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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.

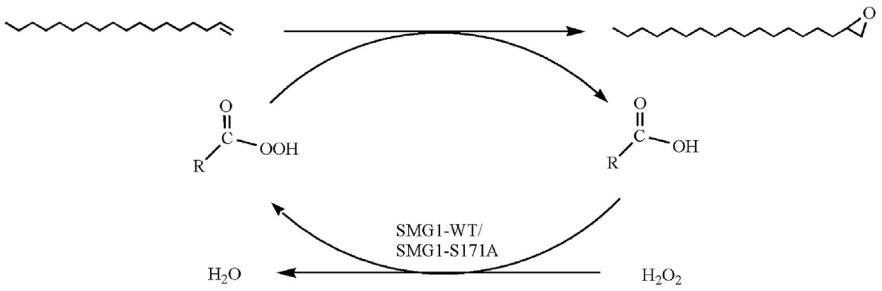
3. Results and discussion

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).

Table 1
The epoxidation reaction catalyzed by SMG1 WT, S171A and H281A mutants.

SMG1 and mutants	Substrates		Oxidant	Active or no	Product
	Double bond donator	Carbonyl/carboxyl donator			
WT	Oleic acid	Oleic acid	H_2O_2	✓	9,10-Epoxystearic acid
	1-Octadecene	Octanoic acid		✓	1,2-Epoxyoctadecane
	1-Octadecene	–		×	–
	1-Octadecene	Propaldehyde		×	–
	1-Octadecene	Pentanone		×	–
S171A	Oleic acid	Oleic acid	H_2O_2	✓	9,10-Epoxystearic acid
	1-Octadecene	Octanoic acid		✓	1,2-Epoxyoctadecane
	1-Octadecene	–		×	–
	1-Octadecene	Propaldehyde		×	–
	1-Octadecene	Pentanone		×	–
H281A	Oleic acid	Oleic acid	H_2O_2	×	–
	1-Octadecene	Octanoic acid		×	–



Scheme 1. Epoxidation of 1-octadecene via perhydrolysis of carboxylic acid. R = Alkyl.

SMG1 WT and its mutants (S171A and H281A) were used in the epoxidation reaction tests. SMG1 WT and S171A did not catalyze epoxidation of 1-octadecene with pentanone or propaldehyde alone, while they were active in the presence of oleic or octanoic acids. This indicates that SMG1 catalyzes peroxidation of carboxyl group exclusively. The indirect mechanism (catalysis of peroxidation and subsequent epoxidation outside the enzyme) is also confirmed as carboxyl group is necessary for the reaction to occur but it does not have to be present on the epoxidized molecule (Scheme 1). The results are consistent with the research reports [10,20]. It was also found that no reaction occurred when the His281 of the catalytic trials was mutated into Alanine. Taken together, our experiments show that SMG1 is able to catalyze formation of peroxides from carboxylic acids exclusively. Moreover, the catalytic Ser171 is not taking part in the reaction. These results indicate a previously undescribed catalytic mechanism of epoxidation: without formation of acyl–enzyme intermediate and specific towards carboxylic acids.

3.2. Enzyme kinetics

Enzyme kinetics for SMG 1 WT and S171A were examined in PBS buffer (pH 6.0). A series of perhydrolysis were followed under pseudo-one-substrate conditions. 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 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. All The following kinetic constants are apparent, since only one substrate was varied. The apparent specificity constant (k_{cat}/K_m^{app}), the apparent Michaelis constant K_m^{app}, and the apparent turnover number k_{cat}^{app}, were calculated for SMG1 WT and S171A.

As shown in Table 2, the k_{cat} value for S171A mutant was slightly higher than that of SMG1 WT while the K_m values for SMG1 WT and S171A were similar with acetic acid as the varied substrate. The k_{cat} value for S171A was 2-fold higher than that of SMG1 WT, and the k_{cat}/K_m constant for S171A was slightly higher than SMG1 WT with hydrogen peroxide as the varied substrate.

It was obvious from the above data that catalytic Ser171 has a negative influence on the enzyme activity. Thus we propose that it is not involved in the peroxidation reaction but its side chain hydroxyl may constitute an either steric hindrance for optimal

Table 2
Steady-state kinetic constants for perhydrolysis of acetic acid catalyzed by SMG1 WT and SMG1 S171A.

Enzymes	Varied substrate	k_{cat} (s^{-1})	K_m^{app} (mM)	k_{cat}/K_m^{app} (s^{-1} M^{-1})
SMG 1	Acetic acid	0.022	1.3	16
	Hydrogen peroxide	0.015	98	0.15
S171A	Acetic acid	0.027	1.3	21
	Hydrogen peroxide	0.030	135	0.22

Table 3
Effects of carbon chain length of carboxylic acids on the epoxidation of 1-octadecene by SMG1 S171A.

