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Acid-catalyzed enzymatic hydrolysis of 1-methylcyclohexene oxide

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Abstract

Limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* DCL14, an enzyme involved in the limonene metabolism of this microorganism, catalyzes the enantioselective hydrolysis of 1-methylcyclohexene oxide. (1*R*,2*S*)-1-Methylcyclohexene oxide was the preferred substrate and it was mainly hydrolyzed to (1*S*,2*S*)-1-methylcyclohexane-1,2-diol, while (1*S*,2*R*)-1-methylcyclohexene oxide was converted more slowly and mainly yielded (1*R*,2*R*)-1-methylcyclohexane-1,2-diol. The reaction proceeded with a high regioselectivity (C1:C2, 85:15). $H_2{}^{18}O$-labelling experiments confirmed that the nucleophile was mainly incorporated at the most substituted carbon atom, suggesting that limonene-1,2-epoxide hydrolase uses an acid-catalyzed enzyme mechanism.

1. Introduction

Enantiopure epoxides are versatile synthons in the total synthesis of more complex biologically active compounds. Both chemical[1,2] and biological[2,3] methods for the production of these epoxides are available, and kinetic resolutions using epoxide hydrolases (EHs) have been studied as these enzymes are stable and cofactor-independent.[4,5]

Hydrolytic epoxide cleavage is either: (i) a base-catalyzed nucleophilic event, without apparent activation of the epoxide ring; or (ii) an acid-catalyzed event, implying an electrophilic activation of the epoxide ring concerted with the nucleophilic attack. Base-catalyzed hydrolysis is by nucleophilic

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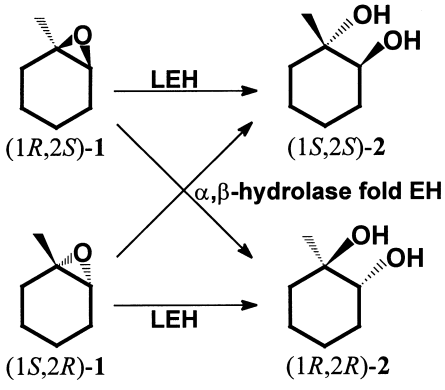
attack predominantly at the less substituted or less hindered carbon atom,[6] while under acidic conditions nucleophilic attack mainly takes place at the more substituted carbon atom, resulting in a stabilization of the carbocation formed in the transition state.[7] Both mechanisms result in a *retention* of configuration at the carbon atom at which the nucleophilic attack does not take place.

Remarkably, the many EHs from various sources, ranging from humans to bacteria, form a homogeneous group of enzymes belonging to the α,β-hydrolase fold superfamily that are structurally and mechanistically similar.[8,9] They all show a base-catalyzed mechanism, resulting in the *trans*-addition of water at the less sterically hindered or less substituted carbon atom.[10]

Recently, we described the novel limonene-1,2-epoxide hydrolase (LEH) from *Rhodococcus erythropolis* DCL14, which does not belong to the α,β-hydrolase fold EHs.[11,12] In this report we describe the regioselectivity of the LEH-catalyzed conversion of 1-methylcyclohexene oxide.

2. Results

1-Methylcyclohexene oxide **1** is a good substrate for LEH.[11] The enzyme preferentially hydrolyzed (1*R*,2*S*)-**1** mainly into (1*S*,2*S*)-1-methylcyclohexane-1,2-diol **2**, while (1*S*,2*R*)-**1** was converted more slowly and mainly yielded (1*R*,2*R*)-diol-**2** (Scheme 1, Fig. 1). From the data presented in Fig. 1, a regioselectivity of C1:C2 (85:15) was calculated. Both enzymatic reactions proceeded with retention of configuration at the less substituted carbon atom, indicating that nucleophilic attack occurred at the more substituted carbon atom (Scheme 1).



