Identification of acrylate, the product of the dehydration of (*R*)-lactate catalysed by cell-free extracts from *Clostridium propionicum*

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Cell extracts from *Clostridium propionicum* harvested in the late log-phase catalysed the dehydration of (*R*)-lactate to acrylate at a maximum rate of 0 06 U/mg protein The unsaturated acid was identified by high-performance liquid chromatography and as *p*-bromophenacyl ester by gas chromatography combined with mass spectroscopy The amount of acrylate formed was dependent on protein and (*R*)-lactate concentrations However, due to product inhibition the yield of acrylate did not exceed 0 5% Like the dehydration of (*R*)-2-hydroxyglutarate to glutaconate the dehydration of (*R*)-lactate to acrylate was inhibited by 1 mM hydroxylamine, 1mM azide, 0 1 mM dinitrophenol, 10 mM EDTA or by exposure to air A radical mechanism is postulated

(R)-*Lactate* *Dehydration* *Acrylate* p-*Bromophenacyl ester* *Oxygen inactivation*
Clostridium propionicum

1. INTRODUCTION

The mechanism of the dehydration of lactate to acrylate, which proceeds apparently against the rule of Markownikoff, remains an intriguing problem. In a previous paper we demonstrated the formation of ^{3}HOH from (*R*)-[3-^{3}-H]lactate for the first time in cell-free extracts. The dehydration had to be performed anaerobically, it required acetyl phosphate and CoASH as cofactors and was inhibited by hydroxylamine, arsenate, azide (1 mM each) or 0.1 mM 2,4-dinitrophenol, although the enzyme or enzyme system was found in the soluble fraction. However, only propionate and no acrylate could be detected as second product Moreover, trapping experiments with [U-^{14}C]lactate and unlabelled acrylate failed. Therefore, the existence of acrylate as a product of the dehydration became questionable [1].

Here we describe different growth conditions for *Clostridium propionicum* yielding a cell-free extract which catalysed the dehydration of (*R*)-lactate much more efficiently. Acrylate which accumulated thereby was identified by high-performance liquid chromatography (HPLC), gas-liquid chromatography and mass spectroscopy.

2. MATERIALS AND METHODS

Sources and media for *C. propionicum* and *Acidaminococcus fermentans* have been described [1,2]. Cells were harvested in the late log-phase. The preparation of cell-free extracts [3] and the dehydratase assays were performed under a strict anaerobic atmosphere [1,4]. (*R*)-Lactate dehydratase was assayed with an extract which was passed through Sephadex G-25 in 20 mM Pipes buffer (pH 7.0) immediately before use in order to remove endogenous short-chain fatty acids and cofactors such as NAD By this treatment the activity of the extract became unstable and was reduced to 10% within 8 h at 0°C The activity of the untreated extract, however, remained stable when stored at −80°C. The formation of acrylate

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was measured at 37°C in a total volume of 0.2 ml containing 60 mM Pipes (pH 7.0), 5 mM acetyl phosphate, 0.5 mM CoA, 75 mM (*R*)-lactate and 0.5 mg extract protein unless otherwise indicated. In some cases DTT and $FeSO_4$ (2 5 mM each) were included to remove the last traces of oxygen. After usually 5 min the reaction was stopped with 10 μl of 4 N sulfuric acid. Precipitation of the protein was completed by further incubation at 0°C for 30 min Finally a standard (1 μmol) was added and the supernatant was analysed for short-chain fatty acids

HPLC of these acids was performed on a RP-18 column (LiChrosorb, Merck, 250 × 4 mm) in 20 mM H_2SO_4 at 1 ml/min A linear gradient of 0–3% CH_3CN was applied after 2 min until 15 min, when it was kept constant for a further 5 min required for elution of the isobutyric acid used as internal standard. The acids were detected at 214 nm They were also separated by gas chromatography on a teflon column (2 × 1000 mm) filled with Chromosorb 104 in nitrogen (30 ml/min) and detected by flame ionisation The temperature was started at 140°C, raised to 158°C (3°C/min), kept constant for 5 min and raised further to 185°C (3°C/min) to detect the internal standard caproic acid.

