JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 202-206

Vol. 129, No. 1
Printed in U.S.A.

Carboxylation of Phosphoenolpyruvate by Extracts of *Neisseria gonorrhoeae*

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Received for publication 16 March 1976

The enzymatic carboxylation of phosphoenolpyruvate by cell-free extracts of *Neisseria gonorrhoeae* was examined and determined to be similar to the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC). This was shown by the irreversibility of the reaction and nucleotide independency. The enzyme was found to have some characteristics different from the other bacterial PEPCs reported. The enzyme showed catalytic activity in the presence of cobalt ions as well as magnesium and manganese ions, was not inhibited by succinate in fresh extracts, and displayed a low Michaelis constant for bicarbonate (0.27 mM), as compared with other PEPCs. The significance of this low Michaelis constant is discussed with respect to the growth of the organism and the importance of this enzyme to protein and nucleic acid synthesis.

It is generally considered that an increased carbon dioxide tension is necessary for the growth of *Neisseria gonorrhoeae* on primary isolation. However, recent studies in our laboratory (21) have demonstrated that the gaseous carbon dioxide requirement can be replaced by the addition of sodium bicarbonate at a concentration that is too low to significantly increase the atmospheric carbon dioxide level, even if a 100% bicarbonate conversion to carbon dioxide was obtained. Also, in this study, it was shown that the majority of the radioactive bicarbonate assimilated by growing cells was incorporated into nucleic acid and protein fractions.

In an attempt to discover why *N. gonorrhoeae* requires supplemental CO_2 or HCO_3, a study of the carboxylation of phosphoenolpyruvate (PEP) purified from cell-free extracts of *N. gonorrhoeae* was initiated. The precursors of both nucleic acid and protein synthesis could be provided by this physiologically significant reaction.

MATERIALS AND METHODS

Reagents. All reagents were commercial preparations: oxalacetic acid (OAA), inosine triphosphate (ITP), and malate (Nutritional Biochemicals Corp.); PEP (tricyclohexylammonium salt) and aspartate (Grand Island Biological Co.); pyruvate, adenosine triphosphate, fumarate, and succinate (Biochemical Laboratories, Inc.); guanosine diphosphate (GDP), adenosine diphosphate (ADP), and inosine diphosphate (IDP) (Sigma Chemical Co.); and $NaH^{14}CO_3$ (New England Nuclear Corp.).

Growth techniques. Clinical specimens of *N. gonorrhoeae* were obtained from Lubbock City-County Health Unit, Lubbock, Tex., and cultured on Thayer-Martin medium. Liquid growth medium was made according to the procedure outlined by Talley and Baugh (21). A closed 2-liter Erlenmeyer flask containing 1 liter of medium was inoculated with a suspension of the organism, and incubated for 16 h in a New Brunswick controlled-environment incubator-shaker set at 37°C and 200 rpm. Two hundred milliliters of this culture was added to five closed 2-liter flasks, containing 1 liter of medium and incubated as before. Each culture was checked again at this point for possible contamination.

Extract preparation. The cells were harvested by centrifugation at room temperature and washed once in phosphate buffer. The washed cells were resuspended in 0.1 M phosphate buffer (4°C) and treated with a Bronwill Biosonik 10-kc sonic oscillator with three 1-min periods of sonication with 5-min intervals between each. The suspension was then centrifuged at 2,500 rpm (1,600 × *g*) for 20 min, and the supernatant was decanted. The cell pellet was resuspended in phosphate buffer, and the sonication process was repeated.

Fifty milliliters of the extract was placed into a flask with a magnetic stirring bar, and 2% protamine sulfate solution was added dropwise until a final concentration of 0.5% was obtained. The precipitate was removed by centrifugation, and the supernatant was dialyzed with five 2-liter volumes of 0.1 M phosphate buffer over a period of 48 h. A saturated solution of ammonium sulfate was then added to the stirred dialyzed extract solution to give a final concentration of 30%. The extract was centrifuged and dialyzed again as previously described.