Scheme 1. Epoxide hydrolase-catalyzed hydrolysis of 1-methylcyclohexene oxide **1** into *trans*-1-methylcyclohexane-1,2-diol **2**

To confirm that the C–O bond of the oxirane ring was cleaved at the tertiary carbon atom, the LEH-catalyzed hydrolysis of epoxide-**1** was performed in ^{18}O-labelled water. Samples were taken after 35 min, when (1*R*,2*S*)-**1** is completely converted with almost no conversion of the (1*S*,2*R*)-**1** enantiomer, and after 5 h, when both enantiomers were almost completely converted (Fig. 1). The products were analyzed by GC–MS using a chiral stationary phase. The mass peak (*m*/*z* 132) of the product formed in ^{18}O-labelled water was 2 mass units higher than that of unlabelled product (*m*/*z* 130). The position of the ^{18}O-label was determined by analysis of the fragmentation pattern, and is based on the initial loss of water from the secondary alcohol, which is much more favourable than loss of water from tertiary alcohols.[13] The mass spectrum of the main product [(1*S*,2*S*)-**2**] after 35 min of reaction showed an initial loss of unlabelled water (*m*/*z* 114), indicating that the ^{18}O-label was inserted at the tertiary carbon atom. The minor product, (1*R*,2*R*)-**2**, contained the ^{18}O-label mainly at the secondary alcohol, as indicated by a loss of labelled water (*m*/*z* 112). The formation of the latter compound can be explained by the fact

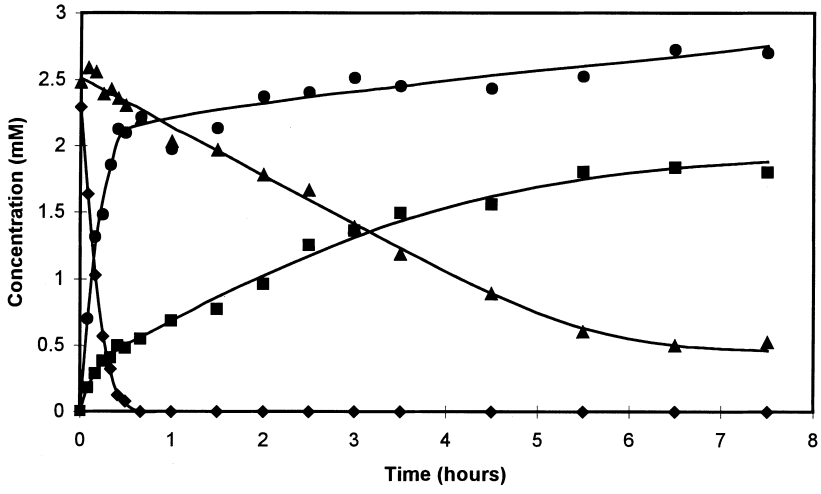


Figure 1. Conversion of 1-methylcyclohexene oxide **1** into *trans*-1-methylcyclohexane-1,2-diol **2** by purified LEH from *R. erythropolis* DCL14. ◆, (1*R*,2*S*)-**1**; ▲, (1*S*,2*R*)-**1**; ●, (1*S*,2*S*)-**2**; ■, (1*R*,2*R*)-**2**

that the LEH-catalyzed conversion of (1*R*,2*S*)-**1** is not absolutely regioselective, and by the much higher reaction rate of LEH with (1*R*,2*S*)-**1** than with (1*S*,2*R*)-**1**. Therefore, most of the (1*R*,2*R*)-**2** formed after 35 min originates from (1*R*,2*S*)-**1** (Fig. 1). In contrast, after 5 h of incubation most of the label in (1*R*,2*R*)-**2** was situated at the tertiary carbon atom, as now the (1*S*,2*R*)-epoxide-**1** has been the main substrate for the formation of (1*R*,2*R*)-**2**. These results with ^{18}O-water are in agreement with those described in Fig. 1.

