On the dehydration of (*R*)-lactate in the fermentation of alanine to propionate by *Clostridium propionicum*

Georg Schweiger and Wolfgang Buckel

Biochemie I, Universität Regensburg, D-8400 Regensburg, FRG

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All the enzymes of the pathway of (*S*)-alanine fermentation to acetate and propionate were detected in cell-free extracts of *Clostridium propionicum*. Among these (*S*)-glutamate dehydrogenase (NAD), (*R*)-lactate dehydrogenase (NAD) and propionate CoA-transferase were purified to apparent homogeneity. Their structures were presumably α_6, α_2 and α_4, respectively. The latter enzyme was specific for short-chain monocarboxylic acids with a pronounced preference for (*R*)-lactate over the (*S*)-enantiomer. The key step of the pathway, the dehydration of (*R*)-lactate required acetyl phosphate and CoASH under anaerobic conditions. It was inhibited by hydroxylamine, arsenate, azide (1 mM each) or by 0.1 mM 2,4-dinitrophenol. Thus it closely resembled the dehydration of (*R*)-2-hydroxyglutarate in *Acidaminococcus fermentans*, although an activation was not necessary.

(S)-Alanine *(S)-Glutamate dehydrogenase (NAD)* *(R)-Lactate dehydrogenase (NAD)*
Propionate CoA-transferase *(R)-Lactate dehydration* *Inhibition by 2,4-dinitrophenol*

1. INTRODUCTION

The reversible dehydration of 2-hydroxy acids is an enzyme-catalyzed reaction of which the mechanism is still not understood. Although a radical was proposed as intermediate in the dehydration of (*R*)-2-hydroxyglutarate to glutaconate, a definite conclusion could not be drawn, since the experiments had to be performed with cell-free extracts rather than with pure enzymes. Hitherto attempts to purify the enzyme have failed [1]. Therefore, the aim of this paper is the characterisation of a second system, the dehydration of lactate to acrylate, in the hope of obtaining a dehydratase less resistant to purification.

In the fermentation of lactate to propionate two pathways are involved. Propionibacteria ferment lactate via succinate [2] whereas in *Megasphera elsdenii* [3,4] and *Clostridium propionicum* [5,6] the key intermediate appears to be acrylate. Although several lines of evidence indicate the existence of this so-called 'direct pathway' in the latter two organisms, the dehydration of (*R*)-lactate has not been observed directly. To study this reaction, *C. propionicum* was chosen, since it displays a fermentation pattern simpler than that of *M. elsdenii*. This paper describes the identification of (*R*)-lactate as intermediate in the pathway from alanine to propionate. In addition a direct assay for the dehydration of (*R*)-lactate is presented.

2. MATERIALS AND METHODS

C. propionicum NCIB 10656, a gift from Dr G.C. Mead, Norwich, England, was grown on a medium containing 0.5% (*R,S*)-alanine, 1% yeast extract, 0.05% cysteine–HCl, 0.4% VRB-salt [7] and 5 mM K–phosphate buffer (pH 7.0). Cell-free extracts for aerobic and anaerobic experiments were prepared as in [10] and [8], respectively.

(*R*)-[3-^{3}H]Lactate and (*R*)-[U-^{14}C]lactate were synthesized from the corresponding (*S*)-alanines. The reaction mixture contained 0.1 M Tris–HCl (pH 7.6), 0.1–2 mM (*S*)-alanine, 5 mM 2-oxoglutarate, 0.2 mM NADH, 50 μCi (*S*)-[3-^{3}H]alanine (New England Nuclear) or 5 μCi (*S*)-[U-^{14}C]alanine

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(Amersham-Buchler), 25 μg (*R*)-lactate dehydrogenase and 50 μg alanine aminotransferase (Boehringer Mannheim). After completion of the reaction residual alanine was separated by passing the mixture through a Dowex AG 50 (H^+) cation exchange column (1 × 5 cm). The neutralized eluate was separated by a Dowex 1-x8 (Cl^-) column (1 × 5 cm) using a 0–10 mM HCl gradient (200 ml). The product eluted at 5–7 mM HCl was adjusted with NaOH to pH 8–9, concentrated, acidified to pH 2 with H_2SO_4 and extracted continuously with diethyl ether. After evaporation of the ether phase to dryness the residue was dissolved in water, neutralized and stored at 4°C.

Carbonic acids were separated on an RP18 HPLC column (LiChrosorb, Merck; 250 × 4 mm) equilibrated with 10 mM H_2SO_4 at a flow rate of 0.8 ml/min and detection at 206 nm.

