Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 358-362

GLYCOLYSIS AND ENTNER-DOUDOROFF PATHWAYS IN *HALOBACTERIUM HALOBIUM*: SOME NEW OBSERVATIONS BASED ON ^{13}C NMR SPECTROSCOPY

Haripalsingh M. Sonawat*, Sudha Srivastava, S. Swaminathan+ and Girjesh Govil

Chemical Physics Group, Tata Institute of Fundamental Research,
Homi Bhabha Road, Bombay-400 005, India

Received October 12, 1990

^{13}C NMR was used to study glucose metabolism in intact cells of *Halobacterium halobium*. Spectra of glucose grown cells incubated with [1-^{13}C] glucose indicate the presence of gluconate as the initial product. The existence of glycolytic pathway is also indicated. In the extracts of these cells an NADP dependent glucose dehydrogenase was detected. Galactose grown cells failed to metabolise glucose but exhibited glucose dehydrogenase activity although about 20-50% less than that for glucose grown cells. Possible explanations of these experiments are discussed.

Although long known as carbohydrate non-utilizers, several extremely halophilic bacteria metabolize carbohydrates (1-6). A modified Entner Doudoroff pathway appears to be the major route of carbohydrate metabolism, in which oxidation at anomeric carbon precedes the phosphorylation step. Glucose oxidation to yield gluconate is catalyzed by an NADP linked glucose dehydrogenase in *Halobacterium salinarium* (7) and *H. saccharovorum* (8). The enzyme is however synthesized only in presence of glucose in growth medium and the organisms show a complete lack of glucose dehydrogenase when galactose is used as carbon source.

Growth of *H. halobium* is stimulated by glycerol, glucose or galactose (9). Crude extracts of this organism grown without any added carbohydrates were shown to exhibit phosphoglucose isomerase, fructose-1,6-diphosphatase and fructose-1,6-diphosphate aldolase (10). However, hexokinase, phosphofructokinase and enzymes of Entner-Doudoroff pathway were reported to be non-detectible.

We have used intracellular ^{13}C NMR spectroscopy to demonstrate, for the first time, the presence of glucose dehydrogenase activity in *H. halobium*

* To whom correspondence should be addressed.

+ Present address: Chemistry Department, Hall Atwater Laboratories, Wesleyan University, Middletown, Connecticut 06457.

0006-291X/90 $1.50

grown in presence of glucose. *In vitro* enzyme assays and NMR experiments have also been carried out on extracts of these cells to support the intracellular experiments.

MATERIALS AND METHODS

[1-^{13}C] Glucose was from MSD Isotopes, peptone (L-37) from Oxoid Ltd., and NADP from Sisco, India. Other chemicals were reagent grade and used as supplied.

H. halobium culture was obtained from the Biochemistry and Food Technology Division of B.A.R.C., India and maintained and propagated in a medium consisting of NaCl 250 g, KCl 2 g, $MgSO_4.7H_2O$ 20 g, and peptone 10 g per litre (11). Glucose or galactose (1%) was used instead of citrate. Cells were grown in a rotary shaker at 39^{o}C and 150 rpm under illumination (6 x 40W fluorescent tubes). 5% of this was used for further propagation. Cells were harvested after 70 hrs. by centrifugation at 3000 g for 30 min, washed once with basal salt medium (without peptone) and taken up in a similar medium prepared in D_2O to get 20% (w/v) cell suspension. For cell-free extract preparation the cells were suspended in 0.1 M tris-HCl buffer pH 7.2 in 1.2 M NaCl, sonicated at 20 KHz and 100W for 15 min at 30% duty cycle in pulsed mode using Branson Sonifier B 30 and centrifuged at 20,000 g for 30 min. The clear supernatent was used as cell-free extract.

NMR experiments were performed in a $^{1}H/^{13}C$ dual probe at 125 MHz on a Bruker AM 500 spectrometer interfaced with an Aspect 3000 computer. The spectra were recorded using a 90^{o} flip angle, with 25000 Hz spectral width and 8K data points corresponding to 0.15 s acquisition time and a digital resolution of 6.78 Hz/Pt. Powergated heteronuclear broadband decoupling of protons was used to minimize dielectric heating. 0.3W power was used during relaxation delay (3 s) and 2.2W for decoupling during acquisition. 200 transients were accumulated for each spectrum. An exponential multiplication leading to an additional linebroadening of 20 Hz was applied prior to Fourier transformation of the FIDs. Experiments were started by adding 10 mg of solid [1-^{13}C] glucose to 0.5 ml of cell suspension or extract in a 5 mm NMR tube maintained at 37^{o}C. The sample was spun at 20 Hz. Resonance assignments were done by adding different metabolites in a solution containing 25% NaCl in D_2O + H_2O and using corresponding peak positions. Sodium 3-trimethylsilyl (2,2,3,3-^{2}H) propionate was used as an external reference.

