Two moles of $O_2$ consumption and one mole of $H_2O_2$ formation during cholesterol peroxidation with cholesterol oxidase from *Pseudomonas* sp. strain ST-200

Noriyuki DOUKYU and Rikizo AONO

Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama, 226-8051, Japan

Cholesterol oxidase from *Pseudomonas* sp. strain ST-200 oxidized cholesterol and cholestanol to 6β-hydroperoxycholest-4-en-3-one and 5α-cholestan-3-one respectively. The former was converted spontaneously to several oxysteroids such as 6α-hydroxycholest-4-en-3-one and cholest-4-ene-3,6-dione, with the consumption of 2 mol of $O_2$ and the formation of 1 mol of $H_2O_2$ for each mole of cholesterol oxidized. An oxidized form of the cholesterol oxidase dehydrogenates cholesterol, probably to the 5-en-3-one derivative. A reduced form of the enzyme, yielded from the cholesterol dehydrogenation reaction, dioxygenated cholest-5-en-3-one to 6β-hydroperoxycholest-4-en-3-one.

Key words: cholestanol, cholest-4-ene-3,6-dione, dioxygenation, FAD, 6β-hydroperoxycholest-4-en-3-one.

INTRODUCTION

Most cholesterol oxidases (cholesterol:oxygen oxidoreductase, EC 1.1.3.6) catalyse the oxidation of cholesterol (cholest-5-en-3β-ol) (Scheme 1A) to cholest-4-en-3-one [1,2]. The cholesterol oxidation step with cholesterol oxidase is the oxidation of the hydroxy group at the C-3 position with the simultaneous isomerization of the Δ2-double bond to produce cholest-4-en-3-one as a final product. Cholesterol oxidases were isolated and characterized from numerous bacterial sources [3]; most of them were shown to contain 1 mol of tightly bound FAD per mol of protein as a prosthetic group [2,4,5].

We isolated a cyclohexane-tolerant and cholesterol-converting bacterium, *Pseudomonas* sp. strain ST-200 (referred to hereafter as ST-200). This organism effectively oxidizes cholesterol dissolved in an organic solvent [6–8]. Extracellular cholesterol oxidase produced by ST-200 has been purified and characterized previously [9]. This enzyme was stable in the presence of organic solvents and showed high activity in the presence of organic solvents. Long-term oxidation of cholesterol with the oxidase generates several products besides cholest-4-en-3-one.

It has been shown that the cholesterol oxidase of *Pseudomonas fluorescens* (Pfl) is responsible for the production of 6β-hydroperoxycholest-4-en-3-one but not cholest-4-en-3-one [10]. Pfl seems to have a dioxygenase activity. A possible scheme of a reaction pathway for the formation of 6β-hydroperoxycholest-4-en-3-one by the oxidase has been proposed on the basis of a structural analysis of the product. However, the stoichiometry of the formation of $H_2O_2$ and the consumption of $O_2$ accompanying the oxidation of cholesterol has not previously been shown for the dioxygenation.

Here we report the identification of oxidized products generated with ST-200 cholesterol oxidase and the stoichiometry of $O_2$ consumption, product formation and $H_2O_2$ formation during the reaction.

MATERIALS AND METHODS

Cholesterol oxidase preparations

ST-200 was grown at 30°C in Luria–Bertani medium consisting of 1% (w/v) Bacto Tryptone (Difco Laboratories, Detroit, MI, U.S.A.), 0.5% Bacto Yeast Extract (Difco) and 1% (w/v) NaCl. The extracellular cholesterol oxidase was purified as described previously [9]. The following commercial cholesterol oxidase preparations were also used in this study: Pfl and the cholesterol oxidases from *Brevibacterium* sp. (Bre) (Sigma Chemical Co., St. Louis, MO, U.S.A.), *Nocardia erythropolis* (Ner) (Boehringer Mannheim Yamanouchi, Tokyo, Japan), *Streptomyces* sp. SA-COO (Str) (Toyobo, Tsuruga, Japan) and *Pseudomonas* sp. (Pse) (Wako Chemical, Osaka, Japan).

