AEROBIC DISSIMILATION OF GLUCOSE BY HETEROLACTIC BACTERIA

III. ALDEHYDE DEHYDROGENASE AND ALCOHOL DEHYDROGENASE OF *LEUCONOSTOC MESAENTEROIDES*

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(Received October 16, 1971)

Properties of CoA-dependent aldehyde dehydrogenase and NAD-linked alcohol dehydrogenase of *Leuconostoc mesenteroides* IFO 3426 were studied, and it was found that the two dehydrogenases cannot be separated from each other, as well as from those of *Escherichia coli* B, by various methods of enzyme fractionation. It was also observed that the dehydrogenases of *Leuconostoc mesenteroides* appeared in a precipitate fraction prepared by centrifugation at $105,000\times g$, and estimated to be associated with intracellular particles containing RNA.

The metabolic pathway of ethanol production from acetyl phosphate in heterolactic fermentation has been believed to be; acetyl phosphate→acetyl-CoA→acetaldehyde→ethanol (1). Although the properties of phosphate acetyl-transferase [EC 2.3.1.8] (1-4) or alcohol dehydrogenase (ADH) [EC 1.1.1.1; 1.1.1.2] (2, 5-7) isolated from heterolactic bacteria have been reported, the existence of acetaldehyde dehydrogenase (ALDH) [EC 1.2.1.10] has not yet been recognized.

To clarify the mechanism of aerobic glucose dissimilation by heterolactic bacteria, on which some unexpected phenomena were reported, studies on basic characteristics of ALDH may be considered to be essential (3). During the course of studies on the purification of ALDH of *Leuconostoc mesenteroides* IFO 3426, we found that ALDH and ADH may be considered as enzymes associated with the same subcellular organelle.

The present paper concerns the partial purification and properties of these

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dehydrogenases in the organism which was recognized to be a typical hetero-lactic coccus.

MATERIALS AND METHODS

Organism. *Leu. mesenteroides* IFO 3426 was used throughout this study and procedures for cultivation and preparation of washed cells were the same as previously reported (9).

Methods. Determination of activities of ALDH was carried out as follows: After incubation (30°, 5 min) of a mixture containing 200 μ moles of dipotassium phosphate (pH 8.0), 5 μ moles of reduced glutathione, 0.5 μ mole of NAD, 12.5 m μ moles of CoA, enzyme (0.003 to 0.006 unit) and deionized water to bring a total volume of 975 μ l, the reaction was started by the addition of 25 μ l of 1 M acetaldehyde. The initial rate of formation of NADH₂ was calculated from the increase of optical density at 340 m μ . For the estimation of ADH activities, 975 μ l of a reaction mixture containing 30 μ moles of potassium phosphate buffer (pH 7.0), 0.1 μ mole of NADH₂ and the enzyme (0.01 to 0.02 unit) was held at 30° for 5 min, followed by the addition of 25 μ l of 50 mM acetaldehyde to start the reaction. From the initial rate of decrease in optical density at 340 m μ , the activity of ADH was estimated. NADH₂ oxidase, NADPH₂ oxidase, NADH₂ peroxidase or NADPH₂ peroxidase activity was assayed by the method reported previously (9). Activities of lactate dehydrogenase and acetate kinase were determined according to the methods of DENNIS and KAPLAN (10) and ROSE *et al.* (11), respectively. The activity of phosphate acetyltransferase was estimated as previously reported (3). All determinations of enzymic activities were made in quartz cuvettes with 1-cm light path, using Hitachi Model 124 spectrophotometer equipped with an automatic recorder, except in the case of phosphate acetyltransferase and acetate kinase. One unit of enzyme activity was defined as the amount of enzyme causing change in one μ mole of substrate per min, and specific activity was defined as units per mg of protein.

Concentration of protein or acetaldehyde was estimated by the method of LOWRY *et al.* (12) or BARKER and SUMMERSON (13), respectively. When samples contained high concentrations of cysteine, they were dialyzed overnight before the determinations of protein. Ultracentrifugation of the enzyme solution was carried out in a Hitachi-65P ultracentrifuge at 5°.

Materials. Acetyl-CoA and acetyl phosphate were purchased from Boeringer and Soehne GmbH. NADP, NADPH₂ and RNase T₁ were obtained from Sigma Chemical Co., Ltd. NAD, NADH₂, GMP, GDP, ATP, ADP, AMP and guanosine were purchased from Kyowa Hakko Co., Ltd. CoA and Chromagel A-2 were obtained from Takeda Chem. Ind. Co., Ltd., and Wako Pure Chemical Co., Ltd, respectively. Other reagents were of analytical grade.

