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A mechanistic study into the epoxidation of carboxylic acid and alkene in a mono, di-acylglycerol lipase

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ABSTRACT

More and more industrial chemistry reactions rely on green technologies. Enzymes are finding increasing use in diverse chemical processes. Epoxidized vegetable oils have recently found applications as plasticizers and additives for PVC production. We report here an unusual activity of the *Malassezia globosa* lipase (SMG1) that is able to catalyze epoxidation of alkenes. SMG1 catalyzes formation of peroxides from long chain carboxylic acids that subsequently react with double bonds of alkenes to produce epoxides. The SMG1 is selective towards carboxylic acids and active also as a mutant lacking hydrolase activity. Moreover we present previously unobserved mechanism of catalysis that does not rely on acyl–substrate complex nor tetrahedral intermediate. Since SMG1 lipase is activated by allosteric change upon binding to the lipophilic–hydrophilic phase interface we reason that it can be used to drive the epoxidation in the lipophilic phase exclusively.

1. Introduction

Epoxides are major key raw materials for various industrial products such as pharmaceuticals, cosmetics, agrochemical and food additives [1]. Biodegradable, epoxidized vegetable oils and their derivatives are environment-friendly, renewable resources, that have recently found industrial applications as plasticizers and additives for PVC to improve the high temperature heat stability [2]. Poly(vinyl chloride) (PVC) is one of the most widely used polymeric materials in the plastics industry, and used in food packaging material [3]. It suffers from poor thermal stability, so heat stabilizers are required in the processing of the polymer [4]. In addition epoxidized plant oils can be used as reactive diluents for paints and as intermediates for polyurethane–polyol production [5].

In industrial catalysis, more attraction has been focused on the enzymatic epoxidation due to its environmental-friendly materials, mild reaction conditions, less side products and toxic regents compared to chemical method [6,7]. Lipases have been reported to be one kind of enzymes that could be used to produce epoxides [8,9]. Although numerous references exist in the technical literature concerning the methods of epoxidation of different olefinic substrates, very few are concerned with the mechanism of epoxidation and its selectivity. Lipases [10] have been used for the indirect epoxidation of alkenes with hydrogen peroxide through the formation of peracids. Subsequently, peracids attack alkenes to produce epoxides outside the enzyme. In 1998, Hofmann proposed a two-step mechanism of the formation of peracid from acetic acid [11]. In the first step, catalytic serine acts as a nucleophile to attack the carbonyl carbon of acetic acid and an acyl–enzyme intermediate is formed. Next, the active site histidine deprotonates hydrogen peroxide to attack the carbonyl carbon of the serine–acyl complex and forms the peracid. Future studies confirmed this mechanism by mass spectrometry and X-ray crystallography [12]. In 2004, Bugg proposed that peracid could also be formed by direct attack of hydrogen peroxide on carboxyl group without the formation of an acyl–enzyme intermediate [13]. In this mechanism, the catalytic serine acts as hydrogen bond donor to stabilize the carbonyl oxygen of acetic acid. Most of the published findings on the indirect epoxidation assumed that catalytic serine is involved in the reaction. However, the serine-free formation of peroxides from aldehydes has been also described [14].

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SMG1 has been biochemically and structurally characterized [15,16]. Its crystal structures in closed form (PDB ID: 3UUE and 3UUF) have been solved, revealing that it has a unique lid fragment with loop conformation and a canonical α/β hydrolase fold core with Ser171–Asp228– His281 as catalytic triad [17]. SMG1 is proved to be a potential catalyst using for synthesis of diacylglycerol [15,18], while its potential in epoxidation has been completely unknown so far.

Here, we report that *Malassezia globosa* lipase (SMG1) is able to catalyze indirect (via peroxyacid) epoxidation of different alkenes without involvement of catalytic serine. In contrast to the published data we prove that the peroxidation of carboxylic acids can be catalyzed by serine-free (thus devoid of hydrolytic activity) mutant of the enzyme. Moreover, structural analysis shows that hydrogen peroxide attacks carboxyl group without the formation of an acyl–enzyme intermediate. The catalytic SMG1-catalyzed peroxidation mechanism relies on precise stabilization of both substrates in the SMG1 active site in a spatial arrangement optimal for the reaction. The reaction is carboxyl-selective and does not occur for aldehydes.

2. Materials and methods

2.1. Chemicals

Oleic acid (99.9% purity) and acetic acid (99.9%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). monochlorodimedone (MCD, 98.0%) was purchased from Alfa Aesar (Beijing, China). 1-Ocatadecene (99.5%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Octanoic acid (99.0%), decanoic acid (99.0%), lauric acid (98.0%), stearic acid (98.0%), propaldehyde (99.5%), pentanone (99.8%) and sodium bromide (99.9%) were purchased from Aladdin® (Shanghai, China). HPLC grade methanol and acetonitrile were from Merck (Darmstadt, Germany). Water was purified with a Millipore (Bedford, MA) Milli-Q water system. The 30% (w/w) hydrogen peroxide was analytical grade.

