PARTICULATE NATURE OF ENZYMES INVOLVED IN THE FERMENTATION OF ETHANOL AND ACETATE BY *CLOSTRIDIUM KLUYVERI*

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Received 7 February 1972

1. Introduction

Clostridium kluyveri ferments ethanol and acetate to butyrate, caproate and H_2 [1]. The fermentation is initiated by the dehydrogenation of ethanol to acetylcoenzyme A via acetyldehyde [2]. It seems unlikely that the evolution of hydrogen, the hydrogenation of acetoacetyl-CoA to butyrate, and the dehydrogenation of ethanol occur in the same compartment. Therefore, the subcellular localization of enzymes involved in butyrate synthesis was investigated. We report here that alcohol dehydrogenase and acetaldehyde dehydrogenase reside in a particulate fraction indicating that dehydrogenation and hydrogenation reactions are spatially separated.

2. Methods

Acetoacetyl-CoA was prepared from diketene and CoA; acetyl-CoA, crotonyl-CoA and butyryl-CoA from the appropriate anhydrides, and coenzyme A by the method of Simon and Shemin [3]. β-Hydroxybutyryl-CoA was synthesized according to Wieland and Rueff [4]. Enzymes and coenzymes were purchased from Boehringer, Mannheim and deoxyribonuclease from Serva, Heidelberg.

Cl. kluyveri ATCC 8527 was grown according to Bornstein and Barker [5]. To prepare lysates 1 g of cell paste was suspended in 10 ml of 0.2 M Tris-HCl buffer pH 7.5 containing 4 mg of lysozyme (EC 3.2.1.17, 17 000 U/mg) and 1 mg of deoxyribonuclease (EC 3.1.4.5, 450 U/mg). The suspension was incubated in a water bath at 35° for 1 hr and then passed through a French press (pressure, 10–12 tons/cm^2). The lysate which was free of whole cells was centrifuged at 35 000 *g* for 20 min, the supernatant decanted and the pellet washed twice with 50 ml of 0.2 M Tris-HCl buffer pH 7.5. After suspending the pellet in 1.5 ml of the above buffer it was stored at 0°. Protein was determined by the methods of La Rivière [6] and of Lowry et al. [7].

Optical assays were carried out in 1-cm cuvettes at 25°. When enzyme activities of the supernatant were determined the reaction mixtures were freed of oxygen by passing purified nitrogen through the cuvette for 15 min. Controls without substrate and with $NADH_2$ were run simultaneously. Assays with membrane fractions could be carried out without these precautions since they did not contain $NADH_2$ oxidase. One unit of activity is defined as the amount of enzyme that catalyzes the consumption of 1 μmole of substrate per min.

The following assays were performed as described: phosphotransacetylase, EC 2.3.1.8 [8]; alcohol dehydrogenase, EC 1.1.1.1 [9]; NAD(P) acetaldehyde dehydrogenase, EC 1.2.1.10 [10]; thiolase, EC 2.3.1.9 [11]; NAD(P) β-hydroxybutyryl-CoA dehydrogenase, EC 1.1.1.35 [12]; butyryl-CoA dehydrogenase, EC 1.3.99.2 [13]; CoA transferase, EC 2.8.3.1 [14]; and hydrogenase, EC 1.12.1.1 [15]. Crotonase, EC 4.2.1.17 was assayed in a reaction mixture containing in a final volume of 1.5 ml: 60 mM sodium veronal buffer, pH 9.4; 0.4 mM NAD; 0.15 mM crotonyl-CoA; 13 μg NAD β-hydroxyacyl-CoA-DH from pig heart, and lysate fraction.

Table 1
Protein of lysate fractions of *Cl. kluyveri* and phosphotransacetylase activity.

Fraction	Protein				Phosphotransacetylase	
	Concentration (mg/ml)	Total (mg)	(%)	Specific Activity (U/mg)	Total (U)	(%)
Whole cells	17	150	99	–	–	–
Lysate	18	152	100	35	5300	100
Supernatant	12	104	60	49	5100	96
1st wash	0.4	6.3	4	23	145	3
2nd wash	0.2	3.0	2	7.5	22	0.4
Particulate fraction	16	24	16	0.15	4	0.08

Data represent mean values from 20 fractionations.

3. Results

Lysates of *Cl. kluyveri* were centrifuged and the pellet was washed twice with buffer to separate all soluble components from the particulate material. Table 1 gives the average protein content of the individual fractions and their phosphotransacetylase activity. Since phosphotransacetylase is present in *Cl. kluyveri* in high activity and solely from its physiological function can be expected to be soluble it was chosen as pilot enzyme for soluble proteins. It can be seen that the particulate fraction contained 16% of the total protein and that it was essentially free of phosphotransacetylase activity. Table 2 summarizes the distribution of enzymes of *Cl. kluyveri* in the supernatant and in the particulate fraction. Four enzyme activities were predominantly associated with the particulate fraction: alcohol dehydrogenase, NAD and NADP-linked acetaldehyde dehydrogenase and NAD β-hydroxybutyryl-CoA dehydrogenase. The supernatant contained a NADP specific β-hydroxybutyryl-CoA dehydrogenase possessing a 30-fold higher specific acitvity as the NAD dependent enzyme and the remaining enzymes necessary for the conversion of acetyl-CoA into butyrate. With the exception of hydrogenase the recovery of the soluble enzymes in the lysate-supernatant was above 92%. A considerable amount of the particulate enzymes was lost during fractionation (24–78%).

