HYDROGENATION OF Δ^2-CARBOXYLIC ACIDS NOT ACTIVATED IN FORM OF ENOYL CO-ENZYME A IN A CELL FREE SYSTEM OF *CLOSTRIDIUM KLUYVERI*

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1. Introduction

Recently we observed that it is possible to reduce a large number of very different Δ^2-unsaturated acids with *C. kluyveri* [1–5]. Electron donors can be crotonate or butyrate [3] as well as hydrogen gas [1,2,4]. As far as the stereochemistry of the hydrogenation products has been determined we observed, with two occasional exceptions, strict stereospecificity [2,3,5]. The questions arose as to whether hydrogenations are possible by cell free systems and if such different substrates like tiglinate, cinnamate [1,5], R- and S-allenecarboxylic aicds [2] and 2-halogenated Δ^2-carboxylic acids [4] are all transformed to the corresponding enoyl-CoA derivatives and then reduced or whether they are hydrogenated in a non-CoA-activated form. We observed in *C. kluyveri* besides the butyryl-CoA dehydrogenase the presence of a reductase which adds hydrogen also in a transmechanism but opposite to that of the butyryl-CoA dehydrogenase [5].

In this paper we report about the hydrogenation of crotonate and tiglinate with hydrogen gas catalyzed by supernatants of lysates of *C. kluyveri*. Furthermore, these lysates hydrogenate under conditions where no acyl-CoA compounds exist. Since free acids are neither substrates nor inhibitors of acyl-CoA dehydrogenase [6] an enzyme not so far described must exist.

2. Materials and methods

All enzymes were from C. F. Boehringer, Mannheim and acyl-CoA derivatives from P. L. Biochemicals, Inc., Milwaukee. The origin and cultivation of *C. kluyveri* on crotonate has recently been described [3,5]. The cells grown on ethanol/acetate were obtained from C. F. Boehringer, Tutzing and propagated according to Stadtman and Barker [7].

Preparation of the lysates: A 40 ml suspension contained 5 g wet packed cells, potassium chloride 0.09 M, phosphate buffer 0.01 M, pH 7.0, 20 mg ($4.4 \cdot 10^5$ U) lysozyme [8] and 2 mg (2000 U) DNase [9]. After shaking for 15 min at 35°C the suspension was centrifuged for 10 min at 15 000 *g*. The protein content was between 10.5 and 12.0 mg/ml.

Incubations without arsenate contained 2.0 ml lysate, 0.75 mg streptomycin and 200 μmol crotonate or tiglinate. For the fermentation experiments lysates of cells grown on ethanol/acetate contained 150 μmol ethanol and 100 μmol acetate. The incubation mixtures were shaken under an atmosphere of hydrogen or nitrogen at 35°C. Incubations with arsenate contained, in addition, arsenate (0.067 M), and 20 U phosphotransacetylase. The substrate was added after a preincubation period of 15 min.

Determination of the rate of hydrolysis of acetyl-CoA in the presence of arsenate in a model system: 5 μl of lysate were added to 1 ml containing acetyl CoA (0.3 mM), acetate (30 mM), arsenate (0.067 M), KCl (0.09 M) and phosphate buffer (0.01 M), pH 7.0. The disappearance of acyl-CoA was measured at 232 nm. Analysis of the incubation products: under an atmosphere of hydrogen or nitrogen aliquots of 0.2 ml were taken and quenched in 1.0 N HCl. After centrifugation 5 μl aliquots were injected in a glc apparatus operated at 140°C with a column (0.3 × 180 cm) filled with 20% neopentylglycol succinate and 2% phosphoric acid on chromosorb P.

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3. Results and discussion

We used lysates of *C. kluyveri* grown on crotonate or on ethanol/acetate [3,5,7].

Table 1 shows that hydrogenation of tiglinate or crotonate is possible by a cell free system. The rate of hydrogenation of lysates during the first 3 hr is about 50% of that of intact cells. This reveals that all enzymes necessary for the hydrogenation are soluble. In agreement with these results are the findings of Korkes [10] which show, that cell-free extracts of *C. kluyveri* catalyze the reduction of pyridine nucleotide with molecular hydrogen.

