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Direct involvement of hydrogen peroxide in bacterial α-hydroxylation of fatty acid

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Abstract We have reported that fatty-acid α-hydroxylase partially purified from *Sphingomonas paucimobilis* required NADH and molecular oxygen. In this study, we found that the reaction was greatly inhibited by catalase. Glutathione and glutathione peroxidase also inhibited α-hydroxylation, but superoxide dismutase and mannitol did not. Replacement of NADH and molecular oxygen by hydrogen peroxide increased the α-hydroxylation activity. In the presence of hydrogen peroxide, molecular oxygen was not required for the activity. These findings suggest that hydrogen peroxide was essential for bacterial α-hydroxylase.

Key words: Fatty acid; α-Oxidation; Hydrogen peroxide; *Sphingomonas paucimobilis*

1. Introduction

The α-hydroxylation of fatty acid is the initial step in fatty-acid α-oxidation. 2-Hydroxy fatty acid was determined as an intermediate in this process in mammals [1,2], plants [3,4], and bacteria [5]. However, the enzymatic properties of fatty-acid α-hydroxylase are little known. Recently, we partially purified fatty-acid α-hydroxylase from *Sphingomonas paucimobilis*, 2-hydroxymyristic-acid-rich bacteria [6]. We demonstrated that the enzyme required molecular oxygen (O_2) and NADH, and confirmed that an oxygen atom of O_2 was incorporated into the reaction product by the use of the ^{18}O technique. Therefore, the α-hydroxylation reaction in *S. paucimobilis* seemed to be a monooxygenase reaction.

In our preliminary study on O_2 requirement, α-hydroxylation activity was found to be activated by glucose and glucose oxidase which were used for removal of the remaining O_2 in the reaction mixture. This observation suggests that hydrogen peroxide (H_2O_2) generated by glucose oxidase affects α-hydroxylation activity. Previously, Stumpf [7] reported that an H_2O_2-generating system was required for the α-oxidation system of cotyledons of germinating peanuts, but also that the direct addition of H_2O_2 was ineffective and the addition of catalase did not show marked inhibition. In a later study, Shine and Stumpf [8] proposed that, in an α-oxidation system, the reductant associated with oxidase such as glucose oxidase was oxygenated and subsequently 2-hydroperoxy fatty acid was formed. However, the precise mechanism, especially the involvement of reactive oxygen, remains unclear. Herein, we present the evidence that free H_2O_2 is directly involved in the bacterial α-hydroxylation reaction of fatty acid.

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2. Materials and methods

2.1. Materials

S. paucimobilis EY2395^T was kindly provided by Dr. Eiko Yabuuchi, Osaka City University Medical School. Catalase, glucose oxidase, L-amino acid oxidase, glutathione peroxidase, and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [1-^{14}C]Myristic acid was obtained from New England Nuclear (Bad Homberg, Germany). Myristic acid and [^{18}O] molecular oxygen were purchased from P-L Biochemicals (Milwaukee, OR, USA) and Amersham International (Bucks, UK), respectively. NADH, glutathione (reduced form), H_2O_2 and mannitol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Determination of activity of fatty-acid α-hydroxylase

Fatty-acid α-hydroxylating activity was determined essentially as described previously [6]. Briefly, the reaction mixture containing Tris-HCl buffer (pH 7.0, 20 μmol), NADH (160 nmol), [1-^{14}C]myristic acid (12 nmol), and the enzyme preparation (30 μg of protein) in a total volume of 0.2 ml was incubated at 37°C for 15 min. When H_2O_2 or an H_2O_2-generating system was used instead of NADH, Tris-HCl buffer (pH 8.0, 20 μmol) was used. The amounts of catalase, glutathione peroxidase, superoxide dismutase, glucose oxidase, L-amino acid oxidase, and other compounds added to the reaction mixture are indicated in the figures and tables.