Isolation of the major products from cholesterol by oxidation with the oxidases

Each of the cholesterol oxidases (1 unit, defined as the amount required to produce 1 μmol of $H_2O_2$ by the oxidation of 1 μmol of cholesterol per min at 30°C, unless described otherwise) was incubated in 200 ml of the reaction mixture [50 mM phosphate buffer, pH 7.0, containing 64 mM sodium cholate, 0.34% Triton X-100 and 0.9 mM cholesterol] at 30°C for 32 h. The reaction mixture was extracted twice with 1 vol. of chloroform. The chloroform extracts were concentrated to approx. 2 ml in a rotary evaporator at room temperature. The residual matter was developed on a 2 mm silica gel 60F254 TLC plate (E. Merck AG, Darmstadt, Germany) with n-hexane/diethyl ether (3:1, v/v) as the mobile phase. After chromatography, each compound was located on the silica plate by UV illumination. The compound was recovered with chloroform from the zone showing UV absorption.

Abbreviations used: Bre, cholesterol oxidase from *Brevibacterium* sp.; Pfl, cholesterol oxidase from *P. fluorescens*; Ner, cholesterol oxidase from *Nocardia erythropolis*; Pse, cholesterol oxidase from *Pseudomonas* sp.; ST-200, *Pseudomonas* sp. strain ST-200; Str, cholesterol oxidase from *Streptomyces* sp. SA-COO.

1 To whom correspondence should be addressed (e-mail raono@bio.titech.ac.jp).
Scheme 1 Schemes for the cholesterol oxidation reactions catalysed with cholesterol oxidases

For a full explanation see the Discussion section. (A) Structure of cholesterol. (B) Oxidation of cholesterol with cholesterol oxidase from Str, Bre or Ner. (C) Oxidation of cholesterol with ST-200 cholesterol oxidase, Pfl or Pse. (D) Oxidation of cholestanol with ST-200 cholesterol oxidase, Pfl or Pse. (E) Oxidation of cholesterol and cholestanol with ST-200 cholesterol oxidase.

Measurement of molecular mass of the oxidized product from cholesterol

Molecular masses of samples were measured by low-resolution electron impact MS with a model QP-5000 mass spectrometer (Shimadzu, Tokyo, Japan). The samples were ionized at 230 °C and 70 eV.

Measurement of the NMR spectrum of the oxidized product

The sample was dissolved in CDCl₃ to a concentration of approx. 5–20 mg/ml. ¹H-NMR spectra were recorded at 20 °C with a 300 MHz NMR spectrometer (Varian; model Mercury-300). ¹³C-NMR spectra were measured with a 50 MHz NMR spectrometer (Varian; model Gemini-200) with complete decoupling. Tetramethylsilane was used as an internal standard for the spectra.

Silica-gel TLC of the cholesterol derivatives

The sample was developed at room temperature on a 0.2 mm silica gel 60F254 TLC plate with n-hexane/diethyl ether (3:1, 1:1 or 1:3, v/v) as the mobile phase. Compounds were located by observation under UV illumination.

HPLC of the cholesterol derivatives

The sample was analysed by reverse-phase chromatography on a column of ODS-1201-H (4.6 mm × 200 mm; Senshu Science, Tokyo, Japan) attached to an HPLC apparatus. The column was eluted with n-hexane/propan-2-ol (1:0.02, v/v) at a flow rate of 1.0 ml/min. The elution was followed by monitoring A₂₅₀ to measure cholesterol and its oxidation products. To measure cholestanol, the elution was monitored with a differential refractive index detector (Shimadzu; model RID-300).

