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SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF PARTICULATE DEHYDROGENASES FROM *CLOSTRIDIUM KLUYVERI*

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SUMMARY

Alcohol, acetaldehyde, NAD^+- and $NADP^+$-dependent β-hydroxybutyryl-CoA dehydrogenases present in a particulate fraction of *Clostridium kluyveri* could be solubilized by incubation in 5 mM Tris buffer at 0 °C. The solubilization was prevented by Mg^{2+}. A reaggregation of the enzymes with the particles occurred at higher buffer concentrations (200 mM).

By various methods of protein fractionation including column chromatography and polyacrylamide electrophoresis a separation of the dehydrogenases could not be achieved. With the exception of $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase the dehydrogenases showed resembling inactivation profiles at 0 and 45 °C.

NAD^+- and $NADP^+$-dependent acetaldehyde dehydrogenase activities were similarly affected by sulfhydryl compounds, changes of pH and storage at 0 and 45 °C, respectively. The NAD^+-dependent activity was about 2.3 times higher than the one with $NADP^+$. In the presence of both pyridine nucleotides enzyme activity reached an intermediate level. NADH caused inhibition of the NAD^+-specific aldehyde dehydrogenase only.

INTRODUCTION

The ethanol–acetate fermentation of *Clostridium kluyveri* is initiated by two dehydrogenation reactions by which ethanol is oxidized to acetyl-CoA. It has been shown that the dehydrogenases involved in these reactions and a NAD^+-specific β-hydroxybutyryl-CoA dehydrogenase are particle bound [1]. The other enzymes participating in the evolution of hydrogen and the synthesis of butyrate are soluble. This seems to indicate that a spatial separation of hydrogenation and dehydrogenation reactions is required in order to make this fermentation process possible.

In this publication we shall describe the solubilization of the particle-bound dehydrogenases and some of their properties.

MATERIAL AND METHODS

Growth of bacteria

C. kluyveri was grown in 20-l carboys according to Bornstein and Barker [2]. After harvesting, the cells were kept at −20 °C.

Enzyme assays

Alcohol dehydrogenase (EC 1.1.1.1) and $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35) were determined as described previously [3, 4].

NAD^+ ($NADP^+$)-acetaldehyde dehydrogenase was assayed in a reaction mixture containing in a final volume of 3 ml: 82 mM sodium Veronal buffer (pH 9.0), 33 mM acetaldehyde, 1.5 mM NAD^+ (3.5 mM $NADP^+$), 100 μM (150μM)CoASH, 1 mM dithiothreitol and protein. In some experiments 5 μg phosphotransacetylase and 100 μM K_2HPO_4 were added.

NAD^+-β-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35) was assayed in a reaction mixture containing in a final volume of 2 ml: 50 mM sodium pyrophosphate buffer (pH 7.6), 0.5 mM NADH, 75 μM acetoacetyl-CoA, 2 mM glutathione and protein.

The reactions were carried out in 1-cm cuvettes at 25 °C. They were started by the addition of enzyme solution and followed at 365 nm in a Zeiss photometer PL 4. One enzyme unit catalyzes the consumption of 1 μmole of substrate per min.

Protein was determined according to Lowry et al. [5] with crystalline bovine serum albumin as standard.

Electrophoresis

Separation gels contained 7% acrylamide, 0.13% bisacrylamide, 50 mM Tris–HCl and 370 mM glycine (pH 9.0). Upper gels contained 59 mM Tris–HCl, 3.1 mM H_3PO_4, 2.5% acrylamide, 0.63% bisacrylamide and 0.5% riboflavine (pH 6.9). Electrophoresis buffer contained 6 g Tris–HCl and 28.3 g glycine/l. Gels were stained with 1% amidoblack in 7% acetic acid and destained in 7% acetic acid.

Enzyme-specific staining methods were performed at 25 °C in the dark in reaction mixtures of a final volume of 10 ml.

Alcohol dehydrogenase: 50 mM Tris buffer (pH 8.0), 0.17 mM NAD^+, 550 mM ethanol, 2.4 mg nitroblue tetrazolium, 0.3 mg phenazine methosulfate.