Acrylate and propionate were converted to their *p*-bromophenacyl ester as in [5] and purified by HPLC (see above) The solvent was 50% CH_3CN The esters were detected at 254 nm For combined gas chromatography and mass spectroscopy (GC-MS) the purified esters were dissolved in diethyl ether (~0.1 M). A Hewlett Packard 5995 instrument was used equipped with a Durband 1701 capillary column (30 m, 190°C) with He (1 ml/min) as carrier gas.

The activity of the enzymes (*R*)-lactate dehydrogenase [1], propionate CoA-transferase [1], (*R*)-2-hydroxyglutarate dehydrogenase [2] and glutaconate CoA-transferase [6] were measured by described procedures. (*R*)-Lactate was determined by the formazan method [2] using purified (*R*)-lactate dehydrogenase [1]. The preparation of ^3H and ^{14}C-labelled lactates was reported previously [1]

3 RESULTS

3.1. *The influence of growth conditions on dehydratase activities*

During the work on (*R*)-lactate dehydratase a considerable variation of activity between different batches of *C propionicum* was observed In some cases the activity was even completely absent although (*R*)-lactate dehydrogenase and propionate CoA-transferase, which also belong to the pathway of alanine fermentation [1], were present in high levels. Finally, the dehydratase activity was found to be dependent on the growth phase of the bacteria Maximum activity was measured during the late log-phase (10 h) whereas the activity decreased to zero during the stationary phase (24 h). At this time only a 30% reduction of the other 2 enzymes was observed. Since all attempts failed to reactivate (*R*)-lactate dehydratase as described for (*R*)-2-hydroxyglutarate dehydratase [4], the following experiments were performed with cells harvested in the late log-phase.

It may be of interest that almost identical results were obtained with *A. fermantans* Whereas (*R*)-2-hydroxyglutarate dehydrogenase and glutaconate CoA-transferase activities remained fairly constant during the growth period of this organism, (*R*)-2-hydroxyglutarate dehydratase activity again showed a maximum in the late log-phase and was absent in the late stationary phase. However, this enzyme was reactivated by incubation with ATP, NADH and $MgCl_2$.

3 2. *Acrylate production from* (R)-*lactate*

On incubation of a cell-free extract prepared from late log-phase cells of *C. propionicum* with (*R*)-lactate, acrylate was formed. It was analyzed by gas chromatography or preferentially by HPLC, since it was detected easily at 214 nm due to the absorption of the double bond The acrylate was converted to its *p*-bromophenacyl ester [5] from which a mass spectrum was obtained. Besides the ions of the molecule (*m/e* 268 and 270, due to the naturally occurring ^{79}Br and ^{81}Br isotopes in a 1 : 1 ratio), the acryloyl ion ($CH_2=CH-CO^+$, *m/e* 55) was the most characteristic one. Furthermore, it was the most prominent ion in the mass spectrum of free acrylic acid but absent in the spectra derived from the *p*-bromophenacyl esters of acetate, propionate and butyrate [7]. Acrylate was only

formed from (*R*)-lactate and not from the (*S*)-enantiomer. This was expected since the lactate dehydrogenase from *C. propionicum* was (*R*)-specific.

The production of acrylate was not linearly dependent on the protein concentration (fig 1) Whereas below 1 mg/ml almost no acrylate would be detected, at 1 6 mg/ml the acid was formed at 80% of the maximum rate The threshold at about 1 mg protein/ml indicates that the dehydratase is composed of more than one factor of high molecular mass. Curiously, the amount of acrylate formed from 75 mM (*R*)-lactate never exceeded 0.35–0.45 mM (figs 1 and 2) regardless whether the protein concentration or the incubation time was increased. Thus only 0.5% of (*R*)-lactate was dehydrated to acrylate although the equilibrium should have been reached at about 20% conversion (K_{eq} = [acrylate]/[lactate] = 0.2–0.3 as compared to the fumarase equilibrium [7]). That the acrylate was indeed derived from (*R*)-lactate and not from an impurity was demonstrated by a control experiment in which (*R*)-[U-^{14}C]lactate (100 000 cpm) was added to the unlabelled substrate. Again only 0.5% [^{14}C]acrylate (500 cpm) could be detected

Since the concentration of (*R*)-lactate remained constant during the experiment of fig.2 (not shown), the termination of the reaction far from equilibrium could only be due to product inhibition as observed with the dehydration assay using [3-^{3}H]lactate as substrate [1]. By varying the concentration of (*R*)-lactate (1.7 mg protein/ml) it was shown that at 75 mM the highest acrylate concentration accumulated (0.37 mM in this experiment).

