ON THE FORMATION OF 3-PHENYLPROPIONATE AND THE DIFFERENT STEREO-CHEMICAL COURSE OF THE REDUCTION OF CINNAMATE BY *CLOSTRIDIUM SPOROGENES* AND *PEPTOSTREPTOCOCCUS ANAEROBIUS*

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1. Introduction

According to the literature 3-phenylpropionate, 4-methylpentanoate, 2-methylpropionate, 2- or 3-methylbutyrate and others are products of many different proteolytic and amino acid fermenting clostridia [1–3]. 4-Methylpentanoate and other volatile acids are also products of propionibacteria [4] or peptostreptococci [5]. Surprisingly the route and the enzymes leading to these products are not known.

We described enoate reductase a conjugated Fe–S flavoprotein which reduces nonactivated α,β-unsaturated carboxylates in a NADH-dependent reaction [6]. The source was *C. La 1* a non-proteolytic clostridium grown on (E)-2-butenoate. We found the same type of enzyme in *C. kluyveri* and the proteolytic *C. sporogenes*. However, their substrate specificity was rather different [7]. During our studies on the occurrence and physiological role of enoate reductases in amino acid fermenting clostridia we suggested an oxidative and a reductive branch to explain the above-mentioned products [7]. We compared the capability of resting cells of *C. sporogenes* as well as *Peptostreptococcus anaerobius* to convert L-phenylalanine, (2*R*)-phenyllactate, phenylpyruvate and cinnamate to 3-phenylpropionate. Nevertheless, the processes seem to be so similiar the sterical course of the reduction of (E) cinnamate to 3-phenylpropionate by *C. sporogenes* and *P. anaerobius* is different. The preparation of two further stereospecifically deuterated 3-phenylpropionates is an additional result.

2. Materials and methods

C. sporogenes (ATCC 3584) and *P. anaerobius* (DSM 20357) were grown as in [7]. For the fermentations depicted in table 1, 3 ml of a suspension containing 100 mM phosphate buffer (pH 7.0), 400 mg wet packed cells, 100 mM substrates and 0.075 mg tetracycline were shaken in Warburg vessels under an atmosphere of N_2 at 35°C. Cells of *P. anaerobius* were not washed since they lost too much of their activity.

High-pressure liquid chromatography on a reversed phase (RP-18, column 4 × 250 mm) was applied for the identification and quantitative determination of substrates and products given in table 1. The eluent was aqueous methanol (30% or 10%) with 30 mM formic acid. ORD measurements were conducted in ^2H_2O (sodium salts, 100 mg/ml) and in C^2HCl_3 (free acid, 80 mg/ml) using a JASCO 5 spectrometer. From these solutions PMR spectra were obtained.

The deuteration of cinnamate with *C. sporogenes* was carried out in 76 ml 100 mM phosphate buffer p^{2}H 7.8 containing 2.6 g freeze-dried cells, 5 mmol sodium (E) cinnamate, 1 mM methylviologen and 1.6 mg tetracycline. The suspension was stirred in a 500 ml double-neck bulb under an atmosphere of H_2 at 35°C. The hydrogen consumption was measured with a Warburg manometer filled with mercury. The reaction was complete after 40 h. The deuteration with *P. anaerobius* was done in a 2 mmol scale with 12 g unwashed wet packed cells. For the isolation of the 3-phenyl-[2,3-^{2}H]propionic acid see section 1.c. [8]. For the conversion of (2*S*,3*R*)-3-phenyl-[2,3-^{2}H]propionic acid to (3*R*)-3-phenyl[3-^{2}H]propionic acid 370 mg of the former were boiled for 5 h under nonhydrous conditions in 0.55 ml methanol containing 60 mg sulphuric acid. After adding of water the ester was extracted into ethyl ether, the latter evaporated, the residue dissolved in 1.75 ml 1 M sodium methox-

ide in methanol and kept at 50°C for 2 h. The isolated ester (150 mg) was hydrolyzed in 1 ml 1 M NaOH for 3 h at 50°C, the solution titrated with 0.1 N HCl to pH 7.5 and the sodium salt of (3*R*)-3-phenyl-[3-^{2}H]-propionate isolated by recrystallization (125 mg).

The exchange procedure was checked by keeping methyl 3-phenylpropionate (0.5 mmol) in 0.5 ml $C^2H_3O^2H$ containing 1 M C^2H_3ONa in a NMR tube. According to PMR both protons of the α-position were exchanged after 2 h at 50°C.