RESULTS

In preliminary experiments, it was found that activities of both ALDH and ADH were always detected in the same fractions obtained by fractionation with ammonium sulfate, streptomycin, protamine, acetone, ethanol, calcium phosphate gel, DEAE-cellulose column chromatography, Sephadex G-200 column chromatography, and ultracentrifugation. These results suggesting that two dehydrogenases may constitute a multienzyme complex or a complex associated with subcellular organelle led us to prepare the enzyme fraction including both the enzymes, but not unfavorable enzymes, *i.e.*, NAD(P)H₂ oxidase or phosphate transacetylase which may interfere in the measurement of activities of ALDH and ADH.

Purification of ALDH and ADH

All operations of enzyme purification were performed at 5° and centrifugation was carried out at $15,000 \times g$ for 20 min, unless otherwise stated.

Washed cells obtained from a 5-litre culture were suspended in 25 ml of 5×10^{-2} M potassium phosphate buffer (pH 7.0) containing 0.1 M cysteine, exposed to sonic oscillation (20 kc) for 60 min, and the supernatant (cell-free extract; 32 ml) was prepared by centrifugation. The supernatant solution thus obtained was diluted with the same buffer to give a protein concentration of 25 mg/ml, and added with 250 mg of streptomycin. After stirring for 5 min, precipitate formed was removed by centrifugation. To the supernatant solution (48 ml) thus obtained was gently added 37 ml of saturated ammonium sulfate solution (pH 7.0), and the mixture was stirred for 30 min. Then the mixture was centrifuged to remove the precipitate, and to the supernatant was added 31 ml of the same ammonium sulfate solution. After 30 min of stirring, the precipitate was collected by centrifugation and dissolved in 10 ml of 5×10^{-2} M potassium phosphate buffer containing 25 mM cysteine (pH 7.0). The solution thus prepared was found to contain the activities of ALDH, ADH, NADH₂ oxidase and phosphate acetyltransferase. To remove the oxidase and transferase, 9 ml of the enzyme solution was applied on a column (2.7 × 85 cm) of Sephadex G-200 and fractionated with the same buffer at a flow rate of 15 ml/hr. As shown in Fig. 1, the activities of ALDH and ADH appeared in void volume fractions with complete separation from NADH₂ oxidase and phosphate acetyltransferase. The fractions Nos. 13–17 (10 ml per fraction) were pooled and insoluble substances were collected by centrifugation at $105,000 \times g$ for 2 hr. The insoluble fraction was resuspended in the potassium phosphate buffer and washed twice by centrifugation under the same condition as described above. The washed insoluble material was suspended in the same buffer and this suspension was used as a partially purified preparation of ALDH and ADH. Activities of NADH₂ oxidase, NADPH₂ oxidase, lactate dehydrogenase, NADH₂ peroxidase, NADPH₂ peroxidase, phosphate acetyltransferase, and acetate kinase were not detectable in this purified preparation. Whole procedure of the purification is summarized in Table 1.

Fig. 1. Separation of ALDH and ADH from both NADH_2 oxidase and phosphate acetyltransferase by Sephadex G-200 column chromatography.

—●— ALDH, —×— ADH, —○— NADH_2 oxidase, —▲— phosphate acetyltransferase, —△— $\text{OD}_{280 \text{ m}\mu}$.

Table 1. Purification of ALDH and ADH.

Fraction	Specific activity (unit/mg protein)		Purification of ALDH (fold)	Yield of ALDH (%)
	ALDH	ADH		
Cell-free extract	0.12	—	1.0	100
Streptomycin supernatant	0.14	—	1.1	85
Ammonium sulfate (45–60% saturation)	0.27	—	2.1	79
Sephadex G-200 eluate	0.31	0.22	2.4	24
105,000 $\times g$ precipitate	0.87	0.32	6.6	23

Properties of ALDH

Requirement for enzyme reaction and substrate specificity. Fig. 2 shows the requirement of CoA, NAD and acetaldehyde for the forward reaction of ALDH, and it was found that NADP was unable to replace NAD under the standard assay conditions. It was also observed that activity of the enzyme decreased to 14% when reduced glutathione was omitted from the reaction mixture. As shown in Table 2, propionaldehyde, butyraldehyde or isobutyraldehyde was found to serve as a substrate as acetaldehyde did, indicating the broad substrate specificity of the enzyme.