Acetaldehyde dehydrogenase: 50 mM Tris buffer (pH 8.0), 0.17 mM NAD^+ ($NADP^+$), 0.1 mM CoASH, 2.4 mg nitroblue tetrazolium, 0.3 mg phenazine methosulfate. NAD^+- and $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase: 50 mM Tris buffer (pH 8.0), 0.17 mM NAD^+ ($NADP^+$), 40 μM 3-hydroxybutyryl-CoA, 2.4 mg nitroblue tetrazolium, 0.3 mg phenazine methosulfate.

Enzymes and chemicals

Acetoacetyl-CoA was prepared according to Simon and Shemin, and 3-hydroxybutyryl-CoA as described by Wieland and Rueff [6, 7].

Enzymes and coenzymes were purchased from Boehringer–Mannheim GmbH (Mannheim, Germany), diketene and 3-hydroxybutyric acid from Schuchardt (München, Germany), nitroblue tetrazolium and phenazine methosulfate from Sigma Chemical Company, St. Louis, U.S.A.

RESULTS

When lysates of *C. kluyveri* cells were prepared by lysozyme treatment and one passage through a French press most of the activity of alcohol dehydrogenase and NAD^+- and $NADP^+$-specific acetaldehyde dehydrogenase was present in the sediment after centrifugation at 40 000 × *g* for 20 min [1]. The pellet contained also a high percentage of the NAD^+-specific β-hydroxybutyryl-CoA dehydrogenase activity and some $NADP^+$-dependent β-hydroxybutyryl-CoA dehydrogenase. The level of the latter enzyme was found to be very high in *C. kluyveri* (13.9 units/mg of protein in lysates) [1]. In order to release these dehydrogenases from the particulate fraction several methods were tried; the most useful one was to wash the particles with buffer of low molarity. Fig. 1 shows that the dehydrogenases were associated with the particulate fraction in 0.2 M Tris buffer. When this fraction was incubated with buffer

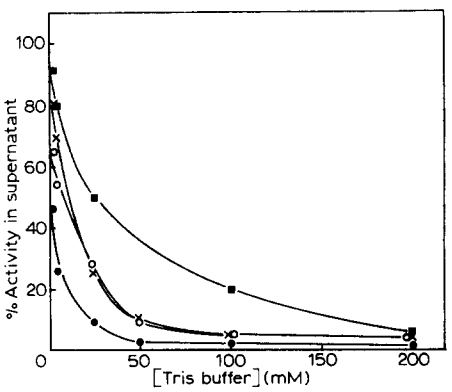

Fig. 1. Effect of Tris buffer concentration on the solubilization of the dehydrogenases. 1 g of cell paste was suspended in 10 ml of 0.2 M Tris–HCl buffer (pH 7.5) containing 2 mg of lysozyme (EC 3.2.1.17) and 1 mg of deoxyribonuclease (EC 3.1.4.5). The suspension was incubated in a water bath at 35 °C for 1 h and then passed through a French press cell (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 centrifugation at 35 000 × *g* for 20 min the pellet was incubated in 10 ml Tris–HCl buffer (pH 7.5) of decreasing molarity at 0 °C for 30 min with shaking. After centrifugation the enzyme activities were determined in the supernatant. ■, alcohol dehydrogenase; ×, NAD- and NADP-acetaldehyde dehydrogenase; ○, NAD^+- and ●, $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase.

of decreasing molarity increasing amounts of the enzyme appeared in the supernatant. It was found that two incubations of the particles with 5 mM Tris buffer for 30 min at 0 °C were sufficient to release most of the alcohol and acetaldehyde dehydrogenase activities. By this procedure NAD^+-β-hydroxybutyryl-CoA dehydrogenase was removed to a high percentage; the $NADP^+$-dependent enzyme behaved differently in that it was present in the supernatant of the first wash in small amounts only (Table I).

There was a considerable increase of the specific activity of the solubilized dehydrogenases. The specific activity of NAD^+-acetaldehyde dehydrogenase in the first wash was 27.6 units/mg of protein and 4 times higher than the one obtained in another purification procedure [6].

TABLE I

SOLUBILIZATION OF THE PARTICLE BOUND DEHYDROGENASES

After centrifugation the pellet of the particles was incubated in 5 mM Tris–HCl buffer (pH 7.5) at 0 °C for 30 min. Centrifugation yielded the first wash supernatant. The pellet was incubated a second time under the same conditions. Protein concentration and enzyme activities were determined in the various fractions.

