AEROBIC DISSIMILATION OF GLUCOSE BY HETEROLACTIC BACTERIA

II. PHOSPHATE ACETYLTRANSFERASE OF *LEUCONOSTOC MESENEROIDES*

SHIGETAKA YASHIMA, KEIICHI KAWAI,
TOMOYUKI KAZAHAYA,
YOSHIRO OKAMI¹ AND YUJI SASAKI

*Department of Microbial Technology, Faculty
of Agriculture, Hokkaido University,
Sapporo, Hokkaido*

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Properties of phosphate acetyltransferase which was partially purified from *Leuconostoc mesenteroides* IFO 3426 were studied, and it was found that some properties of this enzyme are rather similar to those of the transferase from *Clostridium kluyveri* than that from *Escherichia coli* B, and it was also found that aeration did not significantly affect the activity of the transferase *in vivo*.

In the previous work, it was found that neither resting cells of *Lactobacillus brevis* and *L. buchneri* nor pre-aerated resting cells of *Leuconostoc mesenteroides* can dissimilate glucose under static condition, whereas, under aerobic conditions, they are able to produce lactate, acetate and significant amount of ethanol from glucose (1).

On the formation of ethanol or acetate by heterolactic bacteria, the metabolic pathway has been established (2–8; see next page).

In *Leuconostoc mesenteroides* IFO 3426 which is used in this study, acetaldehyde dehydrogenase (acylating CoA) [EC 1.2.1.10], alcohol dehydrogenase [EC 1.1.1.1] and acetate kinase [EC 2.7.2.1] were found to be present (unpublished data), and properties of phosphate acetyltransferase [EC 2.3.1.8] will be described in this paper. Moreover, NADH₂ oxidase and NADPH₂ oxidase were partially purified from this organism, and basic characteristics of these oxidases were previously reported (9).

From the metabolic pathway and the results described above, the mech-

¹ Present address: Institute of Microbial Chemistry, Kamioosaki, Shinagawa-ku, Tokyo.

anism of aerobic formation of acetate seems likely to depend on the action of these oxidases which catalyze the oxidation of reduced pyridine nucleotides resulting in the increased formation of acetate with corresponding decrease in ethanol formation.

It is difficult, however, how to explain the relationship between the ability of aerobic ethanol formation and inability of anaerobic dissimilation of glucose. To elucidate the mechanism of this phenomenon, it is necessary to determine the properties of each enzyme concerned with the ethanol or acetate-forming system, and, especially, studies should be made on both phosphate acetyltransferase and acetate kinase which have reactivity towards acetyl phosphate being estimated as a metabolic intermediate for both acetate and ethanol formation.

Although the evidences for the existence of phosphate acetyltransferase in crude extracts of *L. brevis* (8) and *Leu. mesenteroides* (2), and properties of the transferase in various organisms have been reported (10–22), the details of the enzyme in heterolactic bacteria have not been determined. Present report concerns in the purification and properties of phosphate acetyltransferase in *Leu. mesenteroides* IFO 3426.

MATERIALS AND METHODS

Organism. *Leu. mesenteroides* IFO 3426 was used throughout this study and procedures for the preparation of washed cells were previously reported (9). Aerobic growth of this organism was carried out in a 500-ml Sakaguchi flask containing 100 ml of medium on a reciprocal shaker operating at 122 strokes per min (5.5 cm) at 30°.

Methods. Unless otherwise specified, activity of phosphate acetyltransferase was determined by measuring the rate of decomposition of acetyl phosphate in the presence of CoA and arsenate according to the arsenolytic

method of STADTMAN (23) with omission of cysteine. The reaction mixture contains, in a final volume of 0.45 ml, 5 μ moles of Tris-HCl buffer (pH 8.0), 3 μ moles of acetyl phosphate (Li-K salt), 8 m μ moles of CoA and 0.2 to 0.4 unit of the enzyme. After preincubation of the reaction mixture at 30° for 5 min, the reaction was started by the addition of 50 μ l of 5×10^{-1} M potassium arsenate (pH 8.0), and performed for 10 min at 30°. Then the residual acetyl phosphate was determined as follows: 1 ml of neutralized hydroxylamine was added, and the mixture was allowed to stand for 10 min at room temperature, followed by the addition of 1.5 ml of FeCl₃ solution. Thus, color developed was measured at the wavelength of 540 m μ in a spectrophotometer.