Reversibility. When acetyl-CoA and NADH_2 were used as substrates, de-

Fig. 2. Requirements for ALDH reaction.

Complete reaction mixture contained 200 μ moles of potassium phosphate (pH 8.0), 0.5 μ mole of NAD, 12.5 m μ moles of CoA, 25 μ moles of acetaldehyde and enzyme in a final volume of 1 ml.

A: Complete. B: Complete minus reduced glutathione.

C: Complete minus enzyme, NAD, CoA or acetaldehyde.

Table 2. Substrate specificity of ALDH.

Substrate	Relative activity (%)
Formaldehyde	7
Acetaldehyde	100
Propionaldehyde	115
Butyraldehyde	31
Isobutyraldehyde	14
Benzaldehyde	0

crease of optical density at 232 m μ corresponding to the concentration of acetyl-CoA was detected as shown in Fig. 3, and it was suggested that thiol ester of acetyl-CoA may be cleaved by the action of ALDH in the presence of NADH₂. Moreover, it was recognized that optical density at 340 m μ also decreased during the reaction, and an equimolar relationship between decreases of acetyl-CoA and NADH₂ was calculated. From these results, ALDH of *Leu. mesenteroides* can also catalyze the backward reaction of the following equation:

Fig. 3. Decrease of acetyl-CoA by ALDH in the presence of NADH_2 .

Basal reaction mixture contained 200 μmoles of potassium phosphate buffer (pH 8.0) and 63.4 $\text{m}\mu\text{moles}$ of NADH_2 in a final volume of 1 ml. To A, B or C was added 93, 62 or 31 $\text{m}\mu\text{moles}$ of acetyl-CoA, respectively. The same reaction mixture without acetyl-CoA was used as a blank. The reaction was started by the addition of the enzyme solution. It was calculated that 88, 92 or 100% of acetyl-CoA was decreased in the case of A, B or C, respectively.

Effect of buffer and temperature. As shown in Fig. 4, a remarkable effect of concentration of buffer on the reaction was found and the reaction seemed to require a high ionic concentration. The reaction was completely inhibited when 0.2 M boric acid- Na_2CO_3 buffer (pH 8.0), glycine- NaOH buffer (pH 8.0) or NH_4Cl - NH_4OH buffer (pH 8.0) was used.

It was also found that optimum pH and temperature for the reaction are 8 and 30° , respectively. As shown in Fig. 5, ALDH was inactivated by a heat treatment at 45° for 15 min.

Km values. K_m values for CoA, NAD and acetaldehyde were estimated as 9×10^{-6} M, 1.7×10^{-4} M, and 3.7×10^{-3} M, respectively, from the Lineweaver and Burk's plots.

Effect of various compounds. In the presence of 10^{-3} or 10^{-4} M Na_2HAsO_4 , or 10^{-3} M of AgNO_3 , HgCl_2 or CuSO_4 , 100, 32, 20, 31 or 49% inhibition was observed, respectively, whereas, cations (K^+ , Na^+ , Li^+ , Mg^{2+} , Mn^{2+} , Zn^{2+} , Ca^{2+} , Fe^{2+} and Fe^{3+}), EDTA, *p*-chloromercuribenzoate, iodoacetate, sodium azide and dipyrindyl showed no inhibition at a concentration of 10^{-3} M. At the same concentration, lactic acid, acetic acid, ethanol, acetyl phosphate and pyruvic acid did not show any inhibitory effect on the reaction, as did 10^{-4} M of NADP or NADPH_2 . Although 10^{-3} M of adenine, adenosine, guanine, guanosine, GMP or GDP did not affect the reaction, the same concentration of AMP,

Fig. 4. Effect of phosphate or Tris-HCl buffer on ALDH reaction at different concentrations or pHs.

A: 0.2 M potassium phosphate (pH 8.0). B: 0.2 M potassium phosphate (pH 7.0). C: 0.2 M Tris-HCl (pH 8.0). D: 0.04 M potassium phosphate (pH 8.0). E: 0.04 M potassium phosphate (pH 7.0). F: 0.04 M Tris-HCl (pH 8.0).

Fig. 5. Thermostability of ALDH or ADH.

Purified enzyme preparation was treated for 15 min at temperatures indicated.

—●— ALDH, —○— ADH.

Fig. 6. Reversibility of the reaction catalyzed by ADH.

