INTERMEDIARY METABOLISM OF DIPLOCOCCUS GLYCINOPHILUS

II. Enzymes of the Acetate-Generating System

SIGRID M. KLEIN AND RICHARD D. SAGERS
Department of Bacteriology, Brigham Young University, Provo, Utah

Received for publication July 17, 1961

ABSTRACT

KLEIN, SIGRID M. (Brigham Young University, Provo, Utah) AND RICHARD D. SAGERS. Intermediary metabolism of Diplococcus glycino-philus. II. Enzymes of the acetate-generating system. J. Bacteriol. 83:121-128. 1962—The enzymatic steps in the conversion of glycine to acetate by Diplococcus glycino-philus were examined and the reaction rates of the individual steps compared with the maximal glycine fermentation rate by whole cells. Glycine is oxidatively cleaved to one-carbon units with the alpha carbon being transferred to tetrahydrofolate at the oxidation level of formaldehyde. The activated one-carbon unit is condensed with a second glycine molecule to form serine which is then deaminated to pyruvate. Pyruvate gives rise to acetyl units as acetyl coenzyme A and acetyl phosphate. The latter compound reacts with adenosine diphosphate to yield acetate and adenosine triphosphate, providing the main energy-yielding reaction in the fermentation. The enzyme systems discussed together with their specific activities (μmoles substrate acted upon per hr per mg protein) are as follows: serine aldolase, 9 to 12; serine dehydrase, 180; pyruvate-oxidizing and acetyl unit-generating system, 96; phosphotransacetylase, 96; acetokinase, 3,600. The high activity of the acetokinase system may tend to pull the entire reaction series in the direction of acetate and adenosine triphosphate generation. In all cases the reaction rates of the individual enzyme systems were equal to or significantly greater than the over-all glycine fermentation rate by whole cells (9 μmoles per hr per mg protein). If coupled together, these enzymes could account for the fermentation of glycine to acetate, CO₂, and ammonia and could account for the conversion of 2-labeled glycine to doubly-labeled acetate as demonstrated in previous tracer studies.

In a previous communication (Sagers and Gunsalus, 1961), the oxidative cleavage of glycine to one-carbon units by Diplococcus glycino-philus was described and co-factor requirements for this cleavage defined. A reaction sequence which would allow the fermentation of 2-labeled glycine to doubly labeled acetate was proposed.

The present communication presents evidence to substantiate that proposed pathway by defining the individual enzymatic steps of the glycine to acetate fermentation and establishing a reaction rate for each of the enzyme systems involved. The individual enzyme rates are compared with the maximum rate of glycine fermentation by whole cells.

MATERIALS AND METHODS

Cultivation of cells and preparation of cell-free extracts of D. glycino-philus as well as methods for the determination of ammonia, formaldehyde, pyruvate, and tetrahydrofolate compounds have been described previously (Sagers and Gunsalus, 1961).

The nitrogen content of whole cells was determined by micro-Kjeldahl methods using the indicator system described by Sobel, Mayer, and Gottfried (1944). Protein content of cell-free extracts was determined turbidimetrically by the trichloroacetic acid method of Stadman, Novelli, and Lipmann (1951), using crystalline bovine albumin as a protein standard.

Acetyl coenzyme A was determined as the heat- and acid-stable thioester (Stadman, 1952) by the ferric hydroxamate method of Lipmann and Tuttle (1945).

Acetyl phosphate was prepared from iso-

1 Although Bergey's Manual refers to this organism as Peptococcus glycino-philus, the terminology used in this paper will follow that used in paper I of this series (J. Bacteriol. 81:541-549, 1961).
propenyl acetate by the method of Stadtman and Lipmann (1950) and was determined colorimetrically by the ferric hydroxamate method of Lipmann and Tuttle (1945). Enzymatic reactions involving acetyl compounds were stopped by adding 1 ml of $2.5 \times 10^{-4}$ M neutral parachloromercuribenzoate. Corrections were made for nonenzymatic hydrolysis of acetyl phosphate.

Concentrations of adenosine diphosphate (ADP) solutions were estimated by light absorption at 260 μm (E = $15.4 \times 10^3$) using commercially available 99 to 100% sodium ADP.

