Two moles of O₂ consumption and one mole of H₂O₂ formation during cholesterol peroxidation with cholesterol oxidase from *Pseudomonas* sp. strain ST-200

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Cholesterol oxidase from *Pseudomonas* sp. strain ST-200 oxidized cholesterol and cholestanol to 6 β -hydroperoxycholest-4-en-3-one and 5 α -cholestan-3-one respectively. The former was converted spontaneously to several oxysteroids such as 6-hydroxycholest-4-en-3-one and cholest-4-ene-3,6-dione, with the consumption of 2 mol of O₂ and the formation of 1 mol of H₂O₂ 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 6 β -hydroperoxycholest-4-en-3-one.

Key words: cholestanol, cholest-4-ene-3,6-dione, dioxygenation, FAD, 6 β -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-3 β -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 Δ^5 -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 6 β -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 6 β -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 H₂O₂ and the consumption of O₂ 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 O₂ consumption, product formation and H₂O₂ 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 μ mol of H₂O₂ by the oxidation of 1 μ mol 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 % (v/v) 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.

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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 cholestanol and cholesterol 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 CDCl_3 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). ^{13}C -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 $\times$ 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 A_{215} 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 oxidation activity was assayed by measuring H_2O_2 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 A_{500} during incubation at 30 °C for 5 min. The enzymic activity was calculated from the molar absorption coefficient of quinoneimine (ϵ $5.33 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

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 A₅₀₀ of the mixture was monitored. H₂O₂ formation was calculated from the molar absorption coefficient of the red quinoneimine.

Materials

Cholesterol, cholestanol and cholest-4-en-3-one were purchased from Nacalai Tesque (Kyoto, Japan). 5 α -Cholestan-3-one was purchased from Sigma. 6 β -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 *R_F* 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 *R_F* values of the cholesterol derivatives measured in the three solvent systems. The *R_F* 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 $\lambda_{\max}$ values indicated that these derivatives possessed π -resonance 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 6 β -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 6 β -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, 6 β -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 of derivatives IV, V and VI in the ¹³C-NMR spectra were identical to those reported for 6 β -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 6 β -hydroperoxycholest-4-en-3-one (IV) is spontaneously epimerized to 6 α -hydroperoxycholest-4-en-3-one and reduced to 6 α -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

$\lambda_{\max}$ values were measured in 95% (v/v) ethanol. *R_F* values were measured by silica-gel TLC with n-hexane/diethyl ether (1:3, 1:1 or 3:1, v/v) as mobile phase. The assignments of names to derivatives I and III are putative, as described in the Results section.

Compound	Molecular mass (Da)	$\lambda_{\max}$ (nm)	Solvent ratio (<i>R_F</i>)			Name
			1:3	1:1	3:1	
Cholesterol	386	213	0.49	0.28	0.15	Cholest-5-en-3 β -ol
Derivative I	400	239	0.25	0.09	0.02	6 α -Hydroxycholest-4-en-3-one
Derivative II	400	240	0.36	0.12	0.04	6 β -Hydroxycholest-4-en-3-one
Derivative III	416	240	0.48	0.21	0.08	6 α -Hydroperoxycholest-4-en-3-one
Derivative IV	416	239	0.54	0.30	0.10	6 β -Hydroperoxycholest-4-en-3-one
Derivative V	398	249	0.71	0.40	0.23	Cholest-4-ene-3,6-dione
Derivative VI	394	240	0.71	0.42	0.32	Cholest-4-en-3-one

Figure 1 Oxidation of cholesterol with ST-200 cholesterol oxidase (A) and with Str (B)

Cholesterol oxidase (0.5 unit) was added to the reaction mixture containing 0.9 mM cholesterol (100 ml) and incubated at 30 °C with shaking. Samples (10 ml) were taken from the solution periodically and extracted twice with chloroform (10 ml). The extract was analysed by reverse-phase chromatography. Symbols: ○, cholesterol; ●, 6β-hydroperoxycholest-4-en-3-one; ▲, cholest-4-ene-3,6-dione; ■, cholest-4-en-3-one; △, 6β-hydroxycholest-4-en-3-one. Each point is the mean for duplicate experiments; errors were less than 8%.

