ON THE MECHANISM OF 2-ENOATE REDUCTASE

Elimination of halogen hydracids from 3-halogeno-2-enoates during reduction with NADH

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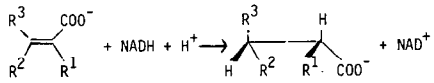
1. Introduction

During our studies about the stereospecific hydrogenation of organic compounds by microorganisms we observed the reductive dehydrohalogenation and hydrogenation of several 3-halogeno-2-enoates by *Clostridia* in the presence of hydrogen gas [1]. Later we discovered in these *Clostridia* a reductase activity which showed a stereochemistry different from that of the butyryl-CoA dehydrogenase [2]. Recently, we described the purification and partial characterization of this hitherto unknown 2-enoate reductase [3]. It turned out to be a conjugated iron-sulphur flavoprotein which reduces nonactivated 2-enoates as follows:



(1)

The groups R can vary to a great extent. Using 3-halogeno-2-enoates such as (*Z*)3-chlorocinnamate, (*E*) and (*Z*)3-bromocinnamate and (*Z*)3-chloro-2-butenoate we observed the complex reaction:

$$RCX{=}CHCOO^- + 2H^+ + 2NADH \longrightarrow RCH_2CH_2COO^- + HX + 2NAD^+ \qquad (2)$$

That means the reduction is accompanied by an elimination of a halogen hydracid. Some results of this reaction are reported since they may shed some light on the mechanisms of reactions (1) and (2). The saturated 3-halogeno acids are not intermediates in reaction (2). Therefore we suggest an initial 1.4 addition of a hydride and a proton as depicted in the scheme.

2. Materials and methods

Enoate reductase was purified as in [3]. According to the indicated references the following acids were synthesized: (*Z*)3-chloro-[4], (*Z*)3-bromo-[5], (*E*)3-bromocinnamic [5], (*Z*)3-chloro-2-butenoic [6], (*R,S*)3-chlorobutyric and (*R,S*)3-bromo-3-phenylpropionic acid [7]. The synthesis of (*R,S*)3-chloro-3-phenylpropionic acid was conducted similar to that in [7]. However, the addition reaction was performed at –45°C in an aqueous solution which was saturated with HCl.

All enzymic reactions were conducted under anaerobic conditions and started by adding the substrates. The initial reaction rates were determined in samples which contained in 2.1 ml total vol. buffer (pH 6.0), citrate 170 mM, sodium phosphate 61 mM, NADH 0.3 mM, enzyme 0.2 U, 34 mM mercaptoethanol and substrate 4 mM.

In order to measure the stoichiometry of the reaction, NADH was 0.41 mM and that of the substrates only 0.14 mM.

The experiments with substoichiometric amounts of NADH contained in 2.1–2.4 ml total vol. buffer and enzyme activity as given above, NADH at 0.28–2.60 mM and 5 μmol substrate. The reactions with (*Z*)3-chloro-2-butenoate were performed in 0.09 M phosphate buffer (pH 6) and 2 μmol substrate.

The high-pressure liquid chromatography (HPCL) analyses were carried out as in [3] and the gas–liquid chromatography (GLC) as in [8].

3. Results

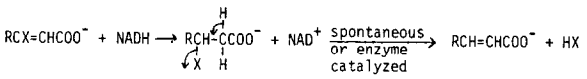
The consumption of NADH by several substrates of

Table 1
Consumption of NADH for the reduction of different substrates and relative initial reaction rates

Substrate	Amount (μmol)	Consumption NADH (μmol)	Relative initial reaction rate
(*E*)cinnamate	1.00	1.0	1.00
(*Z*)cinnamate	–	–	~0.05
(*Z*)3-chloro-cinnamate	1.00	1.9	0.36
(*Z*)3-bromo-cinnamate	1.00	2.0	0.72
(*Z*)3-chloro-2-butenoate	1.00	1.9	–
(*E*)3-bromo-cinnamate	–	–	~0.05