**RESULTS AND DISCUSSION**

To define the reaction sequence in the conversion of glycine to acetate by *D. glycinophilus*, it would be desirable to demonstrate in cell-free extracts the activities of individual enzymes which, if coupled together, would account for the fermentation as carried out by whole cells. It would further be desirable to demonstrate for each of the individual enzymes a specific activity at least as great as the maximal whole-cell fermentation rate, since this over-all rate is determined by the slowest enzyme within the metabolic sequence.

As shown in Fig. 1, the maximal rate of glycine fermentation (indicated by the liberation of ammonia) by freshly grown, washed cells of *D. glycinophilus* is approximately 9 μmoles per hr per mg protein.

The initial steps in the conversion of glycine to acetate by cell-free extracts of *D. glycinophilus* have been described previously (Sagers and Gunsalus, 1961). These steps involve the oxidative cleavage of glycine to one-carbon units with the alpha carbon of glycine being transferred to tetrahydrofolate at the oxidation level of formaldehyde. The carboxyl carbon of glycine is released as CO$_2$.

The next step in the conversion of glycine to acetate by this organism is the condensation of the active one-carbon unit with a second molecule of glycine to form serine. This reaction is catalyzed by the enzyme serine aldolase. The reaction rate was measured by following the rate of formaldehyde disappearance or, when the serine aldolase was coupled to serine dehydrase, by following the rate of pyruvate and ammonia generation. Figure 2 indicates that with 0.03 M glycine plus increasing concentrations of formaldehyde the aldolase reaction proceeded in cell-free extracts at a rate approximating 9 to 12 μmoles per hr per mg protein. Concentrations of formaldehyde greater than 0.01 M caused distinct inhibition of the reaction.

The next step in the metabolic sequence is the deamination of serine to pyruvate by the
FIG. 2. Serine aldolase activity of Diplococcus glycinophilus extracts: Serine synthesis measured by coupling the system to serine dehydrase; increasing amounts of formaldehyde. The 1.0 ml reaction mixture contained in μmoles: KH₂PO₄ (pH 7.3), 20; tetrahydrofolate, 1; glycine, 30; pyridoxal phosphate, 0.5; Na₂S, 0.3; formaldehyde, 2 to 20 as indicated; crude extract (1 mg protein); 36 °C for 1 hr.

FIG. 3. Serine dehydrase: The rate of pyruvate formation from increasing amounts of L-serine by crude extracts of Diplococcus glycinophilus. The 1.0 ml reaction mixture contained in μmoles: KH₂PO₄ (pH 7.3), 100; L-serine, 20 to 300 as indicated; crude extract (0.33 mg protein); 36 °C for 10 min.
**FIG. 4.** Rate of pyruvate degradation with increasing amounts of substrate by crude extracts of *Diplococcus glycinophilus*. The 1.0 ml reaction mixture contained in μmoles: KHPO₄ (pH 7.3), 100; KH₂AsO₄, 40; thiamine diphosphate, 0.1; diphosphopyridine nucleotide, 0.1; coenzyme A, 0.2; triphenyltetrazolium, 30; pyruvate, 2 to 30 as indicated; crude extract (0.25 mg protein); 36 C for 20 min.

**FIG. 5.** Phosphotransacetylase. The rate of acyl-unit transfer from increasing amounts of acetyl phosphate and relationship to the concentration of coenzyme A, using crude extracts of *Diplococcus glycinophilus*. The 1.0 ml reaction mixture contained in μmoles: KHPO₄ (pH 7.3), 100; KH₂AsO₄, 40; coenzyme A (CoA), 0.02, 0.1, 0.2 as indicated; acetyl phosphate, 2 to 20 as indicated; crude extract (0.53 mg protein); 36 C for 10 min.

Enzyme serine dehydrase. Figure 3 shows an approximate saturation curve for L-serine and indicates that pyruvate can be formed at a rate approximating 180 μmoles per hr per mg protein, a rate well above the glycine fermentation rate by whole cells.