Assay of enzymic activity

Cholesterol oxidase activity was assayed by measuring H₂O₂ generation accompanying the oxidation of cholesterol [11], as follows. Cholesterol oxidase was incubated in a reaction mixture consisting of 50 mM sodium potassium phosphate buffer, pH 7.0, 64 mM sodium cholate, 0.34 % (v/v) Triton X-100, 1.4 mM 4-aminoantipyrine, 21 mM phenol, 0.9 mM cholesterol and 1.5 units/ml horseradish peroxidase (Toyobo) at 30 °C. The formation of the red quinoneimine was followed by monitoring A₅₄₀ during incubation at 30 °C for 5 min. The enzymic activity was calculated from the molar absorption coefficient of quinoneimine (ε 5.33 × 10⁴ M⁻¹ cm⁻¹).
Measurement of O₂ consumption accompanying the oxidation of cholesterol

The reaction mixture described above was prewarmed at 30 °C in a sealed tube. The oxidizing reaction was initiated by adding cholesterol oxidase (4 m-units/ml) to the mixture. O₂ consumption was followed by monitoring the dissolved oxygen concentration with a DO meter (Model 53; YSI, Yellow Springs, OH, U.S.A.). The concentration of O₂ was calculated from the saturated O₂ concentration (0.25 mM) at 30 °C [12].

Measurement of H₂O₂ formation accompanying the oxidation of cholesterol

The reaction mixture (prewarmed at 30 °C) was shaken to aerate the mixture. After addition of the oxidase, the A₅₄₀ of the mixture was monitored. H₂O₂ formation was calculated from the molar absorption coefficient of the red quinoneimine.

Materials

Cholesterol, cholestane and cholest-4-en-3-one were purchased from Nacalai Tesque (Kyoto, Japan). 5α-Cholesterol-3-one was purchased from Sigma. 6β-Hydroxycholest-4-en-3-one and cholest-5-en-3-one were products of Steraloids, (Wilton, NH, U.S.A.). Cholest-4-ene-3,6-dione was purified as described previously [6].

RESULTS

Products generated by the oxidation of cholesterol with ST-200 cholesterol oxidase

The extracellular cholesterol oxidase of strain ST-200 oxidized cholesterol to yield at least six chloroform-extractable products after a prolonged incubation. These derivatives were referred to as I–VI in order of their Rᵥ values (Table 1). When the products were isolated from the reaction mixture containing 68 mg of cholesterol as a substrate, approximate recoveries of the derivatives were as follows: I, 0.5 mg; II, 1 mg; III, 0.5 mg; IV, 17 mg; V, 5 mg; and VI, 2 mg. The sum of the recoveries was approx. 40% of the substrate cholesterol. The recovery was low because the silica powder was scraped from only narrow central areas showing UV absorption. The recoveries were probably correlated with productions of the oxidized compounds.

Identification of the cholesterol oxidation products

Table 1 shows Rᵥ values of the cholesterol derivatives measured in the three solvent systems. The Rᵥ values of derivatives II, V and VI agreed with those of the relevant compounds identified by NMR spectrometry as described below. Molecular masses measured for the derivatives are shown in Table 1. The molecular masses of the derivatives were 12, 14 and 30 Da larger than that of cholesterol, indicating that the derivatives were produced from cholesterol by oxygenation and dehydrogenation. The λ₅₄₀ max values indicated that these derivatives possessed π-electron systems due to conjugated double bonds in molecules.

The major products (II, IV, V and VI) were structurally analysed by ¹H-NMR spectrometry. The chemical shift values of signals in ¹H-NMR spectra of these derivatives were as follows: derivative II, identified as 6β-hydroxycholest-4-en-3-one, 0.74 (3 H, s, 18-Me), 0.85 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 0.87 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 4.35 (1 H, dd, J 3.90 and 2.10 Hz, 6-H), 5.82 (1 H, d, J 0.60 Hz, 4-H); derivative IV, identified as 6β-hydroperoxycholest-4-en-3-one, 0.71 (3 H, s, 18-Me), 0.86 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 0.87 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 0.91 (3 H, d, J 6.60 Hz, 21-Me), 4.43 (1 H, dd, J 3.90 and 2.10 Hz, 6-H), 5.89 (1 H, d, J 0.60 Hz, 4-H), 8.26 (1H, br, 6β-OOH); derivative V, identified as cholest-4-ene-3,6-dione, 0.72 (3 H, s, 18-Me), 0.86 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 0.87 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 0.92 (3 H, d, J 6.60 Hz, 21-Me), 6.17 (1 H, s, 4-H); and derivative VI, identified as cholest-4-en-3-one, 0.71 (3 H, s, 18-Me), 0.86 (3 H, d, J 6.30 Hz, 26-Me or 27-Me), 0.87 (3 H, d, J 6.60 Hz, 26-Me or 27-Me), 0.91 (3 H, d, J 6.60 Hz, 21-Me), 5.73 (1 H, s, 4-H). The ¹H-NMR spectra were coincident with those previously reported for the compounds identified as described above [10,13].