Molecular mass determination of enzymes on a Bio-Sil TSK-250 column (300 × 7.5 mm, Bio-Rad) was performed at a flow rate of 1 ml/min in 20 mM K–phosphate (pH 7.0) containing 50 mM Na_2SO_4.

Polyacrylamide gel electrophoresis in the presence of SDS was performed as in [9] at an acrylamide concentration of 9%.

Lactoyl-CoA dehydratase was assayed in analogy to (*R*)-2-hydroxyglutarate dehydratase [1]. The test system contained 66 mM Pipes buffer (pH 7.0), 6.6 mM acetyl phosphate, 0.7 mg/ml CoASH, 3–5 mg/ml protein and 5 μM (*R*)-[3-^{3}H]lactate (2.9×10^5 counts·min^{-1}·$nmol^{-1}$) under strict anaerobic conditions.

Propionate CoA-transferase was measured by the assay for glutaconate CoA-transferase [10] in which glutaryl-CoA was replaced by 0.2 mM propionyl-CoA.

(*R*)-Lactate dehydrogenase and (*S*)-glutamate dehydrogenase were assayed by the formazan method [11] with 1 mM (*R*)-lactate and 10 mM (*S*)-glutamate, respectively.

Alanine aminotransferase was activated by incubation with 100 mM K–phosphate (pH 7.0), 5 mM dithioerythritol, 5 mM pyridoxal phosphate and 10 mM EDTA. After 10 min at 37°C a sample (20 μl) was assayed in a cuvette containing 100 mM K–phosphate (pH 7.0), 100 mM (*S*)-alanine, 0.25 mM NADH, 0.15 U lactate dehydrogenase and 10 mM 2-oxoglutarate.

Propionyl-CoA dehydrogenase was measured in 100 mM Tris–HCl (pH 8.0), 17 μM 2,6-dichlorophenolindophenol, 50 μM Meldolablue and 0.1 mM propionyl-CoA.

Phosphate acetyltransferase was determined with 100 mM Tris–HCl (pH 7.4), 10 mM phosphate, 1 mM 5,5′-dithiobis(2-nitrobenzoate) and 0.2 mM CoASAc.

Pyruvate ferredoxin oxidoreductase was tested as in [13] and acetate kinase assayed according to [14].

3. RESULTS AND DISCUSSION

3.1. *The pathway of alanine fermentation*

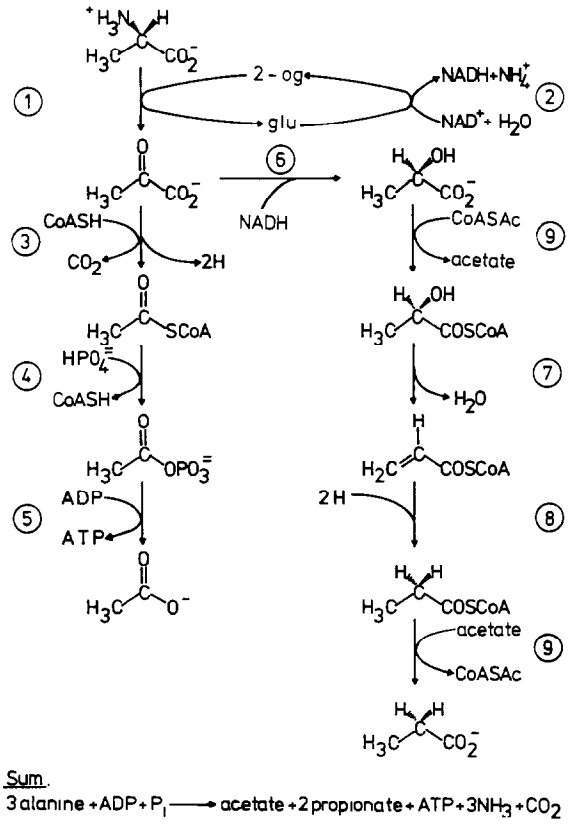
Table 1 lists all the enzymes which could be found in cell-free extracts of *C. propionicum*. From these data a possible pathway of alanine fermentation was deduced (scheme 1). It is remarkable that the conversion of alanine to pyruvate requires two enzymes, alanine aminotransferase (1) and glutamate dehydrogenase (2), whereas a direct dehydrogenation occurs in other Clostridia and certain Bacilli [2]. The enzyme