One unit of enzyme was defined as the amount of enzyme catalyzing the arsenolysis of 1 μ mole of acetyl phosphate per min, and specific activity was defined as units of enzyme per mg of protein. Protein was determined according to the method of LOWRY *et al.* (24).

The preparation and estimation of acyl phosphates were performed by the method of STADTMAN (25). The purities of these compounds were also tested by paper chromatography (26) and it was confirmed that each compound was detected as a single spot.

Materials. Acetyl phosphate and acetyl CoA were purchased from Boehringer & Soehne GmbH. CoA was obtained from Takeda Chemical Ind., Ltd. NADP and NADPH₂ were from Sigma Chemical Co., Ltd. ATP, ADP, AMP, NAD, and NADH₂ were purchased from Kyowa Hakko Kogyo Co., Ltd. Other reagents used were of analytical grade.

RESULTS

Purification of phosphate acetyltransferase

Unless otherwise stated, all procedures were carried out at 0–5°. Washed cells obtained from a 3 liter-culture were suspended in 40 ml of 5×10^{-2} M phosphate buffer (pH 7.0) containing 10^{-3} M cysteine, subjected to sonic oscillation (20 kc) for 60 min, and centrifuged at $15,000 \times g$ for 20 min to remove intact cells and debris. To the cell-free extract thus obtained was added 5 ml of protamine sulfate solution (20 mg/ml: pH 5.0) and the mixture was stirred gently for 30 min. After centrifugation, the supernatant solution was diluted with distilled water to give a protein concentration of 3 mg per ml and fractionally precipitated at 48 and 60% saturation by adding solid ammonium sulfate. The 48 to 60% fraction was collected by centrifugation, dissolved in 15 ml of 5×10^{-2} M phosphate buffer (pH 7.0), and the solution was cooled by immersion in an ice-NaCl bath (–5°C). To this solution, with stirring, cold acetone (–20°C) was gently added until a concentration of 50 per cent (v/v) was reached, and, after centrifugation at $15,000 \times g$ for 5 min at –5°, the supernatant was adjusted to an acetone concentration of 60 per cent (v/v). The precipitate formed was collected by centrifugation as described above and

Table 1. Purification of phosphate acetyltransferase.

Fraction	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Cell-free extract	3.8	1.0	100
Protamine supernatant	9.7	2.5	94
Ammonium sulfate (46–60% saturation)	17.0	4.5	84
Acetone precipitate (50–60%)	50.0	13.3	22
DEAE-cellulose (0.3 M KCl)	155.0	41.3	11
Sephadex G-75	235.0	62.6	10

suspended in 10 ml of 5×10^{-3} M phosphate buffer (pH 7.0), and insoluble materials were removed by centrifugation. The clear supernatant solution thus obtained was applied to a column (1.3 $\times$ 5.6 cm) of DEAE-cellulose which was previously bufferized by 5×10^{-3} M phosphate (pH 7.0). The column was washed with 40 ml of 0.2 M KCl in 5×10^{-3} M phosphate buffer (pH 7.0), followed by elution with 0.3 M KCl in the same buffer at a rate of 0.5 ml per min. The enzyme was recovered in the eluates of tube Nos. 4–6 (6 ml/tube). To remove KCl from the enzyme solution gel-filtration method was employed as follows: 1 ml each of this solution was applied on a column (1 $\times$ 13 cm) of Sephadex G-75 using blue dextran and dinitrophenyl aspartate as indicators. The void volume fractions (blue dextran fraction) were combined and used as a purified preparation (63-fold increase in specific activity).

The whole procedure of purification is summarized in Table 1.