Fractions	Total protein		Alcohol dehydrogenase activity			NAD^+-acetaldehyde dehydrogenase activity			$NADP^+$-acetaldehyde dehydrogenase activity			NAD^+-β-hydroxybutyryl-CoA dehydrogenase activity			$NADP^+$-β-hydroxybutyryl-CoA dehydrogenase activity		
	mg	per cent	Spec. act. (units/mg)	Total activity units	Total activity per cent	Spec. act. (units/mg)	Total activity units	Total activity per cent	Spec. act. (units/mg)	Total activity units	Total activity per cent	Spec. act. (units/mg)	Total activity units	Total activity per cent	Spec. act. (units/mg)	Total activity units	Total activity per cent
Particles	90	100	0.092	8.3	100	4.0	360	100	2.0	180	100	0.14	12.6	100	7.1	640	100
Wash 1	15.4	17	0.49	7.55	91	27.4	422	117	12.0	185	103	0.41	6.3	55	3.9	60	9.4
Wash 2	6.8	7.6	0.04	0.29	3.5	3.5	24	6.7	1.3	8.9	5	0.25	1.76	14	26.8	182	28
Sediment	68	75	0.013	0.91	11	0.04	2.7	0.8	0.026	1.7	0.9	0.006	0.39	37	3.1	211	33

As is evident from Table II Mg^{2+} largely prevented release of the dehydrogenases from the particles. However, $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase again behaved differently than the other dehydrogenases.

To further purify the dehydrogenases chromatographic methods were employed. Fig. 2 shows the elution pattern of protein and of enzyme activities from a DEAE-Sephadex column. Except for $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase the peaks of enzyme activity coincided exactly with the protein peak. Experiments to

TABLE II

EFFECT OF Mg^{2+} ON THE SOLUBILIZATION OF THE DEHYDROGENASES

The particle fraction containing all the dehydrogenases was prepared as described in Fig. 1. Then the particles were incubated in 10 ml of 5 mM Tris–HCl buffer containing increasing concentrations of $MgCl_2$ at 0 °C for 30 min with shaking. After centrifugation the enzyme activities in the supernatants were determined.

$MgCl_2$ (mM)	Total activity in the supernatant (per cent)				
	Alcohol dehydrogenase	NAD^+-acetaldehyde dehydrogenase	NAD^+-β-hydroxybutyryl-CoA dehydrogenase	$NADP^+$-β-hydroxybutyryl-CoA dehydrogenase	Protein (per cent)
0	104	114	98	4.2	25
1	47	59	42	3.3	14
5	11.5	27.8	7.5	1.4	7.6
10	4.2	9.2	4.6	0.7	6.9
20	3.0	6.6	4.2	0.6	5.7
50	6.5	10	7.0	34	6.1

resolve this mixture of dehydrogenases by chromatography on Sephadex G-200 or hydroxylapatite were unsuccessful. Disc electrophoresis of the protein fraction eluted from DEAE-Sephadex revealed the presence of two proteins of different mobility (Fig. 3a). All dehydrogenase activities resided in one protein band as became evident from enzyme-specific staining procedures (Figs 3b–3d). The dehydrogenase mixture, therefore, behaved like an enzyme complex.

The isolated dehydrogenases bound back to the particles when the buffer concentration was increased to 200 mM after release of the enzymes. A corresponding experiment is summarized in Table III. Like in previous experiments, $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase showed a different behaviour.

The enzymes associated in this complex were further characterized. Figs 4 and 5 show the inactivation of the dehydrogenases during incubation at 0 and 45 °C, respectively. Under both conditions $NADP^+$-dependent β-hydroxybutyryl-CoA dehydrogenase was very stable. The other dehydrogenases lost most of their activity. The kinetics of inactivation of the two acetaldehyde dehydrogenase activities was almost identical.

The subsequent experiments, therefore, concentrated on the question whether or not the NAD^+- and $NADP^+$-dependent oxidation of acetaldehyde was catalyzed by the same enzyme entity. First, the specific activities of the enzyme were compared