2.2. Protein production

The SMG1 Ser117Ala and His281Ala mutants were created by site-directed mutagenesis. Both Wild-Type (WT) and mutants lipases were expressed and produced in *Pichia pastoris*, purified by an anion-exchange chromatography [19], and freeze dried.

2.3. Epoxidation reaction

The epoxidation reaction mixture contained double bond substrate (1.0 mM), carbonyl/carboxyl substrate (1.0 mM), 2.5 mg SMG1 lipase (WT, S117A or H281A mutant) and 1.0 mL phosphate buffer (100 mM, pH 6.0). The 2.0 mmol 30% (w/w) hydrogen peroxide was added dropwise to the reaction mixture over a period of 2 h. The reactions were placed at 25 °C, stirred at a speed of 250 rpm. After the addition of the hydrogen peroxide was completed, the reactions continued further for the desired time duration. After 24 h of reaction, samples (20 μL) were withdrawn for the subsequent analysis. Reactions carried out without lipases showed no epoxides as there was no background reaction.

2.4. Effect of different carboxylic acids on epoxidation catalyzed by SMG1 S171A

The epoxidation reaction mixture contained 1-octadecene (1.0 mM) and different carbon chain length of saturated free fatty acids (1.0 mM), 2.5 mg SMG1 S171A and 1.0 mL phosphate buffer (100 mM, pH 6.0). The 2.0 mmol 30% (w/w) hydrogen peroxide was added dropwise to the reaction mixture over a period of 2 h. During the 9 h of reaction, samples (20 μL) were withdrawn periodically for the subsequent analysis.

2.5. Composition analysis

Analysis of the oleic acid and 9,10-epoxystearic acid was performed on high-performance liquid chromatography (Waters 1525) and refractive index detector (HPLC-RID) (Waters-1525, USA), equipped with an RP-C18 column (4.6 mm × 250 mm, 5 μm, Wasters, USA) according to the method [20].

Analysis of the 1-octadecene and 1,2-epoxyoctadecane was performed on an Agilent Technology model 7890 GC, equipped with an HP-5 column (30.0 mm × 0.25 mm, 0.25 μm, Macherey–Nagel, Germany). A temperature program was used to keep the samples in a column oven at 170 °C for 1 min. The temperature was increased to 206 °C at 1 °C/min, for a total run time of 19 min. The split ratio was 30:1. The injector and the flame ionization detector temperatures were set at 250 and 300 °C, respectively. The sample (20 μL), withdrawn from the reaction mixture, was transferred into a centrifuge tube with 980 μL of methanol and 0.5 g sodium sulfate, and mixed by vortex. The mixture was centrifuged at 10,000 × g for 2 min to remove water and protein. The supernatant was filtered through a Millipore membrane (0.22 μm, from Roth) and analyzed by GC. Peaks in GC were identified by comparison of their retention times with reference standards. Acquisition and processing of data were made using the instrument integrated software.

2.6. Steady-state kinetic constants for perhydrolysis of acetic acid

Kinetic constants for perhydrolysis were determined using the MCD assay [12], where the amount of enzyme added was adjusted to give a linear dependence of the reaction rate to enzyme concentration at 25 °C. All reactions contained MCD (0.18 mM) and sodium bromide (90.0 mM). The concentrations of hydrogen peroxide and acetate were varied to give evenly spaced data points on both sides of apparent K_m. For measurement of k_{cat} with hydrogen peroxide as a substrate, the concentration of acetic acid was constant at 1.0 M while the concentration of hydrogen peroxide varied. The pH value of all reaction buffers was adjusted to pH 6.0 before the test. For measurement of k_{cat} with acetic acid as a substrate, the concentration of hydrogen peroxide was constant at 0.8 M while the concentration of acetic acid varied. The pH value of all reaction buffers was adjusted to pH 6.0 before the test.

2.7. Structural analysis

The modeling of the ligand carboxylic acid in the binding site and complex energy minimization has been performed with MAESTRO (Schrödinger LLC) software suite. The structure 3UUE and the model of its open conformation [21] has been used for analysis.

2.8. Statistics

All analytical determinations were carried out in triplicate. The results are reported as the means ± standard deviations (SD) of these measurements.

3. Results and discussion

3.1. Epoxidation of different substrates and H_2O_2 by SMG1 lipases and its mutants

Based on the publications [10,20,22,14], enzymatic epoxidation reaction of different substrates with H_2O_2 was designed (Table 1).