Properties of the phosphate acetyltransferase

Substrate specificity. As shown in Fig. 1, the decomposition of acetyl phosphate catalyzed by this transferase was completely dependent on CoA and arsenate indicating that the reaction may be occurred by arsenolysis of acetyl phosphate as well as the reaction catalyzed by the transferase from *Clostridium kluyveri* (10). The same dependencies were also observed when the crude extract was used as enzyme preparation, and it seems likely that the crude extract from *Leu. mesenteroides* contains CoA at very low concentration. It was confirmed that relative activities of the transferase for acetyl phosphate, propionyl phosphate, butyryl phosphate and valeryl phosphate were found to be 100, 108, 2 and 3, respectively, and that all these reactions also require the presence of CoA and arsenate under the standard assay conditions. When the initial rate of formation of thiol ester from CoA, in the presence of acetyl phosphate, propionyl phosphate, butyryl phosphate or valeryl phosphate, was estimated by measuring the increase of optical density at 232 m μ (23), the relative activity of this enzyme for these substrates was confirmed to be 100,

Fig. 1. Dependency on CoA and arsenate in the transferase reaction. Arsenolytic method was employed.

● Complete, ○ Complete minus CoA, arsenate or enzyme.

Fig. 2. Formation of thiol ester from CoA by the transferase. Reaction mixture contained 1.5 μ moles of acetyl phosphate, 25 μ moles of KCl, 10 μ moles of Tris-HCl buffer (pH 8.0) and 28 (A) or 14 (B) μ moles of CoA in a final volume of 1.0 ml. The reaction was started by addition of enzyme, and a control cuvette contained the same reactants with heat inactivated (100°C, 10 min) enzyme. At the indicated time (C), 100 μ moles of potassium phosphate were added. By calculation using a differential molar extinction coefficient of $4.5 \times 10^6 \text{ cm}^2$ per mole for thiol ester hydrolysis it was found that 95 (A) or 97 (B) % of theoretical value of thiol ester was formed, respectively.

Fig. 3. Decrease of acetyl CoA by the transferase. Reaction mixture contained 100 μ moles of potassium phosphate (pH 8.0), 10 μ moles of Tris-HCl buffer (pH 8.0), and 90 (A), 60 (B), 30 (C) or 12 (D) μ moles of acetyl CoA in a final volume of 1.0 ml. The reaction was started by addition of enzyme. Control cuvette contained all reactants without acetyl CoA. By calculation as described under Fig. 2, it was found that 93 (A), 97 (B), 100 (C) or 102 (D) % of acetyl CoA was hydrolyzed.

Fig. 4. Effect of pH on the transferase activity. Arsenolytic methods were employed at various pHs indicated.

Fig. 5. Effect of temperature on the transferase activity. Arsenolytic methods were employed at indicated temperatures. Reactions were carried out for 10 min (●), and 2 min (○) to prevent heat decomposition of acetyl phosphate.

Fig. 6. Effect of arsenate concentration on the transferase activity. Arsenolytic methods were employed at indicated concentrations of arsenate. Potassium chloride was added to bring all samples to 0.1M with respect to potassium ion.

Fig. 7. Effect of inorganic phosphate concentration on the transferase activity. Arsenolytic methods were employed at various concentrations of inorganic phosphate.

98, 4 or 8, respectively. These values seem to be in fairly good agreement with those obtained by arsenolytic method.

Reversibility of reaction. As shown in Fig. 2, from CoA and excess amount of acetyl phosphate, quantitative accumulation of thiol ester was estimated under the condition indicated. The subsequent decomposition of this thiol ester upon the addition of large amount of potassium phosphate led us to estimate this reaction may be reversible. Moreover, it was also demonstrated that, in the presence of excess amount of potassium phosphate, thiol ester of acetyl CoA was completely decomposed as shown in Fig. 3. From these results, it was concluded that the transferase may be able to catalyze both forward and reverse reactions.

Effect of pH and temperature. The most rapid initial rate of decrease in acetyl phosphate occurred at pH 8.1 as shown in Fig. 4.

Figure 5 shows the reaction rate of acetyl phosphate degradation as a function of temperature.