The next conversion in the sequence forms acetyl units from pyruvate. This conversion...
FiguRe 6. Acetokinase. Saturation curves for adenosine diphosphate, acetyl phosphate, and magnesium chloride. The 1.0 ml reaction mixtures contained 40 μmoles of each of the two substrates held constant, and from 0 to 40 μmoles of the third substrate whose saturation curve was being determined. Crude extract (0.05 mg protein); incubated at pH 7.3, 36°C for 5 min.

FiguRe 7. Pathway of glycine fermentation in Diplococcus glycinophilus. Folate-H₄ (5,6,7,8-tetrahydrofolate); DPN (diphosphopyridine nucleotide); B₆ (pyridoxal phosphate); CoA (coenzyme A); ADP and ATP (adenosine di- and triphosphates, respectively).

probably involves a number of steps. When cell extracts were supplied with pyruvate, catalytic amounts of coenzyme A (CoA), a redox dye (triphenyltetrazolium), and potassium arsenate, pyruvate rapidly disappeared (Fig. 4). In the absence of CoA, pyruvate was not metabolized. In the absence of the dye or in the absence of arsenate, the rate of pyruvate utilization was reduced approximately 90%. As determined in these experiments the maximal rate of pyruvate utilization was approximately 96 μmoles per hr per mg protein, again a rate well above the rate of glycine fermentation by whole cells.

The ability of extracts to carry out the transfer of acetyl units between CoA and phosphate by the enzyme phosphotransacetylase is shown in Fig. 5. In this assay system, acetyl phosphate transfers its acetyl group to CoA. In the reverse direction, when arsenate is substituted for phosphate, acetyl CoA transfers its acyl group to arsenate. The arsenate compound immediately decomposes, leading to an over-all
disappearance of acetyl phosphate. Approximate saturation curves for acetyl phosphate with 0.02, 0.1, and 0.2 μmoles CoA per ml showed an increase in reaction rate with increased CoA. But 0.3 and 0.4 μmoles CoA gave no further increase in rate over 0.2 μmoles. Inhibition occurred with concentrations of acetyl phosphate above 15 μmoles per ml. The maximal observed rate for phosphotransacetylase activity was approximately 96 μmoles per hr per mg protein. Since this rate is the same as that observed for pyruvate disappearance, and since both systems employed the arsenolysis reaction, it is assumed that the limiting reaction in the utilization of pyruvate as described in Fig. 4 was the phosphotransacetylase system.

The final step in the conversion of glycine to acetate by extracts of D. glycinnophilus is the acetokinase reaction in which acetyl phosphate, together with adenosine diphosphate, yields acetate and adenosine triphosphate. The approximate saturation curves for adenosine diphosphate, acetyl phosphate, and magnesium ions shown in Fig. 6 indicate a maximal rate of approximately 3,600 μmoles acetyl phosphate disappearing per hr per mg protein. It is probable that the acetokinase system represents the major energy-yielding reaction in the conversion of glycine to acetate by D. glycinnophilus. The very high specific activity of this enzyme might tend to pull the entire reaction series in the direction of adenosine triphosphate and acetate formation.

From the data presented in this communication it is concluded that cell-free extracts of D. glycinnophilus contain enzymes which, if coupled together, would account for the fermentation of glycine to acetate, CO₂, and NH₃, as outlined in Fig. 7. This fermentation pathway could also account for the conversion of 2-labeled glycine to doubly-labeled acetate as demonstrated in tracer studies by Barker, Volcani, and Cardon (1948).

The fermentation pathway shown in Fig. 7 is similar to the acetate-generating pathway in Clostridium acidi-urici (Sagers, Benzman, and Gunsalus, 1961). X₁ and X₂ represent undefined intermediates in the conversion of pyruvate to acetyl CoA, possibly thiamine diphosphate and 6,8-dithiooctanoate derivatives. Extracts of D. glycinnophilus contain an active diphosphopyridine-linked dehydrogenase for 6,8-dithiooctanoic acid, and thiamine diphosphate stimulates pyruvate utilization under the conditions described in Fig. 4.

ACKNOWLEDGMENT

This work was supported by National Science Foundation grant 7478.

LITERATURE CITED