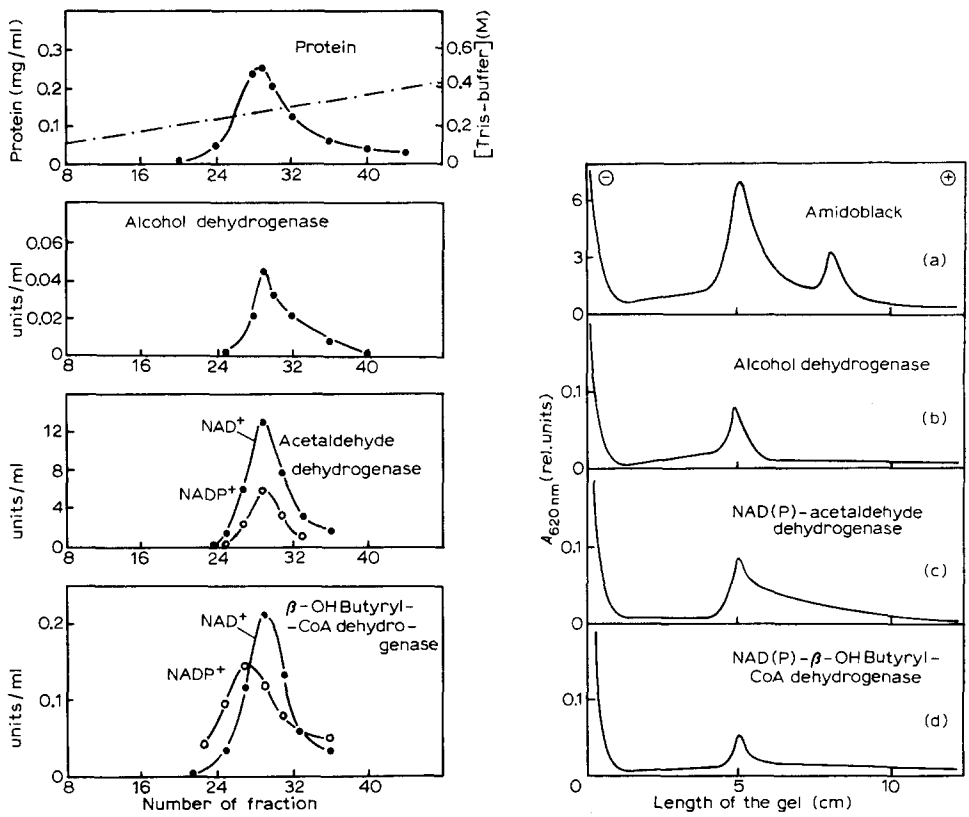

Fig. 2. Elution pattern of protein and enzyme activities from a DEAE-Sephadex column. The solution containing the solubilized enzymes was adjusted to 50 mM with Tris–HCl buffer (pH 7.5) and applied to a DEAE-Sephadex column (1.5 cm × 24 cm) which was equilibrated with the same buffer. The protein was eluted with 100 ml of a linear gradient of buffer from 50 to 500 mM. Fractions of 2.5 ml were collected and analyzed for protein and enzyme activities.

Fig. 3. Polyacrylamide gel electrophoresis of the protein fraction eluted from DEAE-Sephadex. Electrophoresis was carried out in 7% polyacrylamide for 4 h at 15 °C and 3 mA per tube. 50–200 μg protein was applied per tube. (a) Protein peaks as shown in an electropherogram from amidoblack staining. (b) Enzyme activity band of alcohol dehydrogenase ,(c) of NAD^+- and $NADP^+$-acetaldehyde dehydrogenase, (d) of NAD^+- and $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase as shown in electropherograms. For details of electrophoresis and of the enzyme specific staining procedures see Materials and Methods.

with NAD^+, $NADP^+$ or both coenzymes present (Table IV). If two independent aldehyde dehydrogenases were active the addition of NAD^+ and $NADP^+$ should give the sum of the activities with either NAD^+ or $NADP^+$. This was not the case, the specific activity reached a value in between the ones obtained with NAD^+ and $NADP^+$, respectively. Fig. 6 summarizes the effect of various sulfhydryl compounds on enzyme activity and Fig. 7 shows the dependence on the concentration of dithiothreitol. The ratio of the velocities of the NAD^+- and $NADP^+$-mediated reactions (2.3:1) was not significantly altered by varying the sulfhydryl compound or the concentration of dithiothreitol. Also, the effect of pH on activity did not show significant

TABLE III

REAGGREGATION OF THE SOLUBILIZED DEHYDROGENASES TO THE PARTICLES

The dehydrogenases were solubilized as described in Fig. 1. The supernatant of the first incubation (Wash 1) was combined with the pellet of the second incubation and the buffer concentration adjusted to 5 mM and 200 mM, respectively. The activity of the particle suspension containing the solubilized enzymes was set 100%. Then this suspension was incubated for 1 h at 0 °C, centrifuged and the enzyme activities in supernatant and sediment were determined.