Glucose dehydrogenase activity was assayed in a mixture consisting of 0.1 M glucose, 0.1 mM NADP and the cell-free extract in a final volume of 1.0 ml in 0.1 M tris-HCl buffer pH 7.2 in 1.2 M NaCl. The reaction was started by adding the extract. NADPH production at 37^{o}C was monitored at 340 nm. A unit of enzme activity was defined as the amount which produces a µmole of NADPH in 10 min under conditions mentioned above. Protein was estimated by the method of Lowry et al (12) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Experiments on whole cells

Figure 1(a) shows ^{13}C NMR spectrum of *H. halobium* cells grown in glucose and incubated with [1-^{13}C] glucose. The peak at 182 ppm corresponds to the -COOH group of gluconate. Initially no other resonances are observed in this region. Also the signal intensity does not change with time. From these observations it is apparent that the first step, that is, conversion of glucose to gluconate is rapid. A steady state in metabolism is reached within

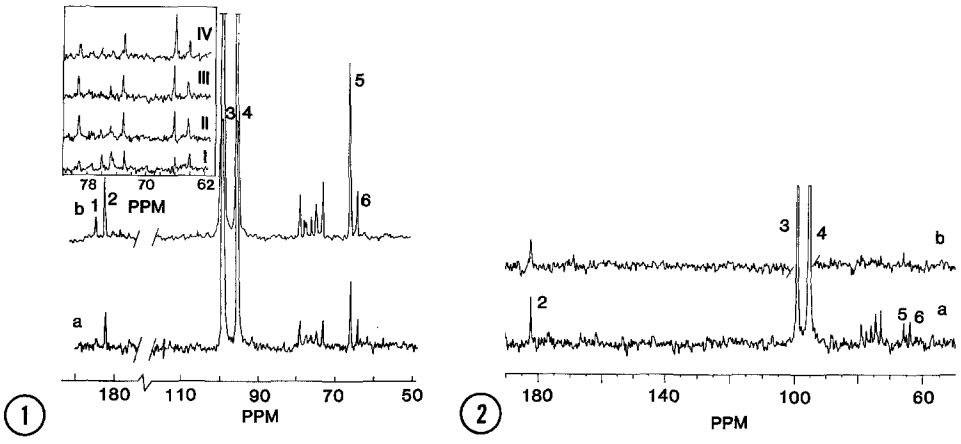

Fig. 1. ^{13}C NMR spectra of *Halobacterium halobium* cells grown in glucose and incubated with 10 mg [1-^{13}C] glucose. (a) Transients accumulated between 10 and 20 min, (b) accumulations obtained after 10 hours incubation (2000 transients). Inset - 60-80 ppm region of the spectrum (different set of experiments) accumulated between I 10-20, II 200-210, III 430-440 and IV 620-630 min. Resonance assignments are (1) [1-^{13}C] of α-keto acid(s), (2) [1-^{13}C] gluconate, (3) and (4) [1-^{13}C] glucose (α and β), (5) [6-^{13}C] fructose-6-phosphate and [6-^{13}C] fructose-1,6-diphosphate, (6) [1-^{13}C] fructose-6-phosphate and [6-^{13}C] glucose-6-phosphate.

Fig. 2. ^{13}C NMR spectra of crude extracts of *Halobacterium halobium* grown in a) glucose and b) galactose, and incubated with [1-^{13}C] glucose. Assignments are as in figure 1.

the first 5-10 min (time difference between addition of labelled glucose and acquisition of first spectrum). Prolonged incubation with [1-^{13}C] glucose gives rise to another signal at 185 ppm which arises from -COOH group of an α-keto acid (Fig 1b). This could either be pyruvate or/and some other α-keto derivative(s) of gluconate. We therefore believe that *H. halobium*, like several other halophilic archaebacteria, initiates glucose metabolism by oxidation of the anomeric carbon followed by Entner-Doudoroff pathway or a modification theroff directly leading to [1-^{13}C] pyruvate generation.

Another feature of the spectrum (Fig 1a and b) is the large number of resonances between 60-80 ppm which usually emanate from ^{13}C directly linked to hydroxyl groups. Some of these peaks could be assigned based on the chemical shifts of possible glycolytic intermediates. For example, the signals at 64.1 and 66.2 ppm represent fructose-6-phosphate C-1 and C-6 respectively. The former overlaps with glucose-6-phosphate C-6 and the latter with fructose-1,6-diphosphate C-6. Signals between 70-80 ppm, though arising from C-3 and C-4 carbons, could not be assigned unambiguously. While the glucose-6-phosphate C-6 + fructose-6-phosphate C-1 (64.1 ppm) resonance does not change, the fructose-6-phosphate C-6 + fructose-1,6-diphosphate C-6 signal