K_m values. All K_m values were calculated from the double reciprocal plots of initial rate of the reaction according to the method of LINEWEAVER and BURK (27). In the standard assay conditions, K_m for CoA was estimated as 2.5×10^{-5} M, and it was found that the apparent rate of arsenolysis of acetyl phosphate is directly proportional to the concentration of CoA within the range of 0.5 to 5 μ moles per 0.5 ml indicating that this enzyme is useful for microdetermination of CoA. The same proportionality was also found

when acetone fraction of this enzyme was substituted for the purified preparation. Value of K_m for arsenate was calculated to be 2.5×10^{-2} M by extrapolation of plots obtained at low concentrations, because high concentration of arsenate inhibited the reaction as shown in Fig. 6, and K_m for acetyl phosphate was determined as 2.8×10^{-3} M. K_m value for acetyl CoA was estimated from the initial rate of thiol ester degradation of acetyl CoA in the reaction system as described in Fig. 3, and it was calculated to be 1.8×10^{-5} M.

Inhibition and stimulation. Recently, it was reported that purified phosphate acetyltransferase from *Escherichia coli* B was inhibited by ADP, ATP and NADH_2 with K_i values of 3.3×10^{-4} M, 8.0×10^{-4} M and 6.6×10^{-5} M, respectively, and stimulated by pyruvate, whereas these compounds did not affect on the activity of phosphate acetyltransferase from *C. kluyveri* (22). The remarkable effects of these compounds on the transferase activity from *E. coli* B are very interesting with respect to the metabolic regulation. It was found, however, that 2×10^{-4} M of NADH_2 , NADPH_2 , NADP or NAD , and 1.0×10^{-3} M of ATP, ADP, AMP, pyruvate, acetaldehyde or ethanol showed no significant effect on the transferase from *Leu. mesenteroides* under the standard assay conditions indicating that the transferase of this organism is rather similar to that from *C. kluyveri* than from *E. coli* B.

In the presence of 10^{-3} M LiCl , NaCl or CuSO_4 , and 10^{-4} M *p*-chloromercuribenzoate or moniodoacetate, 17, 10, 32, 63, or 49% inhibitions were observed, respectively.

From the mechanism of arsenolysis of acetyl phosphate (10), it was expected that inorganic phosphate should act as a competitive inhibitor of the arsenolytic reaction, however, inorganic phosphate did not show the competitive inhibition for the reaction as shown in Fig. 7. The explanation for unexpected behavior of inorganic phosphate should be established in future. It was also found that, at a concentration of 10^{-2} M, cysteine inhibited the reaction slightly (17%) and, at 10^{-3} M, it showed no effect on the reaction.

Effect of oxygen. As previously reported (1), the resting cells of this organism lost the ability of anaerobic dissimilation of glucose by the pre-aeration for 90 min before addition of glucose, and it was also found that the resting cells prepared from aerobic culture, without pre-aeration, did not show their ability of glucose dissimilation under static conditions. To examine the effect of aeration on activity of enzyme, specific activities in crude extract prepared from resting cells pre-aerated or grown with aeration were determined, and it was concluded that pre-aeration for 90 min did not affect the activity of this enzyme and the cells after growth with aeration for 13 hr maintained one half of the activity as cells grown without aeration for the same interval.

Other properties. Activities of both aldehyde dehydrogenase (acylating CoA) and alcohol dehydrogenase of this organism were detected in precipitate fraction of $105,000 \times g$ for 2 hr (unpublished data), whereas the phosphate

acetyltransferase activity was quantitatively recovered in $105,000 \times g$ supernatant.

When the preparation of this transferase was kept at -20° , the activity did not decrease at least for one month.

DISCUSSION

As described in this paper, basic characteristics of phosphate acetyltransferase from *Leu. mesenteroides* IFO 3426 are similar to those of *C. kluyveri*.

On the mechanism of loss of anaerobic dissimilation of glucose, it may be assumed as one of possibilities that pre-aeration of resting cells or aeration during the growth is the cause of inactivation or repression of this enzyme. It is inconvenient, however, for this assumption that activity of the transferase was not affected by aeration *in vivo*. In the same standpoint, studies should be done on aldehyde dehydrogenase and alcohol dehydrogenase with special respect to the sensitivities of these dehydrogenases for aeration.

In elucidating the mechanism of aerobic ethanol formation, properties of this transferase should be reconsidered, in relation to aerobic metabolism of acetyl phosphate, when the basic characteristics of acetate kinase of this organism were clarified, and results obtained will be published in a succeeding paper.

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