The identifications of derivatives IV, V and VI were confirmed by ¹³C-NMR spectrometry. The chemical shift values of signals in derivatives IV, V and VI in the ¹³C-NMR spectra were identical to those reported for 6β-hydroperoxycholest-4-en-3-one, cholest-4-ene-3,6-dione and cholest-4-en-3-one respectively [10,13]. The ¹³C-NMR spectrum of derivative IV confirmed that its C-6 was hydroperoxidized (85.9 p.p.m.) but not hydroxylated (73.3 p.p.m.) [13].

It has been reported that 6β-hydroperoxycholest-4-en-3-one (IV) is spontaneously epimerized to 6α-hydroperoxycholest-4-en-3-one and reduced to 6α-hydroxycholest-4-en-3-one [13,14]. Although the structures of derivatives I and III were not

Table 1 Properties of cholesterol conversion derivatives yielded with ST-200 cholesterol oxidase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular mass (Da)</th>
<th>λ₅₄₀ (nm)</th>
<th>Solvent ratio (Rᵥ)</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:3</td>
<td>1:1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>386</td>
<td>213</td>
<td>0.49</td>
<td>0.28</td>
</tr>
<tr>
<td>Derivative I</td>
<td>400</td>
<td>239</td>
<td>0.25</td>
<td>0.09</td>
</tr>
<tr>
<td>Derivative II</td>
<td>400</td>
<td>240</td>
<td>0.36</td>
<td>0.12</td>
</tr>
<tr>
<td>Derivative III</td>
<td>416</td>
<td>240</td>
<td>0.48</td>
<td>0.21</td>
</tr>
<tr>
<td>Derivative IV</td>
<td>416</td>
<td>239</td>
<td>0.54</td>
<td>0.30</td>
</tr>
<tr>
<td>Derivative V</td>
<td>398</td>
<td>249</td>
<td>0.71</td>
<td>0.40</td>
</tr>
<tr>
<td>Derivative VI</td>
<td>394</td>
<td>240</td>
<td>0.71</td>
<td>0.42</td>
</tr>
</tbody>
</table>

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determined by NMR spectrometry because of their low production, their molecular masses suggested that they were 6α-hydroxycholest-4-en-3-one and 6α-hydroperoxycholest-4-en-3-one respectively.

**Comparison of the predominant derivatives from cholesterol by oxidation with cholesterol oxidases**

Prolonged the oxidation of cholesterol with ST-200 cholesterol oxidase produced several derivatives from cholesterol (Table 1). Pfl or Pse gave derivatives similar to that of ST-200 cholesterol oxidase. With Str, Bre and Ner, cholest-4-en-3-one was the predominant product.

Next, cholesterol (0.9 mM) was oxidized with various cholesterol oxidases (5 m-units/ml) at 30 °C for 3 h. Oxidized derivatives were analysed by silica-gel TLC. The predominant derivative was cholest-4-en-3-one when cholesterol was oxidized with Bre, Ner, or Str. In contrast, the cholesterol oxidases derived from *Pseudomonas* spp. (ST-200 cholesterol oxidase, Pfl or Pse) produced 6β-hydroperoxycholest-4-en-3-one as the predominant derivative.