2.3. Preparation of fatty-acid α-hydroxylase

The fatty-acid α-hydroxylase was partially purified from *S. paucimobilis* by a minor modification of the method described previously [6]. In brief, *S. paucimobilis* EY2395^T was grown in the medium containing 0.5% polypeptone, 0.2% yeast extract, and 1% glucose at 35°C. After cells were harvested, the cells were disrupted by sonication and unbroken cells were removed by centrifugation. The supernatant was centrifuged by 140 000 × *g* for 1 h and then the resultant supernatant was treated with 2.5% streptomycin. After centrifugation, the supernatant was fractionated with ammonium sulfate (AS) and a 33–45% AS fraction was obtained. The AS fraction was dialyzed against 0.15 M potassium phosphate buffer (pH 7.0) overnight, and then applied to a hydroxylapatite column equilibrated with the same buffer. After being washed with the same buffer, the column was eluted with 0.45 M potassium phosphate buffer (pH 7.0). The eluate was concentrated and used as the enzyme preparation. The protein concentration was assayed by the method of Bradford [9] with serum albumin as the standard.

2.4. Analysis of the product by gas chromatography/mass spectrometry (GC/MS)

The reaction mixture (final volume, 1 ml) containing Tris-HCl buffer (pH 8.0, 100 μmol), H_2O_2 (200 nmol), myristic acid (60 nmol), the enzyme preparation (75 μg of protein) was incubated. After the reaction product was methylated with diazomethane, the resultant fatty acid methyl ester was analyzed by GC/MS using JEOL SX-102A (JEOL, Tokyo, Japan) coupled with a gas chromatograph 5890 series II (Hewlett-Packard, PA, USA) with a flame ionization detector and a 30 m × 0.25 μm capillary column SP™ 2380 (Supelco Inc., Bellafonte, PA, USA) as described previously [10].

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Table 1
Effect of scavengers for reactive oxygen species on α-hydroxylation of myristic acid

Scavengers[a] (amount in 0.2 ml)	Specific activity[b] (nmol/min per mg protein)
None	2.76
Catalase (6 units)	0.36
GSH (1 μmol)	2.44
GSH Px (1 unit)	2.49
GSH (1 μmol)+GSH Px (1 unit)	0.04
SOD (10 units)	2.29
Mannitol (20 μmol)	2.79

[a]The reaction mixture (final volume, 0.2 ml) consisted of Tris-HCl (pH 7.0, 20 μmol), NADH (160 nmol), myristic acid (12 nmol), and enzyme preparation (30 μg of protein). The amount of scavengers for reactive oxygen species added to the reaction mixture are indicated above. GSH, glutathione; GSH Px, glutathione peroxidase; SOD, superoxide dismutase.
[b] Data are means of three independent determinants.

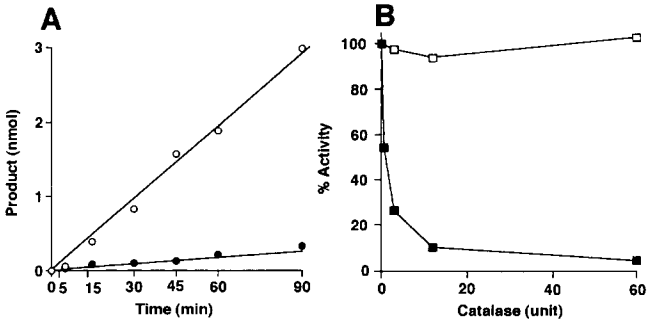

Fig. 1. Effect of catalase on fatty-acid α-hydroxylation. (A) Time course for α-hydroxylation of myristic acid with or without catalase. 60 units of catalase was added to the reaction mixture in a total volume of 0.2 ml. Closed and open circles indicate product with and without catalase, respectively. (B) Effect of catalase on fatty-acid α-hydroxylation activity. Closed and open squares indicate % activity after addition of catalase and boiled catalase, respectively.

3. Results and discussions

To test for the involvement of H_2O_2 in the α-hydroxylation reaction in the presence of NADH and O_2, we added catalase as a scavenger for H_2O_2 to the reaction mixture. Addition of catalase markedly inhibited α-hydroxylation activity (Fig. 1A). Whereas α-hydroxylation activity was inhibited by catalase in a dose-dependent manner (94% inhibition with 60 units catalase), heat-inactivated catalase did not show significant inhibition at any concentration (Fig. 1B). In addition, the inhibitory effect of catalase on fatty-acid α-hydroxylation was prevented by sodium azide (data not shown). These findings indicate that the inhibition of fatty-acid α-hydroxylation by catalase was due to an enzymatic effect rather than to other nonspecific effects. The inhibitory effect was also observed on the addition of glutathione and glutathione peroxidase (Table 1). Superoxide dismutase (superoxide anion scavenger) and mannitol (hydroxyl radical scavenger) did not show any inhibitory effect. These findings strongly suggest that H_2O_2 itself, but not the superoxide anion or hydroxyl radical, is essential for the α-hydroxylation reaction.