Reaction mixture contained 300 μ moles of ethanol, 1 μ mole of NAD, 500 μ moles of potassium phosphate buffer (pH 8.0) and enzyme in a final volume of 3.0 ml. The same mixture without enzyme was used as a blank. The reaction was started by the addition of enzyme, incubated for 60 min at 30° and 300 μ moles of acetaldehyde were added.

ADP or ATP showed respectively 48, 42 or 38% inhibition of the reaction and it was concluded that these substances seemed to inhibit the reaction competitively for NAD from kinetic analysis. The K_i values of AMP, ADP and ATP were calculated as 7.5×10^{-4} M, 2.5×10^{-3} M and 8.6×10^{-3} M, respectively.

Properties of ADH

Stoichiometry, reversibility and substrate specificity. After 60 min of the reaction with NAD and ethanol as substrates, the amount of NADH_2 or acetaldehyde formed was estimated from the molecular absorbcancy coefficient of NADH_2 at 340 m μ or color intensity at 560 m μ developed by the method of BARKER and SUMMERSON (13), respectively, and it was confirmed that 98.8 m μ moles of NADH_2 were produced with formation of 97.8 m μ moles of acetaldehyde. As shown in Fig. 6, NADH_2 thus formed was completely oxidized by the addition of excess amount of acetaldehyde indicating that this dehydrogenase may catalyze reversible reactions. Substrate specificities of the enzyme in the forward and backward reactions are summarized in Table 3, and it was also found that NADP did not function as a coenzyme in this reaction. From these results, the reaction catalyzed by ADH of *Leu. mesenteroides* IFO 3426 may be shown by the following equation:

Table 3. Substrate specificity of ADH.

Substrate	Relative activity (%)
Forward reaction ^a	
Methanol	0
Ethanol	100
Propanol	74
Isopropanol	0
Butanol	139
Isobutanol	0
Phenol	0
Backward reaction ^b	
Formaldehyde	7
Acetaldehyde	100
Propionaldehyde	104
Butyraldehyde	125
Isobutyraldehyde	22
Benzaldehyde	0

^a Each substrate was added at a final concentration of 0.1 M.

^b Each substrate was added at a final concentration of 5 mM.

Effect of pH and temperature. Optimum pH and temperature for the reaction were found to be 7.0 and 45°, respectively. The enzyme was inactivated by heat treatment at 50° for 15 min, as shown in Fig. 5.

Km values. Km values for acetaldehyde, NADH₂, ethanol and NAD were estimated as 6.9×10^{-4} M, 1.0×10^{-5} M, 1.4×10^{-3} M and 2.5×10^{-5} M, respectively.

Effect of various compounds. The reaction was not inhibited in the presence of 10^{-3} M of K⁺, Na⁺, NH₄⁺, Li⁺, Mg²⁺, EDTA, Na₂HAsO₄, iodoacetate, NaN₃ or dipyriddy and 10^{-4} M of Ca²⁺, Zn²⁺, Fe²⁺ or Fe³⁺. The reaction was, however, inhibited by 10^{-3} M *p*-chloromercuribenzoate, 10^{-4} M of CuSO₄ or AgSO₄ with 100, 35 or 16% inhibition, respectively. Although adenine, adenosine, ATP, guanine, guanosine, GMP and GDP did not inhibit the reaction at a concentration of 10^{-3} M, the same concentration of AMP and ADP showed 10 and 19% inhibition, respectively. It was also recognized that 10^{-3} M of lactic acid, acetic acid, ethanol, acetyl phosphate and pyruvic acid showed no inhibitory effect on the reaction.

Association of ALDH and ADH

As shown in Fig. 1, both ALDH and ADH were recovered in the void volume fractions of Sephadex G-200 column chromatography. This finding suggests a large molecular weight for these dehydrogenases. It was also mentioned earlier that activities of the enzymes could not be separated from

Fig. 7. Elution pattern of a Chromagel A-2 column.

—●— ALDH, —×— ADH, —○— OD_{280 mμ}.

Fig. 8. Gel-filtration of RNase-treated enzyme preparation through Chromagel A-2 column.

Purified enzyme preparation (5.0 unit) in 2 ml of phosphate buffer (pH 7.0) was treated with 1 mg of crystalline RNase T₁ at 30° for 60 min, then 0.5 ml of the reaction mixture was applied on a column of Chromagel A-2.