The oxidation of cholesterol with Str or ST-200 cholesterol oxidase was followed by qualitative TLC and quantitative HPLC. ST-200 cholesterol oxidase produced 6β-hydroperoxycholest-4-en-3-one as the derivative appearing first (Figure 1A). This derivative reached a maximum quantity at 6 h; thereafter the amount decreased slightly. After 12 h the amounts of conversion products were as follows: 6β-hydroperoxycholest-4-en-3-one, 59 mol % of the initial cholesterol; cholest-4-ene-3,6-dione, 21 mol %; cholest-4-en-3-one, 9 mol %; 6β-hydroxycholest-4-en-3-one, 5 mol %. To determine whether oxysteroids other than 6β-hydroperoxycholest-4-en-3-one had been formed by enzymic activity or by auto-oxidation, 6β-hydroperoxycholest-4-en-3-one was incubated for 24 h with or without enzyme (results not shown). To exclude the possibility of microbial contamination, toluene droplets were added to the solution. The oxysteroids, such as 6-hydroxycholest-4-en-3-one and cholest-4-ene-3,6-dione, were generated from 6β-hydroperoxycholest-4-en-3-one in the absence of enzyme. The formation rates of these oxysteroids were similar to those in the presence of enzyme, demonstrating that the oxysteroids were formed by auto-oxidation.

Str accumulated exclusively cholest-4-en-3-one even after 12 h (Figure 1B). Although 6β-hydroperoxycholest-4-en-3-one was found in the reaction mixture at 12 h the yield was less than 0.1 %. Other oxysteroids were not detected for at least 12 h.

**O2 consumption and H2O2 formation accompanying the oxidation of cholesterol or cholestanol with cholesterol oxidases**

Generally, cholesterol oxidases catalyse the oxidation of cholesterol with 1 mol of O2 to produce 1 mol of cholest-4-en-3-one and 1 mol of H2O2 [2]. O2 consumption was examined by measuring the decrease in concentration of O2 dissolving in the assay solution before and after the oxidation of cholesterol (Table 2). Approx. 1 mol of O2 was consumed per mol of cholesterol with Bre, Ner or Str. In contrast, 2 mol of O2 were consumed per mol of cholesterol with ST-200 cholesterol oxidase, Pfl or Pse. Each enzyme used in this study consumed 1 mol of O2 to oxidize 1 mol of cholestanol and yield 5α-cholestan-3-one. These results show that the 5-double bond is essential for the excess O2 consumption with the latter oxidases.

H2O2 generated by the oxidation of cholesterol was measured by determining the quinoneimine produced by condensation with H2O2, phenol and aminoantipyrine (results not shown). In the oxidation reaction catalysed with each cholesterol oxidase examined in this study, 1 mol of H2O2 was produced accompanying the oxidation of 1 mol of cholesterol or cholestanol.

We compared the reaction rates of O2 consumption, H2O2 formation and product formation during the oxidation of cholesterol (Table 3). The three reaction rates were almost identical for the oxidation of cholesterol with Str. With ST-200 cholesterol oxidase, the rate of O2 consumption was double those of the formation of H2O2 and of 6β-hydroperoxycholest-4-en-3-one.
Table 2  Consumption of O$_2$ by cholesterol oxidation with cholesterol oxidases

Cholesterol oxidase from *Pseudomonas* sp. strain ST-200

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ST-200</th>
<th>Pse</th>
<th>Pfl</th>
<th>Str</th>
<th>Bre</th>
<th>Ner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.87 ± 0.02</td>
<td>0.67 ± 0.02</td>
<td>0.88 ± 0.03</td>
<td>0.43 ± 0.02</td>
<td>0.43 ± 0.02</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>Cholestanol</td>
<td>0.44 ± 0.02</td>
<td>0.43 ± 0.02</td>
<td>0.43 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.43 ± 0.02</td>
</tr>
</tbody>
</table>

Table 3  Reaction rates of O$_2$ consumption, H$_2$O$_2$ formation and product formation during cholesterol oxidation with ST-200 cholesterol oxidase or Str

Cholesterol (0.9 mM) was incubated with each cholesterol oxidase (4 m-units/ml) at 30 °C. O$_2$ consumption was followed by monitoring dissolved oxygen concentration during the oxidation. H$_2$O$_2$ formation was followed by horseradish-peroxidase-coupled assay. The production of each compound was followed by HPLC analysis. Abbreviation: n.d., not detected.