Tris buffer (mM)	Fractions	Total activity (per cent)				Protein (per cent)
		Alcohol dehydrogenase	NAD^+-acetaldehyde dehydrogenase	β-hydroxybutyryl-CoA dehydrogenase		
				NAD^+	$NADP^+$	
200	Wash 1 + sediment 2	100	100	100	100	100
	Supernatant	25	33	44	15	9.6
	Sediment	70	50	70	93	95
5	Wash 1 + sediment 2	100	100	100	100	100
	Supernatant	92	99	41	2.8	21
	Sediment	14	8.5	19	88	87

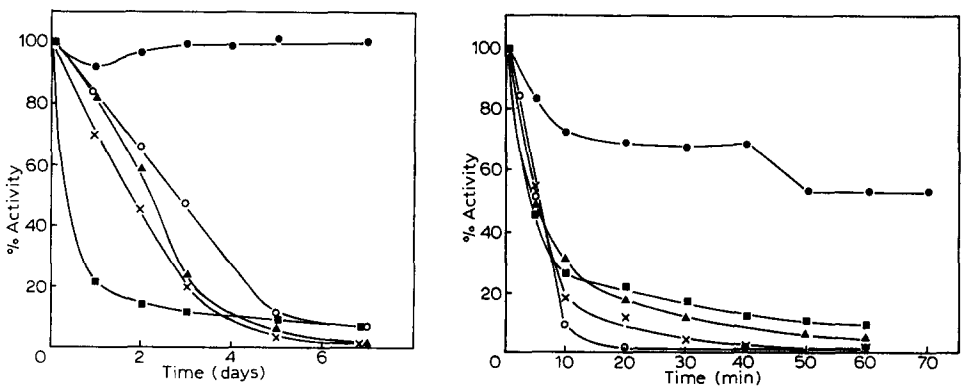

Fig. 4. Inactivation of the dehydrogenases during incubation at 0 °C. The solubilized enzyme mixture was incubated in 5 mM Tris buffer (pH 7.5) at 0 °C. Enzyme activities were measured every 24 h. ■, alcohol dehydrogenase; ×, NAD^+- and ▲, $NADP^+$-acetaldehyde dehydrogenase; ○, NAD^+- and ●, $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase.

Fig. 5. Inactivation of the dehydrogenases during incubation at 45 °C. The solubilized enzyme mixture was incubated in 5 mM Tris buffer (pH 7.5) at 45 °C with gentle stirring. Enzyme activities were determined every 10 min. ■, alcohol dehydrogenase; ×, NAD^+- and ▲, $NADP^+$-acetaldehyde dehydrogenase; ○, NAD^+- and ●, $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase.

differences for the two reactions (Fig. 8). The substrate specificity is given in Fig. 9. With NAD^+ as coenzyme the homologous aldehydes served somewhat better as substrates than with $NADP^+$.

TABLE IV

ACTIVITY OF ACETALDEHYDE DEHYDROGENASE IN THE PRESENCE OF BOTH COENZYMES

The reaction mixture contained in a final volume of 3 ml: 82 mM sodium Veronal buffer (pH 9.0), 33 mM acetaldehyde, 100 μM (150 μM) CoASH, 5 μg phosphotransacetylase, 100 μM K_2HPO_4, 6 μg solubilized protein, NAD^+ and $NADP^+$ as given in the table.

Coenzyme added		Activity (units/ml)
NAD^+ (mM)	$NADP^+$ (mM)	
1.5	0	14.3
0	3.5	5.7
1.5	3.5	10.2

Table V summarizes the K_m values as determined at saturating concentrations of the two fixed substrates. It can be seen that the affinity of the enzyme for NAD^+ was much higher than for $NADP^+$. The K_m value for acetaldehyde was also largely affected by the coenzyme employed, the one for CoASH to a lesser extent.

Differences were also noticed when the effect of NADH and NADPH on enzyme activity was studied. NADPH did not have a pronounced effect on both dehydrogenase activities (Fig. 10). However, NADH strongly inhibited NAD^+-dependent acetaldehyde dehydrogenase activity whereas the $NADP^+$-dependent reaction was not affected (Fig. 11). The inhibition was r oncompetitive as is evident from Fig. 12.