To obtain further evidence for the participation of H_2O_2 in the α-hydroxylation reaction, H_2O_2 or an H_2O_2-generating system consisting of glucose and glucose oxidase, or L-leucine and L-amino-acid oxidase was added to the reaction mixture in the absence of NADH. As shown in Table 2, either H_2O_2 or an H_2O_2-generating system replaced NADH. Even in the absence of O_2, H_2O_2 was capable of fully supporting the α-hydroxylation reaction. α-Hydroxylation activity in the presence of H_2O_2 was greater than that in the presence of NADH and O_2. The K_m value for H_2O_2 was approx. 50 μM. In the α-oxidation system of peanut cotyledons, an H_2O_2-generating system has been reported to be necessary [4,8]. However, the following evidence indicates that the bacterial α-hydroxylation system was different from the plant system: (1) catalase showed an inhibitory effect on the bacterial system, but had no effect on the plant system; (2) unlike the plant system, the bacterial system was activated by direct addition of H_2O_2; and (3) whereas the addition of glutathione and gultathione peroxidase activated the plant system, it inhibited the bacterial system.

The reaction product in the presence of H_2O_2, when myristic acid was used as a substrate, was analyzed by GC/MS. Here, we present the mass spectrum of the product showing the typical fragmentation pattern of 2-hydroxymyristic acid methyl ester (Fig. 2). We further confirmed the formation of 2-hydroxymyristic acid by radio-gaschromatography and HPLC (data not shown). Shine and Stumpf [8] and Huang et al. [11] have reported that 2-hydroperoxy fatty acid was a possible intermediate. However, we did not find any hydroperoxy intermediate.

When ^{18}O labeling experiments were carried out to determine the source of the oxygen atom incorporated into the reaction product in the presence of H_2O_2, the oxygen atom of H_2O_2, rather than labeled oxygen, was found to be incorporated into the product (data not shown). In the presence of NADH and O_2, however, the oxygen atom of O_2 was introduced into the substrate as previously reported [6]. Therefore, these findings susgest that the oxygen atom of H_2O_2 is preferentially incorporated into the bacterial α-hydroxylation system even in the presence of O_2. Oxygenation of a substrate by

Table 2
Replacement of NADH by hydrogen peroxide or hydrogen-peroxide generating systems

Compounds tested[a] (amount in 0.2 ml)	Specific activity[b] (nmol/min per mg protein)
NADH (160 nmol)	3.37
H_2O_2 (40 nmol)	11.89
H_2O_2 (40 nmol, anaerobic[c])	14.60
H_2O_2 (40 nmol)+catalase (60 units)	0.00
Glucose (200 nmol)+glucose oxidase (1 unit)	11.19
L-Leucine (2 μmol)+L-amino acid oxidase (0.03 units)	13.68

[a]Incubations were carried out in the reaction mixtures described in Table 1 except that NADH was replaced by the compounds indicated.
[b]Data are means of three independent determinants.
[c]The reaction mixture was made anaerobic by repeated cycles of evacuation and flushing with nitrogen gas in a Thunberg tube.

