OCCURRENCE AND THE POSSIBLE PHYSIOLOGICAL ROLE OF 2-ENOATE REDUCTASES

Matthias BÜHLER, Hermine GIESEL, Wilhelm TISCHER and Helmut SIMON

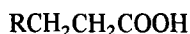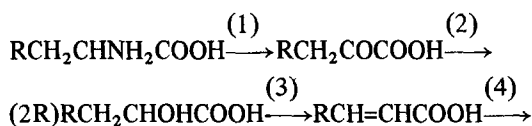*Institute for Organic Chemistry, Technical University Munich, D-8046 Garching, FRG*

1. Introduction

Recently we described the purification and some properties of a hitherto unknown NADH-dependent enzyme from the *Clostridium* species La 1 growing on (E)2-butenoate which reduces the carbon–carbon double bond of α,β-unsaturated carboxylate anions (2-enoates) [1]. We now report a purified enoate reductase from *Clostridium kluyveri* and the occurrence of such an activity in the typical amino acid fermenting species *Clostridium sporogenes*.

So far no physiological role of the reductase is known. In the metabolic schemes of clostridia fermenting (E)2-butenoate or ethanol and acetate such an enzyme was not postulated [2]. Up to 1.5% of the soluble protein of the *Clostridium* sp. La 1 grown under certain conditions consists of the reductase [3,4].

In *C. sporogenes* we found enzyme activities necessary for the following reaction sequence:

$$RCH_2CHNH_2COOH \xrightarrow{(1)} RCH_2COCOOH \xrightarrow{(2)}$$

$$(2R)RCH_2CHOHCOOH \xrightarrow{(3)} RCH=CHCOOH \xrightarrow{(4)}$$

$$RCH_2CH_2COOH$$

$$R=C_6H_5-, p(HO)C_6H_4- \text{ or } (CH_3)_2CH-$$

(2S)-phenyllactate is not a substrate

It is assumed that many clostridia, growing on protein hydrolysates or amino acid mixtures, obtain much of their energy by a coupled oxidation–reduction reaction between suitable amino acids, or amino acids and non-nitrogenous compounds. This reaction scheme, well known as the Stickland reaction [5], contains only reaction (1) but not (2), (3) and (4) of the above

reaction sequence. Furthermore, phenylalanine, tyrosine or isoleucine function in Stickland pairs as reductants. That means they are oxidized and not reduced. However, the formation of phenylpropionate and isocaproate (usually 3- and 4-methylpentanoate are not differentiated) by amino acid fermenting clostridia [6–8] or anaerobic Gram-positive cocci [9] has been often described, but no reports on the involved enzymes seem to exist. Moss et al. [6] not only demonstrated the formation of phenylpropionate from phenylalanine but also found that when suspensions of *C. sporogenes* were incubated with phenylalanine, cinnamate accumulated and was then reduced to phenylpropionate.

2. Materials and methods

(2S)-Phenyllactate was a product of Aldrich Chemical Co., (2R)-phenyllactate was obtained by the stereospecific hydrogenation of phenylpyruvate catalyzed by *Proteus mirabilis* (unpublished). Enoate reductases from *Clostridium* sp. La 1 (DSM 1460) and *C. kluyveri* (DSM 555) were purified as in [1]. For the growth of these clostridia see [3,4]. To the basal medium for *C. sporogenes* (ATCC 3584) 12 mM phenylalanine was added [7]. The same growth conditions were used for *Peptostreptococcus anaerobius* (DSM 20357).

All enzymic reactions were conducted under anaerobic conditions [1] and started by adding the substrates. In order to eliminate traces of oxygen the initial reaction rates of enoate reductases were determined in the presence of glucose, glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6). The samples contained in 2.15 ml 0.2 M phosphate buffer, 85 mM glucose, 4 mU glucose oxidase, 4 U catalase, 33 mM mercaptoethanol, 0.22 mM NADH and ≤23 mM substrate.

The hydrogen uptake in the presence of L-phenylalanine or other substrates by *C. sporogenes* was measured by the Warburg technique. In a vessel shaken at 35°C under an atmosphere of H_2 400 mg wet packed cells were suspended in 3.0 ml 0.1 M phosphate buffer (pH 7.0) containing 75 μg tetracycline hydrochloride and 33 mM of the substrate. The products were quantitatively determined by HPLC as in [1].