<table>
<thead>
<tr>
<th>Cholesterol oxidase</th>
<th>O$_2$ consumption (µmol/min per mg of enzyme)</th>
<th>H$_2$O$_2$ formation (µmol/min per mg of enzyme)</th>
<th>Formation of derivative IV (µmol/min per mg of enzyme)</th>
<th>Formation of derivative VI (µmol/min per mg of enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-200</td>
<td>19.8 ± 0.7</td>
<td>10.3 ± 0.4</td>
<td>8.7 ± 1.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Str</td>
<td>9.4 ± 0.5</td>
<td>9.1 ± 0.5</td>
<td>n.d.</td>
<td>8.4 ± 1.8</td>
</tr>
</tbody>
</table>

Figure 2  Peroxidation of cholest-5-en-3-one with ST-200 cholesterol oxidase and cholestanol

(A) Cholest-5-en-3-one (0.25 mM) was incubated at 30 °C with shaking in reaction mixture without cholesterol oxidase. (B, C) Cholest-5-en-3-one (0.25 mM) was incubated with cholesterol oxidase (4 m-units/ml) in the presence (B) or absence (C) of cholestanol (0.25 mM). At intervals, samples were extracted twice with 1 vol. of chloroform. The chloroform extract was analysed by HPLC. Symbols: ⧿, cholestanol; ⧿, cholest-5-en-3-one; ⧿, 6β-hydroperoxycholest-4-en-3-one; ⧿, cholest-4-ene-3,6-dione; ⧿, cholest-4-en-3-one; ⧿, 6β-hydroxycholest-4-en-3-one; ⧿, 5α-cholestan-3-one. Each point is the mean for duplicate experiments; errors were less than 10%.

Dioxygenation of cholest-5-en-3-one with ST-200 cholesterol oxidase in the presence of cholestanol

To ascertain whether a putative intermediate, cholest-5-en-3-one, would be dioxygenated, cholest-5-en-3-one was incubated with ST-200 cholesterol oxidase (Figure 2). In the absence of the cholesterol oxidase, a small portion of cholest-5-en-3-one was converted into cholest-4-ene-3,6-dione (8 mol% of the initial cholest-5-en-3-one), 6β-hydroperoxycholest-4-en-3-one (3 mol%), cholest-4-en-3-one (2 mol%), and 6β-hydroxycholest-4-en-3-one (1 mol%) (Figure 2A). This conversion is believed to be due to auto-oxidation of the substrate [14]. Only the production of 6β-hydroperoxycholest-4-en-3-one was slightly enhanced when cholest-5-en-3-one was incubated with ST-200 cholesterol oxidase (Figure 2B). The amount of 6β-hydroperoxycholest-4-en-3-one produced was equivalent to 7% of the initial cholest-5-en-3-one concentration after 12 h.

The oxidation of cholest-5-en-3-one and the production of 6β-hydroperoxycholest-4-en-3-one were markedly enhanced when cholest-5-en-3-one was incubated with ST-200 cholesterol oxidase in the presence of cholestanol, added to reduce the oxidase (Figure 2C). After 12 h the amount of 6β-hydroperoxycholest-4-en-3-one was equivalent to 33% of the initial cholest-5-en-3-one added. The O$_2$ consumption corresponded to the sum of the consumption of cholestanol and cholest-5-en-3-one. The yield of H$_2$O$_2$ was equivalent to the amount of cholestanol added. When cholest-5-en-3-one was incubated in the presence of 0.25 mM

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The nucleotide sequence of gene relation between these two types of enzymes is not at all clear at and formed 1 mol of H\textsubscript{2}O. The oxidases used in the present study. Cholesterol oxidases has been reported [16] but not those of the other genes encoding chiometry of O\textsubscript{2} consumption and H\textsubscript{2}O\textsubscript{2}. The results described here suggest that the intermediate from cholesterol to 6\beta-hydroperoxycholest-4-en-3-one is cholest-5-en-3-one rather than the 4-ene isomer.

**DISCUSSION**

ST-200 can oxidize cholesterol dissolved in appropriate organic solvents [6]. The extracellular cholesterol oxidase from ST-200 was purified as an enzyme responsible for the oxidation of cholesterol in a two-phase microbial conversion system consisting of water and organic solvent [9]. ST-200 cholesterol oxidase produced the same derivatives from cholesterol as were observed in the two-phase conversion system for cholesterol with ST-200 cells. These derivatives were identified as shown in Table 1. In a previous study, 6\beta-hydroperoxycholest-4-en-3-one was mis-identified as 6\beta-hydroxycholest-4-en-3-one [6] because its molecular mass was underestimated owing to pyrolysis of the derivative during ionization.