PEP carboxylation studies. Each reaction mixture for Tables 1 and 3 contained 0.3 ml of enzyme extract in 0.1 M phosphate buffer at pH 7.2, 5 μmol of bicarbonate containing 1 μCi of radioactive bicarbonate, the additions indicated, and distilled water to give a final volume of 1 ml in a closed test tube (15 by 100 mm). The final concentration of substrates,

TABLE 1. *Phosphoenolpyruvate carboxylase activity*

Additions	cpm/0.1 ml
Expt i	
None	11
PEP	75
PEP + Mg^{2+}	3,200
PEP + Mn^{2+}	822
PEP + Co^{2+}	1,259
PEP + Mg^{2+} + ADP	2,855
PEP + Mg^{2+} + GDP	3,302
PEP + Mg^{2+} + IDP	3,911
Expt ii	
OAA	11
OAA + Mg^{2+}	28
OAA + PP_i	20
OAA + Mg^{2+} + ITP	22
OAA + Mg^{2+} + ITP + PP_i[a]	6
OAA + Mg^{2+} + ATP	13
OAA + Mg^{2+} + ATP + PP_i	6

[a] PP_i, Inorganic pyrophosphate.

activators, and inhibitors in the reaction mixture was 10 μmol/ml except for the nucleoside di- and triphosphates, which were at a concentration of 5 μmol/ml. The reaction mixtures were incubated in a water bath at 25°C for 30 min. The test for the reversibility of the reactions were carried out according to the methods of Baugh et al. (2, 3).

The reaction mixtures for Table 3 contained 0.1 ml of enzyme extract, and 5 μmol of bicarbonate containing 1 μCi of radioactive bicarbonate, 40 μmol of magnesium, and 10 μmol of PEP, unless otherwise indicated in the column headings. Acetyl coenzyme A and fructose 1,6-diphosphate were added where indicated to give final concentrations of 0.2 and 4 μmol, respectively, in the 1-ml reaction volume.

The reactions were stopped by the addition of 0.1 ml of 12 N HCl. A portion (0.1 ml) was transferred to a scintillation vial and dried by a stream of air for 30 min to remove any residual radioactive bicarbonate. Two milliliters of Ready Solv Solution V (Beckman) was then added to each vial, and the radioactivity was measured with a Beckman LS-150 liquid scintillation counter.

Statistical methods. The data presented are an average of at least three trials. The data for Fig. 1 were analyzed for the best possible straight line by the method of least squares, and the correlation coefficient of these lines was determined.

RESULTS

Table 1 (experiment i) shows the results obtained when the carboxylation of PEP was tested in the presence of various divalent metal ions and nucleoside diphosphates. PEP was actively carboxylated in a divalent metal cation-dependent reaction. The highest enzymatic activity was obtained when the added cation was magnesium. The addition of cobalt and manganese also activated the enzyme, but the activity was significantly reduced from that obtained with magnesium. These results also indicate that the carboxylation reaction does not depend upon the presence of a nucleotide. At the concentrations tested, ADP and GDP had little, if any, effect on the carboxylation reaction. As shown in this experiment, IDP was slightly stimulatory in fresh extracts, but if the extract was stored at 4°C, it later became inhibitory at the concentrations tested.

Table 1 (experiment ii) shows the results obtained when the enzyme extract was tested for an exchange reaction. No radioactive carbon was assimilated when OAA was substituted for PEP. An exchange reaction did not occur with magnesium or any other divalent ions which were active in the carboxylation reaction, nor with the addition of ITP, ATP, inorganic pyrophosphate, or various combinations of these compounds. Concentrations of ADP between 5 and 25 μmol/ml resulted in a linear decrease in PEP carboxylation. A GDP and IDP concentration of 5 μmol/ml caused a 43 and 48% decrease in PEP carboxylation, respectively.

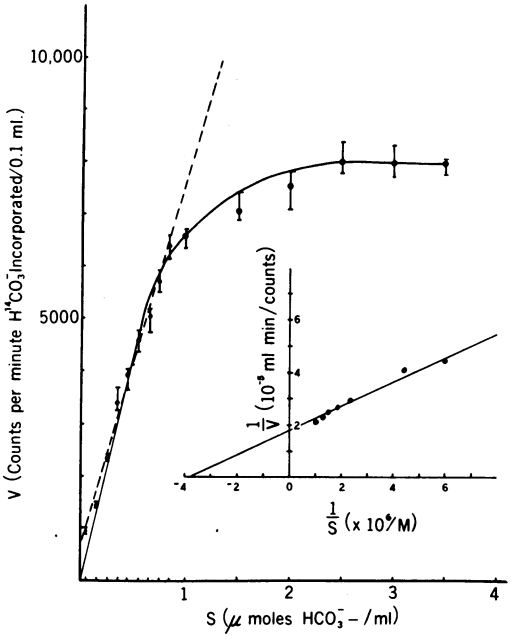

FIG. 1. *PEPC activity at various concentrations of bicarbonate. Reactions were run at 25°C at pH 7.2 in the presence of 10 μmol of PEP per ml and 40 μmol of magnesium per ml. The dashed line in the V versus S plot is the line derived from the data by the statistical method of least mean squares. The vertical error bars represent the high and low values of five experiments, and the point within the bars represents the arithmetic mean of these five values. The line of the Lineweaver-Burk plot was derived from the analysis of the reciprocal values of the V versus S plot by the method of least mean squares.*

The inhibition of the carboxylation of PEP by various tricarboxylic acid cycle intermediates and aspartate was also studied (Table 2). At the concentrations tested, malate, fumarate, and aspartate showed a 95% or greater inhibition of PEP carboxylation.