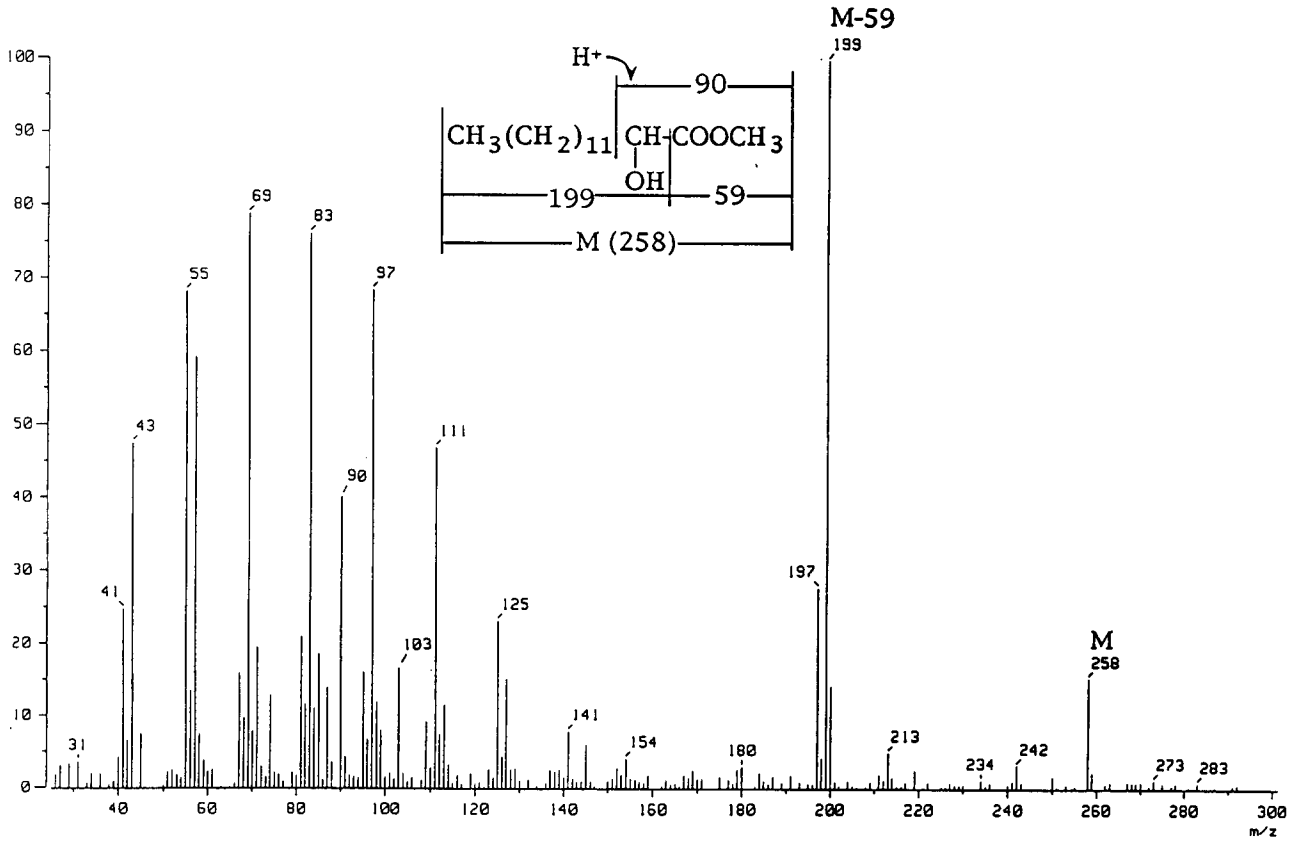

Fig. 2. Mass spectrum of the reaction product of H_2O_2-dependent α-hydroxylation of myristic acid. The mass spectrum of the methylated product gave ions of *m/z* 258 and 199, which were identical to those of methyl-2-hydroxymyristic acid (*M*) and its characteristic fragment (*M*−59), respectively.

an H_2O_2-dependent hydroxylase system has been reported for cytochrome P-450 [12] and methane monooxygenase [13], where a 'peroxide shunt' mechanism was operative. In these systems, O_2, pyridine nucleotide such as NAD(P)H, and reductase are replaced by H_2O_2, and then the oxygen atom of H_2O_2 is incorporated into the product. These reactions are somewhat similar to the bacterial α-hydroxylation reaction, but in the H_2O_2-dependent hydroxylation of these monooxygenases, the K_m value for H_2O_2 was much higher than that in the bacterial α-hydroxylation [14,15]. To our knowledge, there is no evidence that H_2O_2 is essentially required for the ordinary NAD(P)H- and O_2-dependent hydroxylation of these known monooxygenases. Moreover, NADH- and O_2-dependent α-hydroxylation activity in the crude extract of *S. paucimobilis* was completely inhibited by catalase, suggesting that a reductase may be absent and H_2O_2 involves directly, rather than via a 'peroxide shunt', in the bacterial α-hydroxylase reaction. Most recently, we found that H_2O_2 was supplied by NADH oxidase in the α-hydroxylation reaction. Therefore, the mechanism of the bacterial α-hydroxylation is clearly different from those of the known monooxygenase reactions. However, further study is required to elucidate the precise mechanistic role of H_2O_2 in the catalytic cycle of α-hydroxylation of fatty acid.

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