3. Results and discussion

The fermentation broth of *C. sporogenes* as well as of *P. anaerobius* grown on a complex medium supplemented by L-phenylalanine contains as a product besides others 3-phenylpropionate (not shown). The capability of resting cells to convert L-phenylalanine and derivatives under an atmosphere of nitrogen to 3-phenylpropionate is shown in table 1. The partial conversion of (*R*)-phenyllactate, phenylpyruvate and cinnamate to 3-phenylpropionate is a strong hint that these substances or derivatives of them are intermediates in the formation of phenylpropionate. In all cases the reduced products exceed the oxidized ones. The necessary reduction equivalents must derive from endogenous cell material. About half of the phenylpyruvate is converted to phenylacetate. Both organisms behave rather similarly.

Under an atmosphere of hydrogen and the presence of methylviologen (E) cinnamate can be quantitatively hydrogenated to phenylpropionate by whole cells of *C. sporogenes* as well as by cells of *P. anaerobius*. In order to determine the stereochemistry of the hydrogen addition we conducted the hydrogenations in ^2H_2O buffer. The rate for the reduction of (E) cinnamate by C. sporogenes showed a pronounced maximum at pD 7.8 The cells of *P. anaerobius* showed the rate maximum at pD 8.2. The PMR spectra of the products were in agreement with 3-phenyl-[2,3-^{2}H]propionate. The phenylpropionate of the reaction catalyzed by *C. sporogenes* possessed 0.94 atom deuterium each in the 2 and 3 position, and that of the reaction catalyzed by *P. anaerobius* 0.92 atom deuterium in each of the two positions. The ORD curve of both products turned out to be exact mirror images. The product obtained by hydrogenation with *C. sporogenes* was identical with that which we prepared with *C. La 1* and determined as (2*S*,3*R*)-3-phenyl-[2,3-^{2}H]propionate [8,9]. Though the enoate reductases of *C. La 1*, *C. kluyveri* and *C. sporogenes* are different in substrate specificity [7] and physical properties such as isoelectric points, they show an identical stereochemical course.

From the ORD curve we conclude that the reduction of cinnamate in ^2H_2O buffer with *P. anaerobius* led to (2*R*,3*S*)-3-phenyl-[2,3-^{2}H]propionate. However, this is only true if the optical rotation of the diastereomeric (2*R*,3*R*)-3-phenyl-[2,3-^{2}H]propionate is differ-

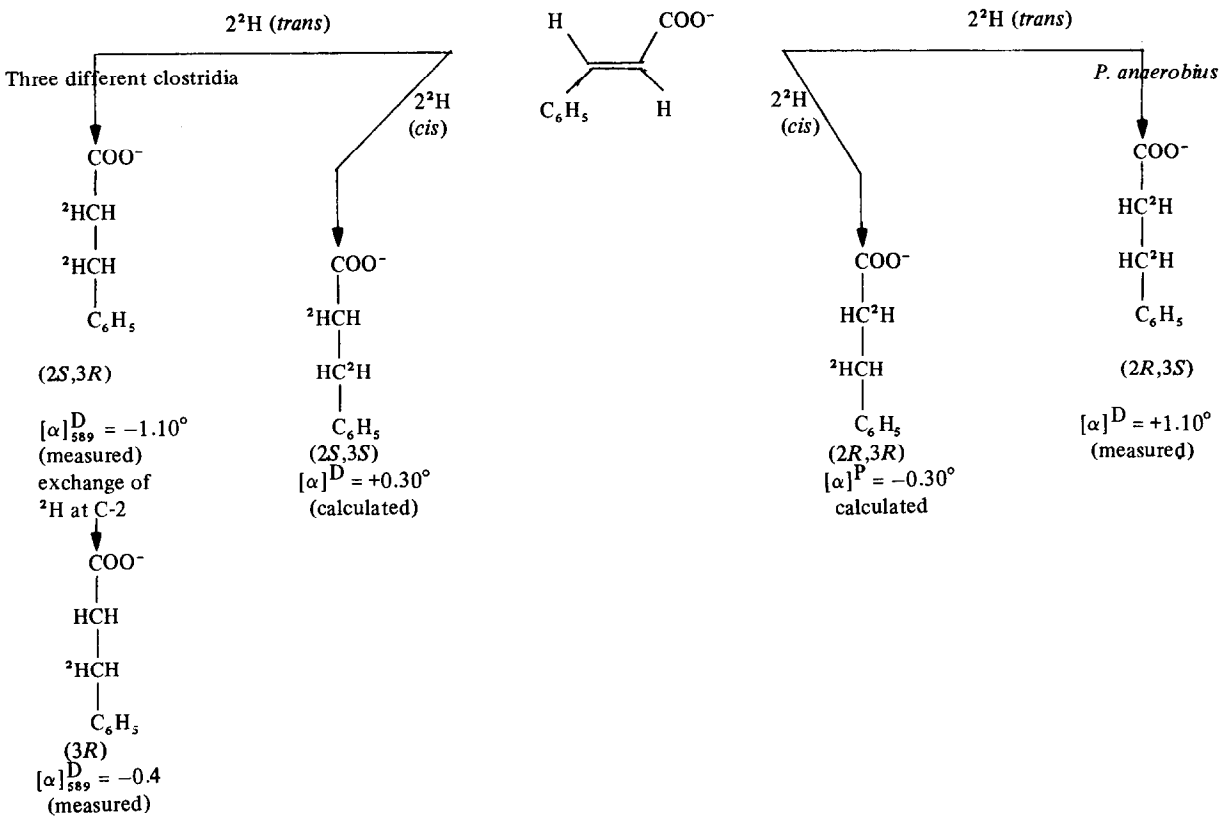
Table 1
Conversion of L-phenylalanine to 3-phenylpropionate and probable intermediates on this route to 3-phenylpropionate by resting cells of *Clostridium sporogenes* and *Peptostreptococcus anaerobius*, respectively