Table 1

Enzyme activities in extracts of *Clostridium propionicum*

No.	Enzyme	EC no.	Activity (units/mg)
1	Alanine:2-oxoglutarate aminotransferase	2.6.1.2	3.8
2	(*S*)-Glutamate dehydrogenase	1.4.1.2	3.1
3	Pyruvate-ferredoxin oxidoreductase	1.2.7.1	0.13
4	Phosphate acetyltransferase	2.3.1.8	1.4
5	Acetate kinase	2.7.2.1	0.2
6	(*R*)-Lactate dehydrogenase	1.1.1.28	2.7
7	(*R*)-Lactoyl-CoA dehydratase	–	1.8×10^{-5}
8	Propionyl-CoA dehydrogenase	–	0.002
9	Propionate CoA-transferase	2.8.3.1	4.6

Enzymes 3 and 7 were assayed in an extract prepared anaerobically. Protein was assayed by the biuret method



Scheme 1. Proposed pathway of alanine fermentation in *Clostridium propionicum*.

activities of the oxidative branch of the fermentation pathway leading to acetate and to the synthesis of ATP were detected in sufficient amounts (3–5). In the reductive branch with the final product propionate high levels of (*R*)-lactate dehydrogenase (6) and propionate CoA-transferase (9) were present. Problems arose with the other two enzymes. The observed activities of lactoyl-CoA dehydratase (7) and propionyl-CoA dehydrogenase (8) were far too low. It is possible that the electron acceptors Meldolablue and dichlorophenolindophenol were not suitable substrates for the oxidation of propionyl-CoA. However in the reverse direction an oxidation of NADH by acrylyl-CoA formed from acetyl-CoA and acrylate with the CoA-transferase, as well as the direct reduction of acrylate could not be detected. A discussion of the key enzyme lactoyl-CoA dehydratase is given in section 3.4.

3.2. *Propionate CoA-transferase*

The dehydration of (*R*)-lactate required acetyl phosphate and CoASH (table 4) indicating the use of lactoyl-CoA rather than the free acid as substrate. Propionate CoA-transferase, a possible candidate for the activation of (*R*)-lactate, was found to be present in high amounts in cell-free extracts of *C. propionicum* (table 1). The substrate specificity of the purified enzyme was determined by adding certain carboxylic acids to the assay (eqs 1,2) in which the formation of CoASH is monitored with 5,5′-dithiobis(2-nitrobenzoate).

$$\text{Acetate} + \text{propionyl-CoA} \rightleftharpoons \text{propionate} + \text{acetyl-CoA} \quad (1)$$

$$\text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \longrightarrow \text{citrate} + \text{CoASH} \quad (2)$$

Any carboxylate substrate other than acetate added to the assay leads to a CoA derivative which cannot generate CoASH in this system. Thereby an apparent inhibition is observed. The results in table 2 show that propionate was found to be the best inhibitor, i.e., the best substrate. Therefore the enzyme was called propionate CoA-

Table 2

Inhibition of the CoA-transferase by carboxylic acid

Group	Carboxylic acid added	Specific activity (units/mg)	Relative activity (%)
1	None	34.1	100
	(*R*)-Lactate	9.1	27
	(*S*)-Lactate	15.8	46
	Acrylate	12.7	37
	Propionate	6.8	20
	Butyrate	9.6	28
2	Fumarate	32.8	96
	Succinate	29.6	87
	(*R*)-2-Hydroxyglutarate	29.6	87
	(*E*)-Glutaconate	32.1	94

Each reaction mixture contained 111 mM K–phosphate (pH 7.0), 11 mM Na–acetate, 0.44 mM 5,5′-dithiobis-(2-nitrobenzoate), 1.1 mM oxaloacetate, 0.19 mM propionyl-CoA, 2.2 units citrate synthase, 94 munits propionate CoA-transferase and 11 mM carboxylic acid as indicated

transferase. Remarkably the transferase was able to discriminate between the enantiomers of lactate with a preference for (*R*)-lactate, the product of the reduction of pyruvate (scheme 1). However, acrylate was only a moderate substrate consistent with the view that the free acid does not occur in the proposed pathway. The transferase appeared to be specific for monocarboxylic acids since all the tested dicarboxylic acids gave no significant inhibition.