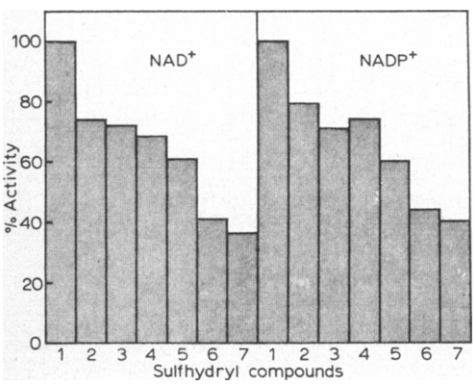

Fig. 6. Effect of various sulfhydryl compounds on enzyme activity. The reaction mixture contained in a final volume of 3 ml: 82 mM sodium Veronal buffer (pH 9.0), 33 mM acetaldehyde, 1.5 mM NAD^+ (3.5 mM $NADP^+$), 5 μg phosphotransacetylase, 100 μM K_2HPO_4, 10 μg solubilized protein with a spec. act. of 7.3 units/mg protein (3.5 units/mg protein) and the following sulfhydryl compounds at concentrations of 1 mM: dithiothreitol (1), cysteine (2), reduced glutathione (3), mercaptoethanol (4), sodium thioglycolate (5), oxidised glutathione (6), without (7).

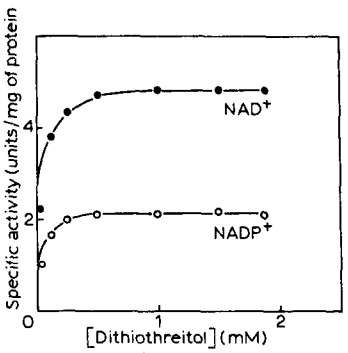

Fig. 7. Effect of dithiothreitol on the activity of NAD- and NADP-acetaldehyde dehydrogenase. The reaction mixture contained in a volume of 3 ml: 82 mM sodium Veronal buffer (pH 9.0), 33 mM acetaldehyde, 1.5 mM NAD^+ (3.5 mM $NADP^+$), 100 μM (150 μM) CoASH, 5 μg phosphotransacetylase, 100 μM K_2HPO_4, 11 μg of protein and varying concentrations of dithiothreitol.

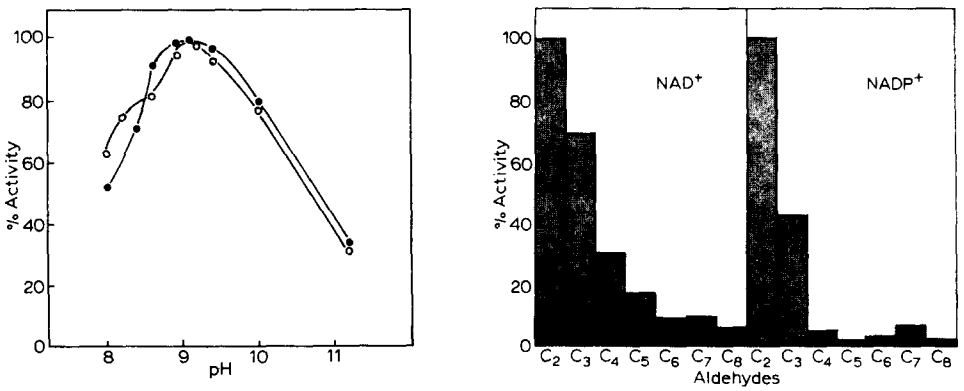

Fig. 8. Effect of pH on the activity of NAD- and NADP-acetaldehyde dehydrogenase. The reaction mixture contained in a volume of 3 ml: 82 mM sodium Veronal buffer of varying pH values, 33 mM acetaldehyde, 1.5 mM NAD^+ (3.5 mM $NADP^+$), 100 μM (150 μM) CoASH, 1 mM dithiothreitol, 5 μg phosphotransacetylase, 100 μM K_2HPO_4 and 10 μg of solubilized protein. ●, NAD^+; ○, $NADP^+$.

Fig. 9. Substrate specificity of the NAD^+- and $NADP^+$-acetaldehyde dehydrogenase. The reaction mixture contained in a volume of 3 ml: 82 mM sodium Veronal buffer (pH 9.0), 1.5 mM NAD^+ (3.5 mM $NADP^+$), 100 μM (150 μM) CoASH, 1 mM dithiothreitol, 5 μg phosphotransacetylase, 100 μM K_2HPO_4, 10 μg solubilized protein and 33 mM of the following aldehydes: acetaldehyde (C_2), propionaldehyde (C_3), butyraldehyde (C_4), valeraldehyde (C_5), capronaldehyde (C_6), enanthaldehyde (C_7), caprylaldehyde (C_8).