Chemically, 1-methylcyclohexene oxide is rapidly hydrolyzed under acid conditions, while under basic conditions hydrolysis proceeded very slowly if at all. The H_2SO_4-catalyzed hydrolysis of **1** in ^{18}O-labelled water confirmed that under acid conditions nucleophilic attack indeed takes place at the tertiary carbon atom, as was concluded from the initial loss of unlabelled water (*m*/*z* 114) from the racemic *trans*-1-methylcyclohexane-1,2-diol products formed.

3. Discussion

1-Methylcyclohexene oxide **1** contains both a secondary and tertiary carbon atom in the oxirane ring, and the EH-catalyzed hydrolysis of this compound gives an indication about the reaction mechanism used by the enzyme. The presently known α,β-hydrolase fold EHs convert **1** with inversion of configuration at the less substituted carbon atom (Scheme 1).[4,14,15] This indicates that nucleophilic attack takes place at the less substituted carbon atom, which is in agreement with the base-catalyzed mechanism of α,β-hydrolase fold EHs.

The data obtained in this study show that, in contrast to the α,β-fold EHs, LEH converts **1** by nucleophilic attack with a very strong preference for the more substituted carbon atom. There are two possible explanations for this phenomenon: (i) LEH uses an acid-catalyzed reaction mechanism; or (ii) LEH uses a base-catalyzed reaction mechanism, but due to the geometry of the active site the attack of the nucleophile has to occur at the more hindered carbon atom. Previously, we obtained indications that LEH uses a reaction mechanism that differs from the α,β-hydrolase fold EHs as: (i) it has a pH-profile which is very different from that of the α,β-hydrolase fold EHs; (ii) it contains none of the highly conserved motifs of the catalytic triad of the α,β-hydrolase fold EHs; and (iii) its natural substrate, limonene-1,2-epoxide, is only significantly hydrolyzed chemically under acid conditions (as is epoxide **1**).[11,12]

All these findings strongly suggest that LEH, in contrast to the α,β-hydrolase fold EHs, uses an acid-catalyzed reaction mechanism. Previously, an acid-catalyzed mechanism for the enzymatic hydrolysis of epoxides has been suggested in a few isolated instances.[16–19] However, in all these studies crude enzyme preparations or whole cells were used, and consequently did not lead to conclusive results. This is the first time that an acid-catalyzed mechanism has been implicated as the mechanism for an EH purified to homogeneity. This is yet another characteristic of LEH, besides its previously described physical, biochemical and structural properties,[11,12] that clearly sets LEH apart from the hitherto described EHs.

4. Experimental

4.1. General

Racemic 1-methylcyclohexene oxide **1** was prepared from 1-methyl-1-cyclohexene with *m*-chloroperoxybenzoic acid as the catalyst.[20] ^{18}O-water (95% isotopic enrichment) was obtained from Aldrich. LEH was purified from the cell extract of cells of *Rhodococcus erythropolis* DCL14 grown on limonene as described previously.[11]

Chromatographic analyses were carried out using a Chrompack CP9000 gas chromatograph equipped with an FID detector. The enantiomers were analyzed on a chiral fused silica cyclodextrin capillary α-DEX 120 column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness; Supelco, Zwijndrecht, The Netherlands). Analyses were performed using N_2 (1.0 ml/min) as carrier gas (injector 200°C, detector 250°C). The temperature program was as follows: 7 min at 70°C, from 70 to 110°C at 10°C/min, then 15 min at 110°C. Chiral GC–MS analyses were carried out on an HP5973 MSD gas chromatograph equipped with the same α-DEX 120 column and temperature program. Carrier gas He (1.0 ml/min), injector 220°C. Electron Impact MS data were obtained at 70 eV. Optical rotations were measured with a Perkin–Elmer model 241 polarimeter.

4.2. Reactions

The reaction mixtures (2 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 5 mM 1-methylcyclohexene oxide **1** and 4.5 μg/ml purified LEH (30°C) in 15 ml vials fitted with Teflon Mininert valves (Supelco Inc.) preventing evaporation of **1**. The vials were placed in a water bath (30°C) and, after different time intervals, a vial was removed from the water bath and the reaction was terminated by the addition of 1 ml ethyl acetate. The vials were vigorously shaken to enable quantitative extraction of **1** and **2**. The ethyl acetate layer was pipetted in a microcentrifuge tube and centrifuged (3 min, 15 000×*g*) to achieve separation of the two layers. Subsequently 1 μl of the ethyl acetate layer was analyzed by chiral GC.