(Estimated error of amounts ≤ ± 5%)

enoate reductase are shown in table 1. Within the experimental error 1 mol cinnamate consumes 1 mol NADH and the three (*Z*)3-halogeno-2-enoates 2 mol, respectively. The initial reaction rates measured by the disappearance of NADH are not very different for the cinnamates with the phenyl group in the *trans* position to the carboxy group. That means the halogen in *cis* position to the carboxy group has no great influence. The rather slow reaction of (*E*)3-bromo-cinnamate may be due to the *cis* standing phenyl group since the (*Z*)cinnamate reacts also distinctly slower than the (*E*)cinnamate. These results could be explained by reaction (2). The question arose whether the corresponding saturated 3-halogeno carboxylates are intermediates of reaction (2) eliminating HX in a spontaneous fashion or catalyzed by enoate reductase (reaction (3)):



(3)

Such an enzyme-catalyzed elimination of HX was observed [9,10] for 3-chloro-aminoacids by D-amino acid oxidase and for 3-chloro-2-hydroxy-propionate by L-lactate dehydrogenase from *Mycobacterium smegmatis* [11]. Both oxidases are flavoproteins.

Tables 2 and 3 show the analysis of intermediates and products during the reduction of (*Z*)3-bromo-

Table 2
Amounts of substrate, intermediate and product after the reduction of 5.0 μmol (*Z*)3-bromocinnamate with substoichiometric amounts of NADH

NADH	(*Z*)3-Bromo-cinnamate	Cinnamate	3-Phenyl-propionate	Sum of columns 2 + 3 + 4
0.60	4.2	0.50	<0.2	4.7
1.15	4.5	0.68	0.2	5.4
2.05	3.7	0.62	0.7	5.0
2.05	3.6	0.64	0.7	4.9
3.35	3.0	0.46	1.4	4.9
3.50	3.1	0.49	1.3	4.9
5.80	1.8	0.53	2.5	4.8

All values are given in μmol. (Estimated error of amounts given in columns 1 and 2, ≤ ± 5%, and ≤ ± 10% in columns 3 and 4, respectively)

Table 3
Amounts of substrate, intermediate and product after the reduction of 2.0 μmol (*Z*)3-chloro-2-butenoate with substoichiometric amounts of NADH

NADH	(*Z*)3-Chloro-3-butenoate	2-Butenoate	Butyrate	Sum of columns 2 + 3 + 4
0.51	1.64	0.08[a]	0.03[a]	1.75
2.15	0.73	0.56	0.70	1.99

[a] These are minimal values due to losses during concentration

All values are given in μmol. (Estimated errors as given in table 2)

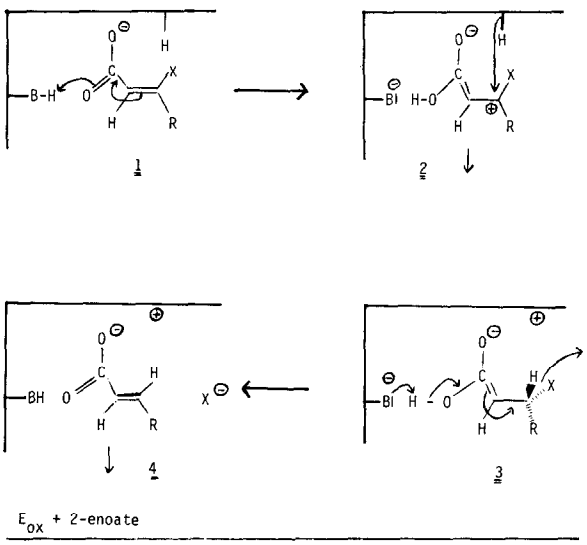
cinnamate and (*Z*)3-chloro-2-butenoate with substoichiometric amounts of NADH.