The effects of the activators acetyl coenzyme A and fructose 1,6-diphosphate are shown in Table 3. If bicarbonate was in low concentrations, acetyl coenzyme A and fructose 1,6-diphosphate increased the carboxylation of PEP fourfold; however, they failed to increase PEP carboxylation with low levels of PEP or magnesium. Even though at low levels of bicarbonate the carboxylation of PEP was increased, it did not reach the carboxylation level at saturated-substrate conditions.

When the effect of the bicarbonate concentration on PEP carboxylation was tested, the reaction displayed Michaelis-Menten kinetics. The greatest activity was obtained at bicarbonate concentrations above 1 μmol (Fig. 1) with considerable bicarbonate uptake at concentrations below 1 μmol. The Michaelis constant for bicarbonate determined by the method of Lineweaver-Burk (11) was 0.27 mM (Fig. 1). The Michaelis constants for PEP and Mg^{2+} were determined to be 4.36 mM and 1.16 mM, respectively.

Significant activity was demonstrated within the pH range of 6.8 to 7.5, with a definite optimum at pH 7.2. The reaction was also temperature dependent; the activity fell sharply as the temperature increased above the optimal temperature of 25°C.

TABLE 2. *Inhibition of phosphoenolpyruvate carboxylase*

Additions	cpm/0.1 ml
None	11
PEP + Mg^{2+}	3,200
PEP + Mg^{2+} + aspartate	65
PEP + Mg^{2+} + malate	100
PEP + Mg^{2+} + fumarate	133
PEP + Mg^{2+} + succinate	3,828

TABLE 3. *Effect of activators on PEP carboxylation*

Activator(s)[a]	cpm/0.1 ml with:			
	Low HCO_3^- (0.05 μmol)	Low PEP (3 μmol)	Low Mg^{2+} (1 μmol)	Saturated conditions
None	391	1,411	514	3,151
AcCoA	1,272	1,426	427	3,572
F1,6DP	1,208	1,560	446	3,200
AcCoA + F1,6DP	1,074	1,639	453	3,408

[a] AcCoA, Acetyl coenzyme A: F16DP, fructose 1,6-diphosphate.

DISCUSSION

The carboxylation of PEP by extracts of *N. gonorrhoeae* required only the presence of bicarbonate and a divalent metal cation. Since the reaction was shown to be irreversible and independent of a nucleoside diphosphate, the PEP carboxylating reaction present in *N. gonorrhoeae* is the same reaction catalyzed by PEP carboxylase (PEPC). The reversible, nucleotide-dependent carboxylation of PEP by PEP carboxykinase, and the reversible, orthophosphate-dependent carboxylation of PEP by PEP carboxytransphosphorylase, were shown not to occur in *N. gonorrhoeae*. In the studies reported by Holton et al. (8), only the activity for PEPC was assayed; the activity of PEP carboxykinase or PEP carboxytransphosphorylase was not checked by assaying for the reversibility of the reaction or the utilization of orthophosphate. Also, the extracts used in their experiments were not reported to have been dialyzed to remove contaminating nucleoside mono-, di-, and triphosphates. Without this precaution, it was not conclusive that any or all of the activity was due to PEPC since in the presence of nucleoside diphosphate PEP carboxykinase can also carboxylate PEP.

The linear decrease in the rate of PEP carboxylation in the presence of increasing concentrations of ADP suggests that ADP is being utilized as a phosphate acceptor, and that the decrease in the bicarbonate incorporated is due to the competition of PEPC with pyruvate kinase for the common substrate PEP.

The inhibition of PEPC in *N. gonorrhoeae* by malate, fumarate, and aspartate is similar to the inhibition of PEPC reported for *Escherichia coli* (6, 19), *Brevibacterium flavum* (12), *Ferrobacillus ferroxidans* (7), and *Salmonella typhimurium* (12–14). Large et al. (10) and Jyssum and Jyssum (9) showed that PEPC from *Pseudomonas* AM1 and *Neisseria meningitidis*, respectively, was inhibited by high concentrations of phosphate ions. Under the conditions of our experiments, no inhibition by phosphate ions was noted for PEPC from *N. gonorrhoeae*.