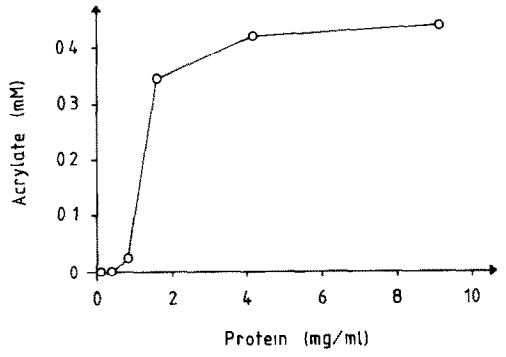

Fig 1 The dependence of acrylate formation on the concentration of cell-free extract protein For further details see section 2

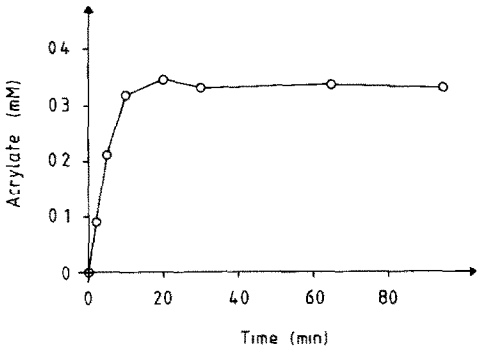

Fig.2 The dependence of acrylate formation on the reaction time The protein concentration was 3 mg/ml

At 25, 125 and 200 mM (*R*)-lactate only 0.16, 0.34 and 0 25 mM acrylate, respectively, was produced Thus, also the substrate was inhibitory at high concentrations. The fastest rate of acrylate formation was 0.06 U/mg protein. This compared well with the other assay in which 0.1 μmol ^{3}HOH min^{-1} mg^{-1} was generated from 75 mM [3-^{3}H]lactate

3.3. *Inhibitors of acrylate formation*

The dehydration of (*R*)-[3-^{3}H]lactate to ^{3}HOH was specifically inhibited by a series of compounds [1]. All with the exception of arsenate also prevented the formation of acrylate (table 1) This demonstrated again that the same reaction was measured with both assays However, the activation rather than the expected inhibition by arsenate reflected presumably the different conditions which had been applied. In the ^{3}H assay, acrylate was immediately reduced to propionate due to the

Table 1

Inhibitors of acrylate formation

Inhibitor	mM	[Acrylate] (μM)	%
None	–	203	100
Hydroxylamine	1 0	0	0
Arsenate	1 0	317	156
2,4-Dinitrophenol	0 1	<1	<0.5
Azide	1 0	0	0
EDTA	10	0	0
Air	–	<1	<0 5

Prior to the addition of (*R*)-lactate the assay mixture (2 3 mg protein/ml) was incubated with the inhibitor for 5 min In the case of air the preincubation was 15 min

presence of high amounts of unpurified cell-free extract with low dehydratase activity Thus, side reactions might have caused the observed inhibition

4 DISCUSSION

The fermentation of acrylate to acetate and propionate was discovered in whole cells of *C propionicum* [8] and in cell-free extracts from *Megasphera elsdenii* [9] Recently Akedo et al [11] reported a biological acrylate production with whole cells of the former organism in which the reduction to propionate was inhibited by 3-butenoic acid. Here we describe for the first time the formation of acrylate from (*R*)-lactate catalysed by a cell-free extract from *C propionicum* from which compounds of low molecular mass have been removed

Under these conditions acrylate was not reduced to propionate. Hence no inhibitor was necessary. However, acrylate inhibited its own formation which resulted in low yields This was not the case with whole cells which were probably able to keep the intracellular acrylate concentration at a low level [10]

The sensitivity of the dehydration of (*R*)-lactate to acrylate towards low concentrations of 2,4-dinitrophenol, hydroxylamine and azide demonstrates the close relationship to the analogous dehydration of (*R*)-2-hydroxyglutarate to *(E)*-glutaconate [4]. The mechanism of action of these inhibitors might be the trapping of free radicals which are probably involved in the reactions [4] This could also explain the extreme oxygen sensitivity of both dehydrations

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