Crude cell extracts were prepared by treating 400 mg wet packed cells in 2.0 ml 0.1 M phosphate buffer (pH 7.0) containing 1.6 mg lysozyme, 200 U DNase and 1 mM EDTA at 35°C for 1 h.

3. Results

Enoate reductase activity is present not only in crude extracts of *Clostridium* sp. La 1 grown on (E)2-butenoate, but also in those of *C. kluyveri* and *C. sporogenes*. Tested with cinnamate as substrate in the presence of 0.2 M hydroxylamine their activity is ~0.1–0.5 U/mg protein. However, their substrate specificity is rather different as can be seen from table 1. The specificities of the enoate reductases from *Clostridium* sp. La 1 and *C. kluyveri* are rather broad but definitely different. The reductase from *C. sporogenes* acts more as a cinnamate reductase.

Resting cells of *C. sporogenes* convert under an atmosphere of hydrogen the following substrates to phenylpropionate in yields of 70–100%: (E)-cinnamate, (2R)phenyllactate, phenylpyruvate and L-phenylalanine. (2S)phenyllactate is not or almost not a substrate. Under the same conditions crude extracts show quite different behaviour: only cinnamate is converted into phenylpropionate, (2R)-phenyllactate does not react at all, and phenylpyruvate is decarboxylated to phenylacetate and to a small extent reduced to phenyllactate.

In crude extracts of *C. sporogenes* the following enzyme activities have been found (U/ml): phenylalanine 2-oxoglutarate aminotransferase (~0.04), NADH-dependent phenylpyruvate reductase (80) and cinnamate reductase (5.5). So far in crude extracts the elimination of water from (2R)-phenyllactate could not be demonstrated. Furthermore, no phenylalanine ammonia lyase could be observed. Also resting cells of *P. anaerobius* catalyze the hydrogenation of cinnamate to phenylpropionate.

4. Discussion

The transformation of an amino acid to the corresponding carboxylic acid and ammonia needs one pair of electrons. The above given sequence could be this

Table 1

Relative reaction rates of enoate reductases from 3 different species of clostridia with several substrates under saturating concentrations at their pH maximum (pH 6.0 for the reductase from *Clostridium* sp. La 1, pH 6.7 and 7.2 for the reductases from *C. kluyveri* and *C. sporogenes*, respectively)

Enoate	Enoate reductase		
	Clostridium sp. La 1[a]	*C. kluyveri*[a]	*C. sporogenes*[b]
(E)-2-methyl-2-butenoate	100	100	<2
(E)-butenoate	280	215	<2
(E)-3-methyl-2-pentenoate	11	23	<2
(Z)-3-methyl-2-pentenoate	11	19	<2
4-methyl-2-pentenoate	128	59	68
(E)-cinnamate	60	200	100
(E)-p-methoxy-cinnamate	41	100	9
(E)-p-nitro-cinnamate	44	52	–
(E)-a-formylamino-cinnamate	21	<2	<2

[a] Enzyme purified to homogenity; the DNA of both clostridia show no hybridization [3]

[b] Supernatant of crude extract

part of an amino acid fermentation which regenerates NAD from NADH. This could arise from a reaction of the following type:

$$RCH_2COCOOH + HX + NAD^+ \longrightarrow RCH_2COX +$$

$$CO_2 + NADH + H^+$$
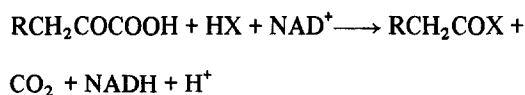
RCH_2COX may be a CoA-ester or another derivative energetically equivalent to ATP if well known reactions are assumed. Because of the reaction catalyzed by the enoate reductase NAD would be regenerated. The enoate as the electron acceptor and the energy-rich intermediate RCH_2COX would be formed from amino acids. The 3 different enoate reductases which have been found in 3 very different clostridia seem to indicate a rather frequent occurrence of this enzyme activity.

Besides the diversities shown in table 1 there is also a similarity in the substrate specificities. Cinnamate and 4-methyl-2-pentenoate which may be derived from phenylalanine and leucine, respectively, are good substrates for all three reductases.

As mentioned in the introduction most of the enzyme activities described herein are not part of the Stickland fermentation. The elimination of water from 2-hydroxy-acids seems to be a complex reaction which could so far only be conducted by whole cells.

The hydrogenation of cinnamate by *P. anaerobius* to phenylpropionate may be a hint for the occurrence of an enoate reductase even in anaerobic bacteria other than clostridia.

References

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