A determination of the time course of product formation from cholesterol with ST-200 cholesterol oxidase showed that 6\beta-hydroperoxycholest-4-en-3-one was produced first with ST-200 cholesterol oxidase, then a small amount of 6\beta-hydroxycholest-4-en-3-one appeared after prolonged incubation, suggesting that 6\beta-hydroperoxycholest-4-en-3-one was the initial product generated by ST-200 cholesterol oxidase (Figure 1). The spontaneous conversion of 6\beta-hydroperoxycholest-4-en-3-one is reported for products with Pil, although it has been proposed that 6\beta-hydroxycholest-4-en-3-one is produced from cholesterol by mono-oxygenation with Psc [15]. We conclude that the oxidase produces 6\beta-hydroperoxycholest-4-en-3-one initially and that other oxysteroids are produced from the initial product.

There are two distinctive types of bacterial enzyme catalysing the oxidation of cholesterol. One forms cholest-4-en-3-one and another forms 6\beta-hydroperoxycholest-4-en-3-one as the major product. The former type catalyses the oxidation of cholesterol to the intermediate, cholest-5-en-3-one, with reduction of O\textsubscript{2} to H\textsubscript{2}O\textsubscript{2} and isomerization of the Δ\textsuperscript{2}-bond to form cholest-4-en-3-one [1,2]. These oxidases consume 1 mol of O\textsubscript{2} and form 1 mol of H\textsubscript{2}O\textsubscript{2} in oxidizing 1 mol of cholesterol. However, the stoichiometry of O\textsubscript{2} consumption and H\textsubscript{2}O\textsubscript{2} formation has not been shown for the latter type of oxidase. The results described here show that this type of cholesterol oxidase consumed 2 mol of O\textsubscript{2} and formed 1 mol of H\textsubscript{2}O\textsubscript{2} in oxidizing 1 mol of cholesterol. The relation between these two types of enzymes is not at all clear at present. The nucleotide sequence of gene choA, coding for Str, has been reported [16] but not those of the other genes encoding the oxidases used in the present study. Cholesterol oxidases derived from bacteria related to *Pseudomonas* probably produce 6\beta-hydroperoxysteroid but not cholest-4-en-3-one.

Scheme 1(B) shows the reactions leading to the formation of cholest-4-en-3-one. The oxidases forming cholest-4-en-3-one from cholesterol have been shown to constitute bifunctional enzymes carrying 3\beta-hydroxysteroid oxidase and Δ\textsuperscript{2}-3-ketosteroid isomerase activities [17]. In Scheme 1(B), the 3\beta-hydroxy group of cholesterol is dehydrogenated with the loss of the 3α-hydrogen and the 3\beta-hydroxy hydrogen, leading to the formation of enzyme-bound cholest-5-en-3-one. Two electron equivalents are transferred to one mol of FAD by the oxidase activity and reduce the coenzyme (FAD.H\textsubscript{2}). The rapid reoxidation of FAD.H\textsubscript{2} with O\textsubscript{2} results in the formation of a flavin hydroperoxide (FAD.H\textsubscript{2}.O\textsubscript{2}), which decomposes rapidly to FAD and H\textsubscript{2}O\textsubscript{2} [18]. The oxidized form of the enzyme catalyses the isomerization of the Δ\textsuperscript{2}-bond of the enzyme-bound cholest-5-en-3-one (transferring the 4β-hydrogen to the 6β position), which leads to the formation of cholest-4-en-3-one. This compound dissociates from the enzyme as a final product. In this oxidation pathway, the consumption of substrates (cholesterol and cholestanol) and O\textsubscript{2} and the formation of H\textsubscript{2}O\textsubscript{2} occur in 1:1:1 stoichiometry.