All experiments shown in table 2 were analyzed by a HPCL apparatus equipped with an ultraviolet-spectrometer, which detects especially sensitive cinnamate and derivatives. But also 3-phenylpropionate can be measured with the indicated accuracy. 3-Bromo-3-phenylpropionate, a possible intermediate, could not be detected. Therefore we synthesized 3-bromo-3-phenylpropionic acid and studied its behaviour. We found its anion extremely unstable in 2 M D_2O phosphate buffer (pH 6.8). It decomposes into two products. Their ratio of formation depends on the pH. At neutral pH the main product is styrene which could be characterized by its smell but also by NMR and HPCL. The second product formed is, 3-hydroxy-3-phenylpropionate. However, cinnamate as a product of HBr elimination from 3-bromo-3-phenylpropionate could never be detected.

Therefore, 3-bromo-3-phenylpropionate can be excluded as a free intermediate in reaction (2) by the following facts: During the enzymic reduction of (*Z*)3-bromocinnamate we were never able to detect a trace of styrene. The spontaneous decomposition of 3-bromo-3-phenylpropionate into styrene is much faster than the enzymic formation of 3-phenylpropionate. However, according to table 1, 2 mol NADH are consumed by 1 mol (*Z*)3-bromocinnamate. As can be seen from table 2, the stoichiometry is correct in between the experimental error for all experiments. 3-Hydroxy-3-phenylpropionate which could also be formed to a certain extent from 3-bromo-3-phenylpropionate, is no substrate for the enoate reductase. As shown in table 3 no 3-chlorobutyrate could be detected as an intermediate in the reduction of (*Z*)3-chloro-2-butenoate. By control experiments it was carefully checked which percentage of 3-chlorobutyrate eliminates HCl during the injection into the gas chromatography. Under the conditions used, 15–20% of 3-chlorobutyrate are converted to 2-butenoate. Due to the high sensitivity of GLC operating with a flame ionization detector <0.04 μmol 3-chlorobutyrate of the experiment shown in table 3 would have been detected. In contrast to 3-bromo-3-phenylpropionate, 3-chlorobutyrate is completely stable under the conditions of the enzymic test. It turned out that (*R,S*)3-chlorobutyrate is no substrate for the enoate reductase. Therefore, the path as depicted by reaction 3 can be excluded by several lines of evidence. We have to assume that the halogen-free 2-enoate leaves the enzyme (table 2, 3). A direct substitution of the halogen by a hydride ion is rather unlikely. There is no example of such a substitution on a sp^2 carbon atom under comparable conditions. Also an elimination of HX from the 3-halogeno-2-enoate under formation of a triple bond is very unlikely since 3-phenylpropinate applied under different concentrations is no substrate but an inhibitor of enoate reductase [12].

The observations above lead to the conclusion that the first hydrogenation step is coupled to the elimination of HX. This could occur in the following fashion (scheme 1): A proton-mediated substrate activation occurs by the initial enzymic protonation of one of the oxygens of the carboxy group $\underline{\underline{1}}$, leading to a positivation of C-3 as depicted by the mesomeric form $\underline{\underline{2}}$. This species takes up a hydride from the reduced enzyme ($\underline{\underline{2}} \rightarrow \underline{\underline{3}}$) and stabilizes itself by elimination of HX ($\underline{\underline{3}} \rightarrow \underline{\underline{4}}$). The complex $\underline{\underline{4}}$ of 2-enoate and the enzyme in the oxidized state decomposes into the 2-enoate and the enzyme. One could assume that the enzyme has to be reduced before 2-enoate can be

Scheme 1
Suggested mechanism of 2-enoate reductase



X = halogen such as Br or Cl

bound again. We would like to suggest this mechanism as a possibility in view of the well-established requirement for proton-mediated substrate activation before hydride transfer in the saturation of the Δ^4-double bond of 3-oxo-4-en-steroids by several reductases [13] and our experimental facts.

Acknowledgements

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