To confirm the association of certain dehydrogenases with particulate fractions, lysates were cen-

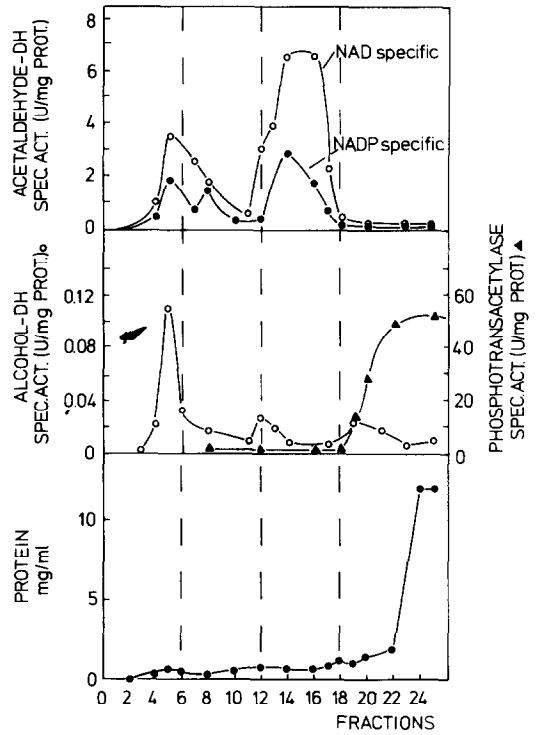

Fig. 1. Fractionation of a lysate of *Cl. kluyveri* on a Tris-buffered discontinuous sucrose density gradient. 3 ml each of 80, 60, 40 and 20% sucrose (w/v) in 0.2 Tris-HCl buffer, pH 7.5, and 1 ml lysate were layered in a cellulose-nitrate tube (1.4 cm in diameter and 9.5 cm long). Centrifugation in a Beckman: Spinco ultracentrifuge (Model L 2-65 B; SW 40 rotor) at 28000 *g* for 30 min at 0°. Fractions 1–6, 80% sucrose; 7–12, 60% sucrose; 13–18, 40% sucrose; 19–24, 20% sucrose.

Table 2
Localization of enzymes involved in the ethanol–acetate fermentation of *Cl. kluyveri*.

Enzyme	Lysate			Supernatant			Particulate fraction		
	Specific activity (U/mg prot.)	Total (U)	(%)	Specific activity (U/mg prot.)	Total (U)	(%)	Specific activity (U/mg prot.)	Total (U)	(%)
Alcohol dehydrogenase	0.06	9.6	100	0	0	0	0.08	2.4	22
NAD acetaldehyde dehydrogenase	1.46	260	100	0.06	7.6	3	5.3	150	58
NADP acetaldehyde dehydrogenase	0.70	126	100	0.04	5.0	4	3.2	92	72
Thiolase	16.5	2500	100	21.5	2300	92	0.17	4.1	0.16
NAD β-hydroxybutyryl-CoA dehydrogenase	0.51	92	100	0.1	13	14	0.85	24	27
NADP β-hydroxybutyryl-CoA dehydrogenase	13.9	2100	100	19.6	2040	98	4.4	105	5
Crotonase	42.0	6390	100	57.0	5940	93	0.3	4.3	0.07
Butyryl-CoA dehydrogenase	42.0	7400	100	66.0	7250	98	6.0	150	2.3
CoA-transferase	0.015	2.1	100	0.02	2.2	105	0	0	0
Hydrogenase	1.2	168	100	1.2	118	70	0.006	0.13	0.08

The experimental conditions are described in Methods.

trifuged in a discontinuous sucrose gradient and the distribution of protein and enzyme activities in the fractions obtained was determined (fig. 1). Phosphotransacetylase and other soluble enzymes were present in the supernatant and in 20% sucrose. Alcohol dehydrogenase resided mainly in the 80% sucrose solution which also contained some activity of the NAD and NADP acetaldehyde dehydrogenases. Most of the activity of these enzymes, however, was present in the 40% sucrose fractions. NAD β-hydroxybutyryl-CoA dehydrogenase (not shown) behaved like the acetyldehyde dehydrogenases.

4. Discussion

The ethanol/acetate fermentation of *Cl. kluyveri* [10] is initiated by two oxidative steps, the dehydrogenation of ethanol to acetaldehyde and of the latter to acetyl-CoA. Both reactions have to proceed in a rather reductive environment, under conditions which normally favour the opposite reaction, the reduction of acetyl-CoA to ethanol. Therefore, it seems advantageous or even necessary for *Cl. kluyveri* to spatially separate these dehydrogenases from the enzymes involved in butyrate synthesis from acetyl-CoA and in hydrogen formation. The arrangement of the oxidative portion of the ethanol/acetate fermentation in a membrane system is indicated by the particulate nature of the alcohol and the acetaldehyde dehydrogenases. This should create more favorable conditions for these reactions since acetaldehyde is formed in the microenvironment of the acetaldehyde dehydrogenases which metabolize it further. Presumably the arrangement of the two dehydrogenation reactions in a membrane system together with the highly active acetyl-CoA-consuming enzymes of *C. kluyveri* render the oxidative reactions possible, even when the $NAD(P)H_2/NAD(P)$-ratio is high.

The individual enzymatic reactions implicated in butyrate formation by *Cl. kluyveri* have been studied

in detail by Barker and coworkers [16, 17]. In addition to the known enzymes of the fermentative metabolism of *Cl. kluyveri* we report here that this microorganism contains a very active NADP β-hydroxybutyryl-CoA dehydrogenase. The specific activity of this enzyme in crude extracts is 13 U/mg of protein. It therefore belongs to the most active enzymes of *Cl. kluyveri* and its presence indicates that $NADPH_2$ plays an essential role in the butyrate fermentation by this organism.

Acknowledgement

This work was supported by a grant of the "Deutsche Forschungsgemeinschaft".

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