Propionate CoA-transferase was purified from an aerobically prepared cell-free extract in 10 mM Pipes (pH 7.0) and 100 mM $MgCl_2$. It was dialyzed against 10 mM Pipes (pH 7.0) and pumped on a DEAE–Sephacel column (5 × 2.5 cm) equilibrated in the same buffer. The enzyme was eluted by a linear gradient (200 ml, 50–400 mM NaCl) at about 250 mM NaCl (100% recovery, 3.8-fold purification). After fractionation with ammonium sulfate (80–100% saturation; 31%, 11-fold) the enzyme was chromatographed on a Sephacryl S-300 column (1.6 × 96 cm) in 10 mM Pipes (pH 7.0). The active fractions (30%, 18-fold) were rechromatographed on the DEAE–Sephacel column as mentioned above. The resulting enzyme (11%, 98-fold, 157 units/mg) remained stable for several months at 4°C when stored as a suspension in saturated ammonium sulfate. The small impurities of this enzyme preparation could be removed by chromatography on Blue Sepharose CL-6B (12 × 1 cm) in 10 mM K–phosphate (pH 6.5). The pure enzyme (fig.1) was eluted with a linear NaCl gradient (80 ml) at 300 mM. However, during this last step 80% of the activity and 65% of the specific activity were lost.

The molecular mass of the CoA-transferase was determined to be 224 kDa, and that of the subunit to be 67 kDa. These data led to a quaternary structure of presumably α_4 (table 3). In comparison, the CoA-transferase from *M. elsdenii* with an almost identical substrate specificity is composed of only two subunits but of similar size (62 kDa). Bacterial CoA-transferases with other substrate specificities such as glutaconate CoA-transferase contain two different subunits of about half the size (34 and 32 kDa) with a $(\alpha\beta)_4$ structure [10].

3.3. *(R)-Lactate and (S)-glutamate dehydrogenases*

(*R*)-Lactate dehydrogenase was purified from *C. propionicum* for preparation of labelled (*R*)-lactates to assay the dehydration (see section 2). (*S*)-Glutamate dehydrogenase copurified until it was separated from (*R*)-lactate dehydrogenase in the last step. Cell breakage and the first chromatography on DEAE–Sephacel were performed under conditions similar to those used for the CoA-transferase, except that all the buffers contained additional dithiothreitol, K_2EDTA and $MgCl_2$ (1 mM each) which were necessary to stabilize the dehydrogenase activity. The eluate from the anion exchanger was fractionated with ammonium sulfate (50–80% saturation), dialysed and rechromatographed on DEAE–Sephacel. After a further dialysis the second eluate was applied on a Blue Sepharose CL-6B column (15 × 2.5 cm). Both enzymes were eluted with 1 mM NADH. The final separation was performed on a Sephacryl S-300 column (95 × 2.5 cm) from which (*S*)-glutamate dehydrogenase (25 units/mg, 12-fold purification, 15% yield) eluted first followed by (*R*)-lactate dehydrogenase (72 units/mg, 28-fold, 19% yield). Both enzymes were pure as judged by gel electrophoresis in the presence of SDS (fig.1). Molecular masses of the complete enzymes and their subunits are given in table 3.

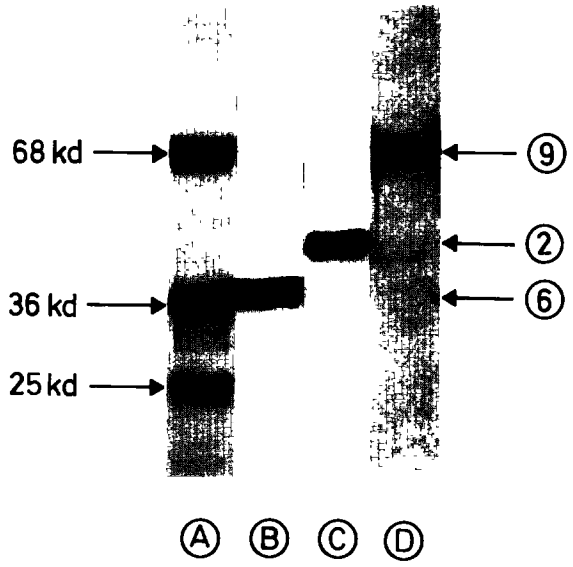


Fig.1. Polyacrylamide gel electrophoresis of the purified enzymes. Lane A: standards (see table 3), 5 μg each; lane B, 15 μg (*R*)-lactate dehydrogenase; lane C, 10 μg (*S*)-glutamate dehydrogenase; lane D, 5 μg propionate CoA-transferase; the gel was stained with Coomassie brilliant blue G-250.