—●— ALDH, —×— ADH, —○— OD_{280 mμ}.

each other by various methods of enzyme fractionation. It seemed to be possible from the results described above that ALDH and ADH may exist as a multienzyme complex or enzymes associated with the same subcellular organelle *in vivo*. To assess this point, fractionation of these enzymes with a column (1.6×32 cm) of Chromagel A-2 was tested. Chromagel (agarose)

A-2 is usually used for separation of substances having 4–10 million molecular weight. Void volume (fraction Nos. 5 to 10 in Fig. 7) was estimated by using blue dextran as an indicator. The purified preparation of enzyme (0.5 ml) was applied on the column of Chromagel A-2 which had been bufferized with 10^{-2} M of potassium phosphate buffer (pH 8.0) and eluted with the same buffer at a flow rate of 10 ml/hr at 5°. As shown in Fig. 7, the activities of the two dehydrogenases were detected in void volume fractions and fraction Nos. 11 to 25 with a constant ratio of activity in each fraction. It was also found that ultraviolet absorption spectrum of the eluate in each fraction was similar to that of RNA, suggesting the possibility of association of ALDH and ADH with subcellular particles containing RNA. As shown in Fig. 8, the molecular weight of purified enzyme was confirmed to shift to a lower weight by treatment of the enzyme by RNase T₁ at 30° for 1 hr. When the enzyme preparation was treated without RNase T₁ under the same conditions, elution profile of the column chromatography was the same as shown in Fig. 7. From this fact, it seemed to be possible that artificial degradation of the associated enzyme did not occur under these treating conditions. The enzyme of lower molecular weight obtained by the RNase treatment and Chromagel A-2 column chromatography was found to be again emerged in void volume fraction of Sephadex G-200 column chromatography.

DISCUSSION

It has been reported that the reaction catalyzed by ALDH of *Clostridium kluyveri* requires the presence of SH-compound and high ionic strength, and that the reaction is inhibited by sodium arsenate (14). Inhibition by sodium arsenate has also been found on ALDH of *Pseudomonas fluorescens* (15), yeast (16) and beef liver (17). These enzymic properties seem likely to be similar to those of ALDH of *Leu. mesenteroides*.

With respect to ALDH and ADH of *Escherichia coli* B, it has been reported that activities of the dehydrogenases cannot be separated from each other by various fractionation methods (18). As mentioned in this paper, ALDH and ADH of *Leu. mesenteroides* could not be separated from each other, and the ratio of ALDH to ADH activity seemed to remain relatively constant in the enzyme fractions prepared by various methods of protein purification. Moreover, it seems that both dehydrogenases of the organism may be associated with the same intracellular particles containing RNA. As one of possibilities, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase may also be associated with an intracellular organelle to which both ALDH and ADH seem to bind, resulting in the efficient coupling of pyridine nucleotides which have been estimated to be reduced by the action of former two dehydrogenases and to be oxidized by latter dehydrogenases in anaerobic heterolactic fermentation. Although activities of NADH₂ oxidase and NADPH₂ oxidase were detected in the organism (9), it was previously reported that lactic

acid formation by this organism was hardly affected by oxygen, while, under aerobic conditions, ethanol production decreased with a corresponding increase in acetate production indicating the existence of some mechanism of specific protection of lactic acid-forming system against the effect of oxygen (8). It is very interesting that ALDH and ADH of the organism were detected as insoluble enzymes suggesting the possibility of different localization of lactic acid dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase from ALDH, ADH, NADH₂ oxidase or NADPH₂ oxidase.

Recently, it was reported that ADH of *Leuconostoc mesenteroides*, the strain number of which was unfortunately not mentioned, is composed of NAD-dependent and NADP-dependent enzymes which are tightly bound together, and molecular weight of the ADH was calculated to be 160,000 (7). With the strain of IFO 3426 used here, however, we found that partially purified NAD-linked ADH, which was found in the insoluble fraction of intracellular particles, does not contain NADP-linked ADH. Although faint activities of NADP-linked ADH were detected in the crude cell-free extracts of this strain, details were not determined in the present study. As previously reported, 6-phosphogluconate dehydrogenase and 2-oxogluconate 6-phosphate decarboxylase were detected as a bound enzyme in some strains, or free enzymes in other strains of heterolactic bacteria, and these facts indicated that there is a strain-specific localization of these enzymes in these bacteria (19–21). Further studies on the intracellular localization of ALDH and ADH in various strains of heterolactic bacteria may be very interesting with respect to physiological meaning of the relationship between the localization and function of these enzymes.

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