TABLE V

K_m VALUES OF ACETALDEHYDE DEHYDROGENASE

Substrate	K_m value (M)
NAD^+	$4.0 \cdot 10^{-4}$
$NADP^+$	$1.5 \cdot 10^{-3}$
Acetaldehyde (NAD^+)	$4.5 \cdot 10^{-4}$
Acetaldehyde ($NADP^+$)	$1.5 \cdot 10^{-3}$
CoA (NAD^+)	$3.3 \cdot 10^{-5}$
CoA ($NADP^+$)	$2.0 \cdot 10^{-5}$

DISCUSSION

The four particulate dehydrogenases involved in the ethanol–acetate fermentation of *C. kluyveri* could be solubilized by washing the particles with 5 mM Tris–HCl buffer at 0 °C. This indicated that the enzymes were connected to their matrix by weak ionic bonds [7]. In accordance with this an increase of the buffer concentration led to an extensive reaggregation of the enzymes with their matrix, and the solubilization was strongly inhibited by the addition of Mg^{2+}.

A comparable behaviour was described by Abrams and coworker [8, 9] and by Muñoz et al. [10] for the ATPases from membrane preparations of various microorganisms. By washing the membranes with Tris buffer of low molarity a great deal of the enzymes could be solubilized accompanied by a rise of the specific activity up to 50-fold. The addition of Mg^{2+} prevented both, the solubilization and the reaggre-

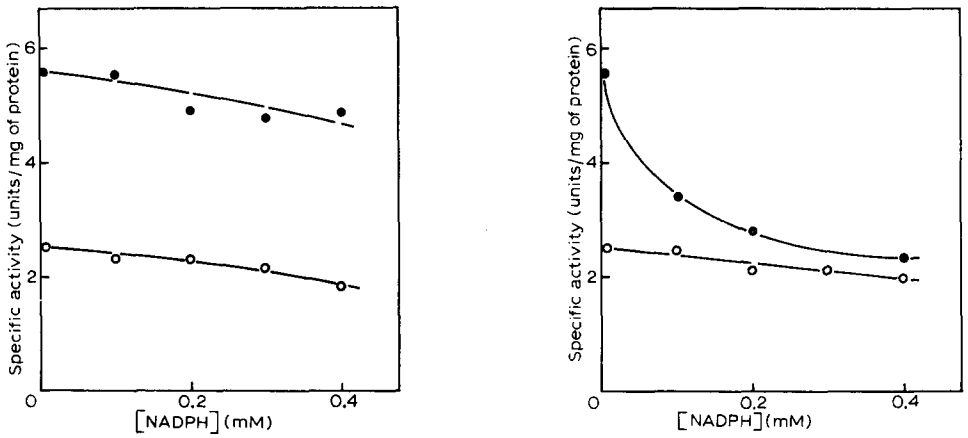

Fig. 10. Effect of NADPH on the activity of the NAD^+- and $NADP^+$-aldehyde dehydrogenase. The reaction mixture contained in a volume of 3 ml: 82 mM sodium Veronal buffer (pH 9.0), 33 mM acetaldehyde, 1.5 mM NAD^+ (3.5 mM $NADP^+$), 100 μM (150 μM) CoASH, 1 mM dithiothreitol, 5 μg phosphotransacetylase, 100 μg K_2HPO_4, 10 μg solubilized protein and varying concentrations of NADPH. ●, 1.5 mM NAD^+; ○, 3.5 mM $NADP^+$.

Fig. 11. Effect of NADH on the activity of the NAD^+- and $NADP^+$-aldehyde dehydrogenase. The reaction mixture contained the same components as in Fig. 9 and varying concentrations of NADH. ●, 1.5 mM NAD^+, ○, 3.5 mM $NADP^+$.

gation. Ellar et al. [11] reported that the solubilization of the NADH dehydrogenase from *Micrococcus lysodeikticus* membranes was drastically lowered by the addition of 0.5% glutaric aldehyde. Nakagawa et al. [7] showed that the β-fructofuranosidase from tomato cell walls could be solubilized by incubation in 10 mM phosphate buffer at alkaline pH. Under acidic conditions the enzyme remained firmly attached to the wall.