The following results prove the total hydrolysis of acyl-CoA derivatives [11]: In a model system 5 μl of lysate containing arsenate and phosphotransacetylase hydrolyzed acetyl-CoA with a rate of 1.2 μmol/min. This means that in the presence of arsenate 2 ml of an incubation mixture is able to hydrolyze 480 μmol of acetyl-CoA per minute. Corresponding assays with butyryl-CoA or crotonyl-CoA instead of acetyl-CoA gave hydrolyzation rates of 5 μmol butyryl-CoA/min and 280 μmol crotonyl-CoA/min in 2 ml of lysate. Therefore after 15 min preincubation with arsenate and phosphotransacetylase no significant concentration of an acyl-CoA compound should exist.

Table 1
Comparison of the hydrogenation activity of intact cells of *C. kluyveri* grown on crotonate and supernatants of lysates

Incubation	Products, in μmol	
	3 hr	7 hr
Lysate + tiglinate	75	140
Intact cells + tiglinate	138	182
Lysate + crotonate	115	218
Intact cells + crotonate	202	223

200 μmol tiglinate or crotonate were incubated together with 0.4 g wet packed cells or the lysate corresponding to the same amount of cells under an atmosphere of hydrogen.

As shown in table 2 cells grown on ethanol/acetate hydrogenate tiglinate and crotonate in the absence of acyl-CoA compounds with initial rates only slightly diminished when compared with lysate containing acyl-CoA compounds. The same is true for lysates of cells grown on crotonate, but the difference in rate between the control experiment and that with arsenate is higher than the difference in rate with lysates of cells grown on ethanol/acetate. An explanation would be that under the conditions of the control experiment with lysates from cells grown on crotonate, tiglinate or crotonate are hydrogenated by two enzymes, the

Table 2
Hydrogenation of crotonate or tiglinate with hydrogen gas catalyzed by lysates of *C. kluyveri* in the absence or presence of the acyl-CoA-hydrolyzing system

Exp. No.	Substrate	Incubation time (hr)	Control-incubation products[a] (μmol)	Incubation with acyl-CoA-hydrolyzing system products[a] (μmol)
1	Tiglinate	2	25	27
		5	72	64
		23	177	182
2	Crotonate	1	34	27
		4	100	72
		17	187	198
3	Tiglinate	3	22	20
		28	123	68
4	Crotonate	3	69	21
		28	92	84

[a] The product of the hydrogenation of tiglinate is 2-methylbutyrate and that of crotonate is butyrate.

Experiments 1 and 2 were conducted with cells grown on ethanol/acetate and experiments 3 and 4 with cells grown on crotonate.

Table 3
Fermentation by lysates under an atmosphere of nitrogen with and without the acyl-CoA-hydrolyzing system

Exp. No.	Substrate	Incubation time (hr)	Control-incubation products[a] in μmol	Incubation with acyl-CoA-hydrolyzing system products[a] in μmol
1	Crotonate	1	None	None
		4	7	1
2	Ethanol/	1	5[b] + 9[c]	None
	acetate	4	18 + 26	None
3	Crotonate	3	74 + 102[d]	2 + 2[d]
		28	74 + 136	16 + 30

[a] The product of the fermentation of crotonate is butyrate and those of ethanol/acetate are butyrate and caproate.
[b] Butyrate
[c] Caproate
[d] Acetate
Experiments 1 and 2 were conducted with cells grown on ethanol/acetate and experiment 3 with cells grown on crotonate.

butyryl-CoA dehydrogenase and the reductase which does not reduce CoA-activated acids. *C. kluyveri* cells grown on ethanol/acetate however are not able to ferment cronateto [12] and therefore the butyryl-CoA dehydrogenase, which is present in the lysates, has no substrate.

Table 3 shows that lysates of cells grown on ethanol/acetate ferment crotonate only in negligible quantities. The presence of arsenate inhibits the fermentation of ethanol and acetate or reduces it drastically.

The hydrogenations in the presence of arsenate do not exclude another kind of activation. On the other hand, from an energetic point of view, a hydrogenation without activation should be feasible. So far only a few rather special examples for the reduction of Δ^2-monocarboxylic acids by pyridine nucleotide dependent enzymes, such as β-nitroacrylate to β-nitropropionate [13] and *o*-coumarate to melilotate [14] are known.

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