(66.2 ppm) intensity increases with time (inset fig 1). The appearence of these resonances simultaneously with that of gluconate can be reconciled with the assumption that the hexose part of glycolysis operates in parallel with the Entner-Doudoroff route. Moreover, the observation of glucose-6-phosphate C-6, frucotse-6-phosphate C-6 and fructose-1,6-diphosphate C-6 implies scrambling of the label at triosephosphate isomerase stage and reversal of glycolysis. It may be mentioned here that phosphoglucose isomerase, fructose-1,6-diphosphate aldolase and fructose-1,6-diphosphatase and activities of most of the other enzymes involved in triosephosphate part of glycolysis, but not hexokinase and phosphofructokinase, have been demonstrated in *H. halobium* grown in medium containing salts-peptone-citrate (10). Crude extract from *H. saccharovorum* has been shown to contain a hexokinase activity (8). Our results indirectly indicate the presence of all the enzymes involved in the hexose part of glycolysis. In addition reversal of glycolysis suggests the existence of fructose-1,6-diphosphatase. However, signal indicative of [3-^{13}C] pyruvate, which should be the end product of glycolysis starting from [1-^{13}C] glucose, is not observed. Therefore the only function of these enzymes might be the reversal of glycolysis as has been suggested earlier (10). The hexose intermediates corresponding to a reversed glycolysis, with [1-^{13}C] pyruvate originating from Entner-Doudoroff pathway, are expected to be labelled at 3 and/or 4 C positions and these would resonate between 70-80 ppm. The presence of some signals in this region (Fig. 1) indicates the possibility of such an alternative. In galactose grown cells incubated with [1-^{13}C] glucose, signals of gluconate or glycolytic intermediates are not detectible. Hence we infer that no glucose uptake occurs in these cells.

Experiments on cell extracts

Spectra of crude extracts of cells grown in presence of glucose or galactose and incubated with [1-^{13}C] glucose exhibit a -COOH signal at 182 ppm (Fig. 2a and b). An NADP dependent glucose dehydrogenase activity has been detected in both the cases. The activity in galactose grown cells is 50-80% of that of cells grown in glucose. Different cross acivities with glucose or galactose as substrate are observed (Table 1). The specific activity with galactose is only 27% of the activity with glucose in glucose grown cells whereas the enzyme from galactose grown cells is 110% more active with glucose than with galactose as substrate. Regardless of the source (glucose or galactose grown cells) or the substrate used, very little activity is observed when NAD is used as coenzyme. The galactose based $NADP^+$ reduction in extracts of galactose grown cells observed in our experiments is distinct from the galactose dependent reduction of dichlorophenolindolphenol activity in particulate fraction in *H. saccharovorum* (13).

Table 1. NADP reduction activities (μmoles/mg protein/10 min) in crude extracts of *H. halobium* grown in presence of glucose or galactose

Cells grown in	Substrate	
	Glucose	Galactose
Glucose	85.4	23.0
Galactose	39.1	35.7

CONCLUSION

Intact cell NMR is a non-destructive technique which circumvents the problems associated with enzyme stability in an *in vitro* milieu, especially so in the case of halophilic organisms. The present study has provided evidence for the existence of Entner-Doudoroff and glycolytic enzymes in *H. halobium* cells grown in presence of glucose. Indications are that a reversed glycolysis is also operative in these cells. Whole cells grown in galactose do not metabolize glucose even though the extract of these cells exhibit glucose dependent NADP reduction. At present, attempts are being made to characterize the glucose dehydrogenase and galactose or glucose dependent NADP reduction activities. NMR experiments are being carried out on whole cells to further elaborate the role of glycolytic part of metabolism in *H. halobium.*

ACKNOWLEDGMENT

The help provided by 500 MHz FTNMR National Facility supported by the Department of Science and Technology and located at T.I.F.R., Bombay is gratefully acknowledged.

REFERENCES

1. Gonzalez, C., Gutierrez, C., and Ramirez, C. (1978) Canad. J. Microbiol. 24, 710-715.
2. Rodriguez-Valera, F., Juez, G., and Kushner, D. J. (1983) Syst. Appl. Microbiol. 4, 369-381.
3. Mullakhanbhai, M. F. and Larsen, H. (1975) Arch. Microbiol. 104, 207-214.
4. Ginzburg, M., Sachs, L. and Ginzberg, B. Z. (1970) J. Gen. Physiol. 55, 187-207.
5. Tomlinson, G. A. and Hochstein, L. I. (1976) Canad. J. Microbiol. 22, 587-591.
6. Tomlinson, G. A. and Hochstein, L. I. (1972) Canad. J. Microbiol. 18, 698-701.
7. Aitken, D. M. and Brown, A. D. (1969) Biochim. Biophys. Acta 177, 351-354.
8. Tomlinson, G. A., Koch, T. K. and Hochstein, L. I. (1974) Canad. J. Microbiol. 20, 1085-1091.
9. Gochnauer, M. B. and Kushner, D. J. (1969) Canad. J. Microbiol. 15, 1157-1165.
10. D'Souza, S. E. and Altekar, W. (1983) Ind. J. Biochem. Biophys. 20, 29-32.
11. Oesterhelt, D. and Stoekenius, W. (1974) Mets. Enzymol. 31, 667-678.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
13. Hochstein, L. I. (1978) In Energetics and Structure of Halophilic Microorganisms (S. R. Caplan and M. Ginzburg Eds.) pp 397-414, Elsevier/ North-Holland Biomed. Press.