Experiments to separate the dehydrogenases by chromatography on Sephadex G-200, DEAE-Sephadex, hydroxylapatite and polyacrylamide electrophoresis were

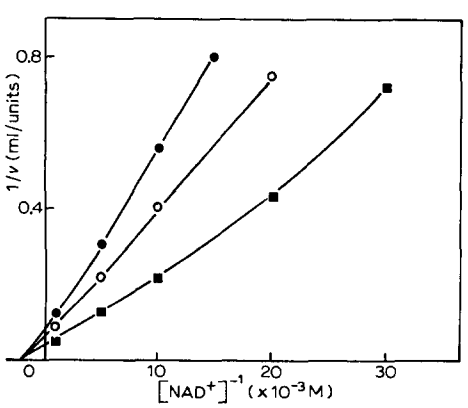

Fig. 12. Lineweaver–Burk plot of the effect of NADH on NAD^+-aldehyde dehydrogenase. The experimental conditions were the same as in Fig. 9. ●, 0.46 mM NADH; ○, 0.23 mM NADH; ■, without NADH.

not successful. With the exception of the $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase the peaks of enzyme activity coincided exactly for all dehydrogenases. During the incubation of the enzymes at 0 and 45 °C, respectively, the $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase was very stable, whereas the inactivation profile of the other enzymes was very much alike. Moreover, $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase behaved differently in that it appeared in the soluble fraction mainly during the second incubation with buffer of low molarity. From these findings it can be concluded that alcohol, acetaldehyde and NAD^+-β-hydroxybutyryl-CoA dehydrogenases are integrated in a multienzyme complex and that the $NADP^+$-specific β-hydroxybutyryl-CoA dehydrogenase is more loosely associated with this complex.

The fixation of the dehydrogenases to particles together with their association in a multienzyme complex guarantees a close cooperation of the individual enzymes and may favour the oxidation of ethanol in the strongly reduced environment of *C. kluyveri*. A homogeneous distribution of the reactants is prevented, and the local concentration of NADH could increase to such an extent that the evolution of hydrogen is made possible [12].

Kazahaya et al. [13] isolated a particle-bound enzyme complex from *Leuconostoc mesenteroides* consisting of alcohol and acetaldehyde dehydrogenase. It was found that the two activities could not be separated from each other by various methods of enzyme fractionation. Purified acetaldehyde dehydrogenase from *Escherichia coli* contained 20% of the original alcohol dehydrogenase activity and all attempts to separate these activities were unsuccessful [14]. Therefore, functional complexes of these two enzymes seems to occur in a number of fermentative microorganisms. The significance of the occurrence of NAD^+-β-hydroxybutyryl-CoA dehydrogenase in the complex isolated from *C. kluyveri* is not known.

The resemblance of the NAD^+- and $NADP^+$-dependent acetaldehyde dehydrogenase activities is remarkable. The ratio of their activities remained constant during purification and incubation at 0 and 45 °C, respectively. The pH optima were identical and the same degree of stimulation was observed upon addition of dithiothreitol and other sulfhydryl compounds. Furthermore, when the assay mixtures contained NAD^+ and $NADP^+$ simultaneously enzyme activities were not additive. The velocity of the 2.3 times faster reaction with NAD^+ decreased to an intermediate level after the addition of $NADP^+$. Differences were also apparent. The K_m value for acetaldehyde increased by a factor of three when NAD^+ was replaced by $NADP^+$. The relative activity of the enzyme with homologous aldehydes was somewhat higher when NAD^+ was used as coenzyme. Furthermore, it was reported by Burton and Stadtman [6] that an enzyme preparation purified to a specific activity of 7 units/mg of protein by an entirely different procedure had lost its ability to react with $NADP^+$. Taking all the results together it seems that NAD^+- and $NADP^+$-specific acetaldehyde dehydrogenases have distinct active sites but also some structural elements in common which are important for the expression of enzyme activity.

From the reduced coenzymes NADH only caused inhibition of acetaldehyde oxidation and the NAD^+-dependent reaction only was impaired. An increase of the NADH level in *C. kluyveri*, therefore, would favour $NADP^+$-dependent acetaldehyde oxidation. That NADPH plays a role in the fermentative metabolism of *C. kluyveri* is indicated by the presence of a very active $NADP^+$-β-hydroxybutyryl-CoA dehydrogenase [1].

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