The reaction mixtures for the incubation with ^{18}O-water consisted of 250 μl ^{18}O-H_2O, 10 μl of a 500 mM potassium phosphate buffer (pH 7.0), 5 μl of a purified LEH solution (0.67 mg/ml) and 2 μl of a 1-methylcyclohexene oxide:acetone (1:9) mixture in 4 ml vials fitted with Teflon Mininert valves. After 30 min and 5 h of incubation at 30°C, the reaction was terminated by the addition of 250 μl of ethyl acetate, and treated as described above. Subsequently, 1 μl of the ethyl acetate layer was analyzed by chiral GC–MS.

The reaction mixtures for chemical acid- or base-catalyzed hydrolysis of 1-methylcyclohexene oxide **1** consisted of 100 μl H_2O or ^{18}O-H_2O, 2 μl 2N H_2SO_4 or 4N NaOH and 1 μl 1-methylcyclohexene oxide **1** in 4 ml vials fitted with Teflon Mininert valves. After 60 min at room temperature, the reaction was

terminated by the addition of 100 μl of ethyl acetate, and 1 μl of the ethyl acetate phase was analyzed by chiral GC–MS.

4.3. Product identification

The enantiomers of 1-methylcyclohexene oxide **1** and 1-methylcyclohexane-1,2-diol **2** were identified by determining the optical rotation of the remaining epoxide isomer and the formed diol[15] at 55% conversion. The reaction medium was first extracted with hexane for selective extraction of epoxide **1**, and was subsequently extracted with ethyl acetate for quantitative extraction of the diol product. The positive $[\alpha]_D$ for both the 'enriched' epoxide **1** and the diol **2** at 55% conversion established the configurations as depicted in Fig. 1.[14,15] Moreover, the configurations of the compounds were confirmed by comparing their GC data with those of the α,β-hydrolase fold EH-catalyzed hydrolysis of **1**.[4]

4.4. (1R,2S)-1-Methylcyclohexene oxide *1*

MS: *m*/*z* 112 [M^+] (2), 111 (5), 97 (100), 83 (10), 69 (25), 55 (41), 43 (84); t_R=7.80 min (α-DEX 120, temperature program).

4.5. (1S,2R)-1-Methylcyclohexene oxide *1*

MS: same as for (1*R*,2*S*)-**1**; t_R=7.98 min (α-DEX 120, temperature program).

4.6. (1S,2S)-1-Methylcyclohexane-1,2-diol *2*

MS: *m*/*z* 130 [M^+] (8), 112 [M^+-H_2O] (22), 97 (25), 84 (15), 71 (100), 58 (38), 43 (68); t_R=22.72 min (α-DEX 120, temperature program).

4.7. (1R,2R)-1-Methylcyclohexane-1,2-diol *2*

MS: same as for (1*S*,2*S*)-**2**; t_R=23.07 min (α-DEX 120, temperature program).

4.8. On secondary carbon atom ^{18}O-labelled 1-methylcyclohexane-1,2-diol *2*

MS: *m*/*z* 132 [M^+], 112 [$M^+-H_2{}^{18}O$], and the other major fragmentation peaks (97, 84, 71, 58, 43) were the same as those in unlabelled 1-methylcyclohexane-1,2-diol **2**.

4.9. On tertiary carbon atom ^{18}O-labelled 1-methylcyclohexane-1,2-diol *2*

MS: *m*/*z* 132 [M^+], 114 [M^+-H_2O], and the other major fragmentation peaks (99, 86, 73, 60, 45) were 2 higher than those in unlabelled 1-methylcyclohexane-1,2-diol **2**.

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