Table 3

Molecular masses of the purified enzymes

Name	Whole enzyme (kDa)	Subunit (kDa)	Whole enzyme / Subunit	Possible structure
Propionate CoA-transferase	224	67	3.4	α_4
(*R*)-Lactate dehydrogenase	65	37	1.8	α_2
(*S*)-Glutamate dehydrogenase	229	43	5.3	α_6

For gel electrophoresis in the presence of SDS the following standards were used: bovine serum albumin 68 kDa, glycerinaldehyde-6-phosphate dehydrogenase 36 kDa, α-chymotrypsinogen 25 kDa. For gel filtration the following standards were applied: thyroglobulin 670 kDa, immunoglobulin G 150 kDa, ovalbumin 45 kDa, myoglobin 17.5 kDa and cyanocobalamin 1355 kDa

Table 4

Effectors of ^{3}HOH elimination from (*R*)-[3-^{3}H]lactate

Substance added	Concentration (mM)	Relative activity (%)	Influence on hydroxyglutarate dehydration as tested in [1]
Standard assay		100	
Standard assay without CoASH and acetyl phosphate		3	
ATP	0.63	8	Stimulation
$MgCl_2$	6.25	52	Stimulation
NaCl	91.0	50	Inhibition
NADH	0.006	87	Stimulation
FAD	0.63	13	n.d.
Acrylate	3.12	5	n.d.
Propionate	3.12	21	n.d.
NH_2OH	0.91	10	Inhibition
Na_3AsO_4	0.91	13	None
2,4-Dinitrophenol	0.091	13	Inhibition
NaN_3	0.91	14	Inhibition
K_2EDTA	9.1	8	Inhibition
Oxygen	–	10	Inhibition

The compounds ATP to propionate were added directly to the standard assay. The inhibitors hydroxylamine to EDTA were added 2 min before the reaction was started with substrate. In the last experiment the incubation mixture was exposed to air for 15 min prior to the addition of the substrate

3.4. *The dehydration of (R)-lactate*

The dehydration of (*R*)-lactate in cell-free extracts of *C. propionicum* was assayed by the same method as developed for the dehydration of (*R*)-2-hydroxyglutarate [1]. Thus (*R*)-[3-^{3}H]lactate was used as a substrate and the tritiated water formed during the reaction was measured. The labelled compound was prepared from commercially available (*S*)-[3-^{3}H]alanine by the consecutive action of alanine aminotransferase and

(*R*)-lactate dehydrogenase from *C. propionicum* (see sections 2 and 3.3).

Using this assay relatively high rates of ^3HOH formation (2000 counts·min^{-1} released per min) were obtained. However, due to the low substrate concentration (5 μM), the absolute rates (units/mg) were very low (table 1). Like (*R*)-2-hydroxyglutarate dehydratase, the enzyme or enzyme system required acetyl phosphate and CoASH for activity (table 4). However, activation with ATP, NADH and $MgCl_2$ was not necessary. Moreover ATP and $MgCl_2$ were inhibitory and NADH had no significant influence on the reaction rate. Hydroxylamine, arsenate, azide (1 mM each) and 0.1 mM dinitrophenol, which were found to prevent the fermentation of lactate and acrylate in cell-free extracts of *M. elsdenii* [3], also inhibited the dehydration of (*R*)-lactate in the present system at the same surprisingly low concentrations. Since with the exception of arsenate these compounds were also effective in the dehydration of (*R*)-2-hydroxyglutarate, a close relationship of both systems was demonstrated. In addition both dehydratases were inhibited by relatively high concentrations of EDTA (10 mM), probably due to Fe^{2+} tightly bound to the enzymes [8].

By using (*R*)-[U-^{14}C]lactate as a substrate and analysing the reaction mixture by high-performance liquid chromatography on a reversed-phase column only [^{14}C]propionate but not acrylate could be detected, although the retention times of both compounds differed by 1.3 min. Trapping of [^{14}C]acrylate with unlabelled acrylate failed since this acid was shown to be inhibitory. However this experiment might indicate that free acrylate is not a necessary intermediate as shown in scheme 1. Furthermore the propionate CoA-transferase with a preference for (*R*)-lactate could be involved in the dehydration of (*R*)-lactate. Thus the actual substrate might be lactoyl-CoA while the product acrylyl-CoA is immediately reduced to propionyl-CoA without forming free acrylate. Therefore the enzyme should be called tentatively lactoyl-CoA dehydratase. However, the enzyme listed as EC 4.2.1.54 [12] is certainly different from that described here, since it is assayed under aerobic conditions.

In summary this paper describes the direct measurement of the dehydration of (*R*)-lactate in cell-free extracts from *C. propionicum*. The mechanism of the reaction is closely related to the dehydration of (*R*)-2-hydroxyglutarate although certain differences have been detected.

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