Organism	Substrate	Products [μmol]			
		(*R*)-Phenyllactate	Cinnamate	3-Phenylpropionate	Phenylacetate
C. sporogenes	L-Phenylalanine[a]	<2	<0.1	41	5
C. sporogenes	Cinnamate	4	33	57	<2
C. sporogenes	Phenylpyruvate	8	<0.1	41	43
C. sporogenes	(*R*)-Phenyllactate	11	<0.1	65	11
P. anaerobius[b]	L-Phenylalanine[c]	<2	3	86	28
P. anaerobius[b]	Cinnamate	0	8	116	14
P. anaerobius[b]	Phenylpyruvate	0	1	47	48
P. anaerobius[b]	(*R*)-Phenyllactate	17	6	82	19

[a] 62 μmol phenylalanine were not converted
[b] Washed cells showed nearly no activity. Attached medium and endogenous material may be the reason for a balance above 100 μmol
[c] No phenylalanine was left

Phosphate buffer (3 ml, 100 mM, pH 7.0) containing 100 μmol substrate and 400 mg wet packed cells were shaken under an atmosphere of nitrogen for 19 h at 35°C

Scheme 1



The measured optical rotations $[\alpha]^D$ of (2*S*,3*R*)-3-phenyl-[2,3-²H]propionate and (3*R*)-3-phenyl-[3-²H]propionate lead to the assumption that the (2*S*,3*S*) and (2*R*,3*R*) forms of 3-phenyl-[2,3-²H]propionate have an $[\alpha]^D$ = +0.30° and −0.30°, respectively. Therefore the product of the reduction by *P. anaerobius* must be the (2*R*,3*S*) form

ent from the (2*R*,3*S*)-derivative. Both forms could not be differentiated if the deuterium at carbon-3 would not or only slightly contribute to the rotation. Therefore we determined the contribution of the deuterium at carbon atom 2 and 3 of (2*S*,3*R*)-3-phenyl-[2,3-²H]propionate to the observed optical rotation.

The deuterium atom at C-2 of (2*S*,3*R*)-3-phenyl-[2,3-²H] propionate can be quantitatively exchanged with sodium methoxide in methanol as proved by *pmr* spectroscopy. By an experiment conducted with non-labelled 3-phenylpropionate and sodium methoxide in $C^2H_3O^2H$ it was checked, that only the protons at carbon atom 2 were quantitatively substituted by deuterium. Since there is no exchange at carbon atom 3, a racemization can be excluded. After the exchange reaction of methyl (2*S*,3*R*)-3-phenyl-[2,3-²H]propionate to methyl-[3*R*]-3-phenylpropionate the ester was converted to (3*R*)-3-phenyl-[3-²H]-propionate and its optical rotation determined (scheme 1).

Due to the rather similiar properties of ¹H and ²H it should be possible to calculate the optical rotations of the (2*S*,3*S*) as well as the (2*R*,3*R*) forms of 3-phenyl-[2,3-²H]propionate by adding the contributions of ²H in 2- and 3-position. According to scheme 1, in the case of the (2*S*,3*S*) form this would be:

$$[\alpha]^D_{(2S,3S)} = [\alpha]^D_{(2S)} + [\alpha]^D_{(3S)} = -0.70 + (+0.40)$$

$$= -0.30$$

Therefore the conclusion that the deuteration of cinnamate by *P. anaerobius* led to (2*R*,3*S*)-3-phenyl-[2,3-²H]-propionate seems to be correct. The *threo* forms (2*S*,3*S*) and (2*R*,3*R*) can be excluded. That means cinnamate is also reduced in a *trans* fashion but different from the mode of clostridia. The latter add hydrogen atoms in a *trans* fashion to the *si*-faces of

both C-2 and C-3 of cinnamate and *P. anaerobius* adds to the *re*-faces. That means the reductions are enzymatically different though the route seems to be so similiar.

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