Reaction time (h)	Conversion of 1-octadecene (%)			
	C8:0	C10:0	C12:0	C18:0
1	2.0 ± 0.8	6.0 ± 1.0	8.0 ± 1.0	9.0 ± 1.1
3	3.0 ± 0.9	6.0 ± 1.1	15.0 ± 1.6	19.0 ± 1.8
5	5.0 ± 1.4	7.0 ± 1.3	15.0 ± 1.8	27.0 ± 2.1
7	6.0 ± 1.9	8.0 ± 1.4	23.0 ± 2.6	31.0 ± 2.9
9	10.0 ± 2.1	10.0 ± 1.7	27.0 ± 2.8	30.0 ± 1.7

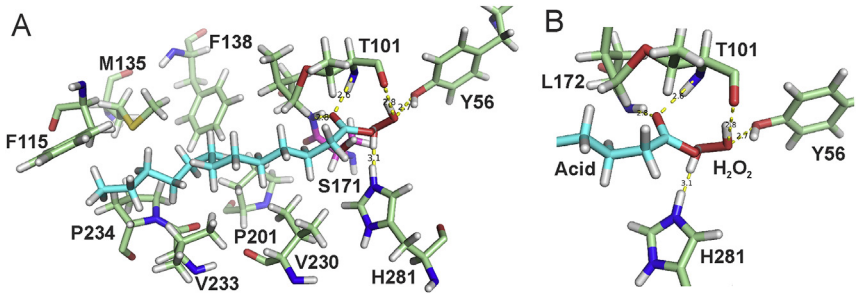


Fig. 1. The model of SMG1 WT catalyzed peroxidation. (A) Long-chain carboxylic acid (blue) is bound in the hydrophobic groove of the lipase (green). The lid region must "open" to allow substrate entry. The binding is not specific and allows diverse aliphatic chains to be bound. (B) Magnified view of the active site. Carbonyl oxygen of the acid is bound in the oxyanion hole formed by amide nitrogens of Thr101 and Leu172. H_2O_2 molecule is stabilized by a network of hydrogen bonds between Thr101 carbonyl, Tyr56 hydroxyl and imidazole ring of His281 belonging to the catalytic triad. The bond network brings H_2O_2 molecule into close proximity of carboxyl group thus positioning it optimally for peroxide formation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

substrate binding or competes for hydrogen bond of His281. This hydrogen bond should bind hydrogen peroxide instead (see Section 3.4).

3.3. *Effects of carboxylic acids with different carbon chain length on epoxidation*

SMG S171A was used to evaluate the effects of carboxylic acids with different carbon chain lengths on epoxidation of 1-octadecene. The carboxylic acids were octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0) and stearic acid (C18:0). The results are shown in Table 3. It was obvious that SMG1 lipase catalyzed preferentially long-chain carboxylic acids peroxidation. The maximal conversion of 1-octadecene was achieved at 7 h of reaction when C18:0 was used as substrates.

3.4. *Structural model of the peroxidation mechanism*

For the modeling of peroxidation mechanism we have used a model of SMG1 lipase with an "open" lid [21]. The opening of the lid is necessary for reconfiguration of the oxyanion hole of the enzyme. The proposed mechanism is presented in Fig. 1. The SMG1 lipase has a preference for long-chain carboxylic acids (see Section 3.3) therefore we reason that the aliphatic chain is bound to the long hydrophobic groove that is formed in the "open" conformation. This stabilizes the acid with its carboxyl in the active site (Fig. 1A). Carbonyl atom of the acid is then bound in the oxyanion hole of the enzyme by two hydrogen bonds to the amide nitrogens of Leu172 and Thr 101 (Fig. 1B). The H281A mutation indicated that histidine is essential for enzymatic activity while Ser171 has negative effect. We therefore reason that His281 takes part in stabilization of hydrogen peroxide as proposed before for Baeyer–Villiger oxidation by Carlqvist et al. [14]. The Tyr56 hydroxyl group and Thr101 carbonyl offer additional hydrogen donor–acceptor system that stabilizes hydrogen peroxide in the active site. Together the SMG1 provides stabilization of both substrates in the configuration that is optimal for formation of the peroxide group. Interestingly, the formation of acyl–enzyme intermediate is not probable as the Ser171 is not present in the active mutant. It is also unlikely that the tetrahedral intermediate is formed as the distance between carbon of the carboxyl group and oxygens of peroxide are too big for bond formation. The essential His281 side chain can form hydrogen bonds both to peroxide and carboxyl oxygen, therefore it most likely intermediates in the formation of the peroxide.

Interestingly, the proposed mechanism can only occur when SMG1 is in its "open" conformation. When "closed" the oxyanion hole is malformed as seen in the crystallographic structure (PDB ID: 3UUE). Thus the peroxidation can only occur upon allosteric activation of the enzyme at the lipophilic–hydrophilic phases interface. We propose that this mechanism can be used for selective epoxidation in the hydrophobic phase of the mixture.

4. Conclusion

We have observed catalytic peroxidation activity of the SMG1 lipase and its mutant devoid of hydrolase activity. The experiments confirm that the epoxidation is a two-phase (indirect) reaction with enzymatic catalysis of peroxyacid formation and subsequent enzyme-independent epoxidation. The SMG1 lipase uses so far undescribed mechanism of peroxidation that is not relying on the acyl–enzyme complex and tetrahedral intermediate formation. Unlikely to previously described mechanisms we observe specificity towards carboxylic acid and no activity against aldehydes. Moreover, SMG1 catalyzes the reaction in the lipophilic phase. The epoxidized molecule might be itself a carrier of the carboxylic group thus reducing the number of system components and allowing production of epoxidized fatty acids. We believe that our results will enable future industrial application of the lipases for peroxide production in heterogenous (lipophilic/hydrophilic) mixtures.

Conflict of interest

None.

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