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 cholesterol 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 cholesterol 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 cholesterol.

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 O₂ by cholesterol oxidation with cholesterol oxidases

Cholesterol or cholestanol (0.45 μ mol) was used as substrate. O₂ consumption was determined by measuring the dissolved oxygen concentration before and after the oxidation with 4 m-units/ml cholesterol oxidase at 30 °C for 30 min. Results are means $\pm$ S.D. for three independent experiments.

Substrate	Cholesterol oxidase consumption of O ₂ (μ mol)					
	ST-200	Pse	Pfl	Str	Bre	Ner
Cholesterol	0.87 $\pm$ 0.02	0.87 $\pm$ 0.02	0.88 $\pm$ 0.03	0.43 $\pm$ 0.02	0.43 $\pm$ 0.02	0.44 $\pm$ 0.02
Cholestanol	0.44 $\pm$ 0.02	0.43 $\pm$ 0.02	0.43 $\pm$ 0.01	0.43 $\pm$ 0.01	0.43 $\pm$ 0.01	0.43 $\pm$ 0.02

Table 3 Reaction rates of O₂ consumption, H₂O₂ 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. O₂ consumption was followed by monitoring dissolved oxygen concentration during the oxidation. H₂O₂ formation was followed by horseradish-peroxidase-coupled assay. The production of each compound was followed by HPLC analysis. Abbreviation: n.d., not detected.

Cholesterol oxidase	Rate (μ mol/min per mg of enzyme)			
	O ₂ consumption	H ₂ O ₂ formation	Formation of derivative IV	Formation of derivative VI
ST-200	19.8 $\pm$ 0.7	10.3 $\pm$ 0.4	8.7 $\pm$ 1.6	n.d.
Str	9.4 $\pm$ 0.5	9.1 $\pm$ 0.5	n.d.	8.4 $\pm$ 1.8

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; $\square$, cholest-5-en-3-one; $\bullet$, 6 β -hydroperoxycholest-4-en-3-one; $\blacktriangle$, cholest-4-ene-3,6-dione; $\blacksquare$, cholest-4-en-3-one; $\triangle$, 6 β -hydroxycholest-4-en-3-one; $\blacktriangledown$, 5 α -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), 6 β -hydroperoxycholest-4-en-3-one (3 mol %), cholest-4-en-3-one (2 mol %) and 6 β -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 6 β -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 6 β -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 6 β -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 6 β -hydroperoxycholest-4-en-3-one was equivalent to 33 % of the initial cholest-5-en-3-one added. The O₂ consumption corresponded to the sum of the consumption of cholestanol and cholest-5-en-3-one. The yield of H₂O₂ was equivalent to the amount of cholestanol added. When cholest-5-en-3-one was incubated in the presence of 0.25 mM

H_2O_2 , the formation of 6β -hydroperoxycholest-4-en-3-one or other oxysterols did not increase (results not shown). This result excludes the possibility that H_2O_2 accumulating in the reaction mixture oxidized the substrate.