The cholesterol oxidation pathway was proposed for Pil by Teng and Smith [10], although stoichiometric examination was not performed for O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}. Here we propose a cholesterol oxidation pathway with ST-200 cholesterol oxidase, Pil or Pse (Scheme 1C) on the basis of the pathway with Pil, the balance of O\textsubscript{2} used and H\textsubscript{2}O\textsubscript{2} generated to oxidize cholesterol or cholestanol, the possible peroxidation of cholest-5-en-3-one in the presence of cholestanol, and the lack of activity of the enzyme towards cholest-4-en-3-one as substrate. As a first step shown in Scheme 1(C), the 3\β-hydroxysteroid oxidase activity of the 6\β-hydroperoxide-forming oxidase also forms FAD.H\textsubscript{2} and enzyme-bound cholest-5-en-3-one as intermediates, as with enzymes forming cholest-4-en-3-one (Scheme 1B). FAD.H\textsubscript{2} is oxidized with O\textsubscript{2} to FAD.H\textsubscript{2}.O\textsubscript{2}, from which O\textsubscript{2} is transferred to the 6β position of enzyme-bound cholest-5-en-3-one but not 5α-cholestan-3-one with a saturated 5-bond (Scheme 1D). The O\textsubscript{2} transfer reaction is unique to cholesterol peroxidation. Then the 4β-hydrogen is transferred to the 6-peroxy group of a dioxygenated intermediate, leading to the formation of 6\β-hydroperoxycholest-4-en-3-one, which dissociates from the enzyme. FAD.H\textsubscript{2} is again oxidized with O\textsubscript{2} to FAD.H\textsubscript{2}.O\textsubscript{2}, which decomposes to FAD and H\textsubscript{2}O\textsubscript{2} in the absence of cholest-5-en-3-one on the enzyme. When cholestanol is used as a substrate, FAD.H\textsubscript{2} and enzyme-bound 5α-cholestan-3-one are formed. The 3-enolic derivative dissociates from the enzyme without further modification (Scheme 1D). FAD.H\textsubscript{2} is oxidized to FAD through the formation of FAD.H\textsubscript{2}.O\textsubscript{2}. In the presence of cholest-5-en-3-one as a substrate coexisting in the reaction mixture, a portion of cholest-5-en-3-one binds with the enzyme instead of 5α-cholestan-3-one and is peroxidized with FAD.H\textsubscript{2}.O\textsubscript{2} (Scheme 1E).

In the oxidation pathway shown in Scheme 1(D), the stoichiometries of the consumption of substrates and O\textsubscript{2} and the formation of H\textsubscript{2}O\textsubscript{2} are different when cholesterol and when cholestanol are substrates: 1 mol of cholesterol is oxidized to 6\β-hydroperoxycholest-4-en-3-one with 2 mol of O\textsubscript{2}, forming 1 mol of H\textsubscript{2}O\textsubscript{2} whereas 1 mol of cholestanol is oxidized to 5α-cholestan-3-one with 1 mol of O\textsubscript{2} and forms 1 mol of H\textsubscript{2}O\textsubscript{2}. The possible intermediate cholest-5-en-3-one is oxidized to 6\β-hydroperoxysterol, consuming 1 mol of O\textsubscript{2} with the reduced form of the enzyme. Our results on O\textsubscript{2} consumption and H\textsubscript{2}O\textsubscript{2} formation support this cholesterol oxidation pathway, although a detailed analysis will be needed of the transfer of hydrogen and the chemical changes of FAD intermediates.

The extracellular cholesterol oxidase isolated in this study produced 6\β-hydroperoxycholest-4-en-3-one as a major product. This product was converted to various compounds (Table 1). ST-200 cells, producers of this enzyme, oxidize effectively cholesterol dissolved in organic solvent and accumulate cholest-4-en-3,6-
dione [6]. The production of cholest-4-ene-3,6-dione is rapid compared with that of 6β-hydroperoxycholest-4-en-3-one. Although 6β-hydroperoxycholest-4-en-3-one is spontaneously converted to several steroids including cholest-4-ene-3,6-dione [15], some unidentified biological process might be involved in the conversion. In addition, ST-200 does not grow on cholesterol or 6β-hydroperoxycholest-4-en-3-one, suggesting that cholesterol is not a natural substrate for this enzyme.

REFERENCES


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