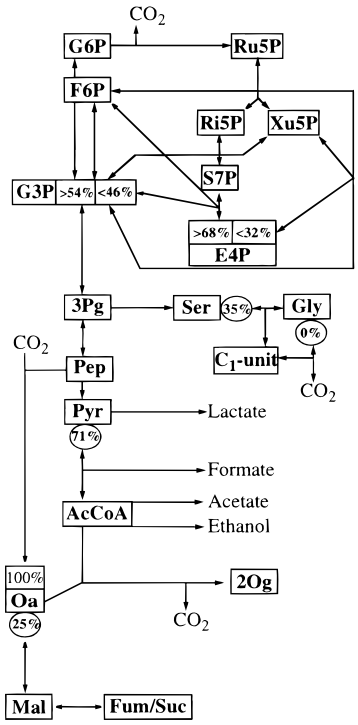

Figure 2. Topology of the network of active pathways and ratios of fluxes as derived from fractional ^{13}C-labeling of wild-type *E. coli* cells under severe oxygen limitation in a fed-batch culture.[14] The fractions of molecules (in %) given in square boxes are synthesized via the fluxes pointing at them.[13,15−17] Numbers in ellipses indicate the amount of reversible interconversion of the molecules. Abbreviations: G6P, glucose-6-P; F6P, fructose-6-phosphate; Ru5P, ribulose-5-phosphate; Ri5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; S7P, seduheptulose-7-phosphate; E4P, erythrose-4-phosphate; G3P, glyceraldehyde-3-phosphate; 3Pg, 3-phosphoglycerate; Pep, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl-CoA; Oa, oxaloacetate; 2Og, 2-oxoglutarate; Mal, malate; Fum, fumarate; Suc, succinate; Ser, serine; Gly, glycine.

from pentoses (Figure 2; Table 1),[13] the data agree with the view that both the oxidative and the nonoxidative branch of the pentose phosphate pathway contribute significantly to pentose synthesis.

In contrast to previous studies performed under strictly anaerobic conditions,[13,18] the glycine cleavage pathway[19] was not detected in microaerobic cells (Figure 2; Table 1), supporting that a high CO_2 concentration is needed to enforce reversible manifestation of this reaction.[13] As in previous studies of *E. coli*[13] and *Bacillus subtilis*,[16] the reversible cleavage of serine into glycine and a C_1-unit was observed (Figure 2; Table 1).

In conclusion, in *E. coli* cells under the microaerobic conditions applied here,[14] respiration is driven solely by reducing equivalents arising from glycolysis. This result is at variance with normally assumed aerobic configurations and illustrates the importance of direct experimental characterization of individual pathway activities, especially under extreme growth conditions.

Acknowledgment. Financial support was obtained from the ETH, the Swiss Priority Program in Biotechnology (SPP BioTech), and the Swedish Research Council of Engineering Sciences (TFR).

Supporting Information Available: Tables of relative abundances of intact C_2 and C_3 fragments in the biosynthesis of fractionally ^{13}C-labeled amino acids (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA983786Y

(14) Fed-batch cultivations of *E. coli* MG1655 carrying the plasmid pUC19 were performed in duplicates in Sixfors bioreactors as described in ref 22. The air flow rate was set to 0.4 volume of gas per volume of liquid per minute, so that the cultures became microaerobic (DO equal to or below 0.02 mmol/L) at the beginning of the early exponential phase. The fed-batch mode was started with 1 mL/h of feed medium[22] containing 10% [$^{13}C_6$]-glucose and 90% unlabeled glucose when A_{600} reached 2−2.5, and 2 mL/h when A_{600} reached 3.5−4, and was maintained constant at 2 mL/h until A_{600} reached 5−5.5. The DO was monitored with a pO_2 electrode (Ingold, Inc.) and the exhaust gases (CO_2 and O_2) with an emission monitor (Brüel and Kjear, Emissions Monitor Type 3427). The DO fell under the detection limit within 6 h after inoculation, but exhaust gas data indicated that respiration continued. Glucose and ethanol concentrations were monitored. A Boehringer Mannheim Acetate Kit (No. 148 261) was used to measure the acetate concentration in bioreactor samples. Metabolite analyses of formate, succinate, and D-lactate were performed enzymatically.[23] The generation of byproducts during the ^{13}C-labeling period was normalized to the dry cell weight (DCW): formate 57.8 mmol/g DCW, acetate 41.6 mmol/g DCW, ethanol 10.3 mmol/g DCW, D-lactate 5.8 mmol/g DCW, succinate 4.3 mmol/g DCW. After harvest of the cells the cellular protein was isolated and hydrolyzed, and 2D [^{13}C,^{1}H]-COSY NMR spectra were recorded as described previously.[13,16] Metabolic flux ratios were calculated from ^{13}C multiplet fine structures.[13,15−17]

(15) Szyperski, T.; Bailey, J. E.; Wüthrich, K. *Trends Biotechnol.* **1996**, *14*, 453−459.

(16) Sauer, U.; Hatzimanikatis, V.; Bailey, J. E.; Hochuli, M.; Szyperski, T.; Wüthrich, K. *Nature Biotechnol.* **1997**, *15*, 448−452.

(17) Szyperski, T. *Q. Rev. Biophys.* **1998**, *31*, 41−106.

(18) Wüthrich, K.; Szyperski, T.; Leiting, B.; Otting, G., in *Frontiers and New Horizons in Amino Acids Research*; Takai, K., Ed.; Elsevier: Amsterdam, 1992; pp 41−48.

(19) Neidhardt, F. C.; Curtiss, R.; Ingraham, J. L.; Liu, E. C. C.; Low, K. B.; Magasamik, B.; Reznikoff, W. G.; Riley, M.; Schaechter, M.; Umbarger, H. E., Eds. *Escherichia coli* and *Salmonella typhimurium*; American Society for Microbiology: Washington, 1996; Vol. 1.

(20) Knappe, J.; Sawers, G. *Microbiol. Rev.* **1990**, *75*, 383−398.

(21) Amarasingham, C. R.; Davis, B. D. *J. Biol. Chem.* **1965**, *240*, 3664−3667.

(22) Kallio, P. T.; Tsai, P. S.; Bailey, J. E. *Biotechnol. Prog.* **1996**, *12*, 751−757.

(23) Bergmeyer, H. U. *Methods of enzymatic analysis*; VCH Publishers: Deerfield Beach, FL, 1985; Vols. VI and VII.