These results indicate that an oxidized form of ST-200 cholesterol oxidase has no isomerase activity to generate Δ^4 -3-ketosteroid from a Δ^5 -3-isomer and a low dioxygenase activity for the formation of 6β -hydroperoxycholest-4-en-3-one from cholest-5-en-3-one. A reduced form of the enzyme has a high dioxygenase activity towards cholest-5-en-3-one with O_2 . Cholest-4-en-3-one incubated with the oxidase in the presence or absence of cholestanol was not converted into 6β -hydroperoxycholest-4-en-3-one or other oxysteroids. These results suggest that the intermediate from cholesterol to 6β -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, 6β -hydroperoxycholest-4-en-3-one was misidentified as 6β -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 6β -hydroperoxycholest-4-en-3-one was produced first with ST-200 cholesterol oxidase, then a small amount of 6β -hydroxycholest-4-en-3-one appeared after prolonged incubation, suggesting that 6β -hydroperoxycholest-4-en-3-one was the initial product generated by ST-200 cholesterol oxidase (Figure 1). The spontaneous conversion of 6β -hydroperoxycholest-4-en-3-one is reported for products with Pfl, although it has been proposed that 6ζ -hydroxycholest-4-en-3-one is produced from cholesterol by mono-oxygenation with Pse [15]. We conclude that the oxidase produces 6β -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 6β -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_2 to H_2O_2 and isomerization of the Δ^5 -bond to form cholest-4-en-3-one [1,2]. These oxidases consume 1 mol of O_2 and form 1 mol of H_2O_2 in oxidizing 1 mol of cholesterol. However, the stoichiometry of O_2 consumption and H_2O_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_2 and formed 1 mol of H_2O_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 6β -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 3β -hydroxysteroid oxidase and Δ^5 -3-ketosteroid isomerase activities [17]. In Scheme 1(B), the 3β -hydroxy group of cholesterol is dehydrogenated with the loss of the 3α -hydrogen and the 3β -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_2). The rapid reoxidation of FAD.H_2 with O_2 results in the formation of a flavin hydroperoxide ($\text{FAD.H}_2\text{O}_2$), which decomposes rapidly to FAD and H_2O_2 [18]. The oxidized form of the enzyme catalyses the isomerization of the Δ^5 -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_2 and the formation of H_2O_2 occur in 1:1:1 stoichiometry.

The cholesterol oxidation pathway was proposed for Pfl by Teng and Smith [10], although stoichiometric examination was not performed for O_2 and H_2O_2 . Here we propose a cholesterol oxidation pathway with ST-200 cholesterol oxidase, Pfl or Pse (Scheme 1C) on the basis of the pathway with Pfl, the balance of O_2 used and H_2O_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 3β -hydroxysteroid oxidase activity of the 6β -hydroperoxide-forming oxidase also forms FAD.H_2 and enzyme-bound cholest-5-en-3-one as intermediates, as with enzymes forming cholest-4-en-3-one (Scheme 1B). FAD.H_2 is oxidized with O_2 to $\text{FAD.H}_2\text{O}_2$, from which O_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_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 6β -hydroperoxycholest-4-en-3-one, which dissociates from the enzyme. FAD.H_2 is again oxidized with O_2 to $\text{FAD.H}_2\text{O}_2$, which decomposes to FAD and H_2O_2 in the absence of cholest-5-en-3-one on the enzyme. When cholestanol is used as a substrate, FAD.H_2 and enzyme-bound 5α -cholestan-3-one are formed. The 3-one derivative dissociates from the enzyme without further modification (Scheme 1D). FAD.H_2 is oxidized to FAD through the formation of $\text{FAD.H}_2\text{O}_2$. 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 $\text{FAD.H}_2\text{O}_2$ (Scheme 1E).

In the oxidation pathway shown in Scheme 1(D), the stoichiometries of the consumption of substrates and O_2 and the formation of H_2O_2 are different when cholesterol and when cholestanol are substrates: 1 mol of cholesterol is oxidized to 6β -hydroperoxycholest-4-en-3-one with 2 mol of O_2 , forming 1 mol of H_2O_2 , whereas 1 mol of cholestanol is oxidized to 5α -cholestan-3-one with 1 mol of O_2 and forms 1 mol of H_2O_2 . The possible intermediate cholest-5-en-3-one is oxidized to 6β -hydroperoxycholest-4-en-3-one, consuming 1 mol of O_2 with the reduced form of the enzyme. Our results on O_2 consumption and H_2O_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 6β -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-ene-3,6-

dione [6]. The production of cholest-4-ene-3,6-dione is rapid compared with that of 6 β -hydroperoxycholest-4-en-3-one. Although 6 β -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 6 β -hydroperoxycholest-4-en-3-one, suggesting that cholesterol is not a natural substrate for this enzyme.

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