The results of Table 3 indicate that the activators acetyl coenzyme A and fructose 1,6-diphosphate can increase the efficiency of PEP carboxylation at low bicarbonate concentrations (Table 3, column 1), but not at low PEP or Mg^{2+} concentrations (Table 3, columns 2 and 3). This is shown by the almost fourfold increase in bicarbonate incorporated at low bicarbonate concentrations with an activator present. There

was no increase in activity of PEPC with low PEP or Mg^{2+} in the presence of an activator. The level of carboxylation at saturated conditions is not reached at low levels of bicarbonate in the presence of an activator, because the amount incorporated is dependent upon the limited presence of bicarbonate. It should also be noted that the activators did not increase the level of carboxylation at higher substrate concentrations (Table 3, column 4). Therefore, the activators provide a mechanism of increasing the carboxylation of PEP if the bicarbonate concentration should be less than saturating conditions. This property of PEPC from *N. gonorrhoeae* is different from that of the PEPC from *E. coli* (6), in which the activator acetyl coenzyme A increased the plateau of saturation with respect to PEP. Canovas and Kornberg (4) and Maeba and Sanwal (14) have reported that acetyl coenzyme A lowers the K_m of PEPC from *E. coli* and *S. typhimurium*, respectively, for the substrate PEP. In *N. gonorrhoeae*, acetyl coenzyme A and fructose 1,6-diphosphate have a similar effect with respect to bicarbonate uptake and conversion to oxalacetic acid. It can be assumed that the activators are increasing the affinity of the enzyme for bicarbonate (or lowering the K_m for bicarbonate). This can be assumed because, if the activators affected the V_{max} of the reaction, there would be an increased incorporation of bicarbonate even at saturated conditions.

The Michaelis constant of the enzyme for bicarbonate reveals that the enzyme has a very great affinity for bicarbonate. This constant of 0.27 mM shows that the enzyme has greater affinity than the PEPC reported for other organisms: six times that of *E. coli* (1.75 mM) (18): five times that of *Thiobacillus thiooxidans* (1.3 mM [20]); and three times that of *S. typhimurium* (1.1 mM) (14). The Michaelis constants of the enzyme for PEP and Mg^{2+} (4.36 and 1.16 mM, respectively) were very similar to the PEPC of *E. coli* (5.5 and 0.98 mM, respectively) (4).

The proposal by Talley and Baugh (21) that *N. gonorrhoeae* was utilizing bicarbonate and not carbon dioxide is supported by the findings of Maruyama et al. (15) that bicarbonate and not carbon dioxide was the reactive substrate of PEPC. The K_m of the enzyme for bicarbonate would have to be low if the carboxylation of PEP is to be accomplished during the fermentation of glucose at 37°C when the acids of fermentation would decrease the pH of the environment and cause disfavorable conditions for the maintenance of bicarbonate in solution. However, during glucose metabolism, when the pH does decrease, the intermediate of glucose metabolism, fructose 1,6-diphosphate, would be present to activate the enzyme, so that even at low bicarbonate concentrations the carboxylation of PEP can occur. Morse et al. (16) have reported the presence of fructose 1,6-diphosphate during glucose metabolism by *N. gonorrhoeae*.

Since Morse et al. (16) have reported that *N. gonorrhoeae* preferentially utilizes glucose as a carbon source before any other substrate, the carboxylation of PEP would appear to be extremely important to the organism. This is the only known pathway *N. gonorrhoeae* can utilize for the synthesis of OAA, and for the synthesis of the aspartic acid family of amino acids and pyrimidines, during glucose metabolism, since the tricarboxylic acid cycle has been reported to be inactive during glucose metabolism.

The results from the kinetic experiments indicate that PEPC of *N. gonorrhoeae* can carboxylate PEP at extremely low concentrations of bicarbonate. Therefore, it would be very doubtful that the characteristics of this enzyme would be the cause for the requirement of an increased carbon dioxide or bicarbonate environment for the growth (of some strains) of *N. gonorrhoeae*. Other carboxylating reactions will have to be investigated before the actual cause of this requirement can be determined.

ACKNOWLEDGMENTS

We wish to thank Dan Dennison, Laboratory Director of the City-County Health Department Laboratory, Lubbock, Tex., for his technical assistance in the primary isolation and for the clinical specimens we obtained from his laboratory.

We also wish to especially thank D. W. Thayer, Texas Tech University, Lubbock, Tex., for providing chemicals and equipment necessary for our studies.

This research was supported by The Institute of Epidemiology and Infectious Diseases, and Office of the Dean, Graduate School, Texas Tech University.

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