VOLUME 119, NUMBER 5
FEBRUARY 5, 1997

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Isomerization, But Not Oxidation, Is Suppressed by a Single Point Mutation, E361Q, in the Reaction Catalyzed by Cholesterol Oxidase

Nicole S. Sampson*,† and Ignatius J. Kass

Contribution from the Department of Chemistry, State University of New York, Stony Brook, New York 11794-3400

Received July 3, 1996[⊗]

Abstract: The putative active site base of cholesterol oxidase from *Streptomyces* has been removed by site-directed mutagenesis and the mutant enzyme characterized. When glutamate-361 is mutated to a glutamine, the isomerization chemistry catalyzed by cholesterol oxidase is suppressed and the intermediate cholest-5-ene-3-one is isolated. The specific activity for oxidation is 20-fold slower than the wild-type reaction, though the specific activity for isomerization is 10 000-fold slower. Furthermore, incubation of cholest-5-ene-3-one with the E361Q cholesterol oxidase resulted in the production of cholest-4-ene-6β-hydroperoxy-3-one (6%), cholest-4-ene-3,6-dione (32%), cholest-4-ene-6β-ol-3-one (36%), and cholest-4-ene-6α-hydroperoxy-3-one/cholest-4-ene-6α-ol-3-one (13%), in addition to cholest-4-ene-3-one (13%). Measurement of reaction stoichiometry eliminated the possibility that H_2O_2 or the C4a-hydroperoxy flavin was the oxygenation agent. It is proposed that cholest-4-ene-6-hydroperoxy-3-one is the product of radical chain autoxidation and that cholest-4-ene-3,6-dione and cholest-4-ene-6-ol-3-one are decomposition products of the hydroperoxy steroid radical. The characterization of the E361Q mutant chemistry has illuminated the importance of intermediate sequestration in enzyme catalysis. The mutant enzyme will be used to obtain information about the structure of the enzyme in the presence of the reaction intermediate. Moreover, the altered activity of E361Q cholesterol oxidase will facilitate its application in studies of cell membranes.

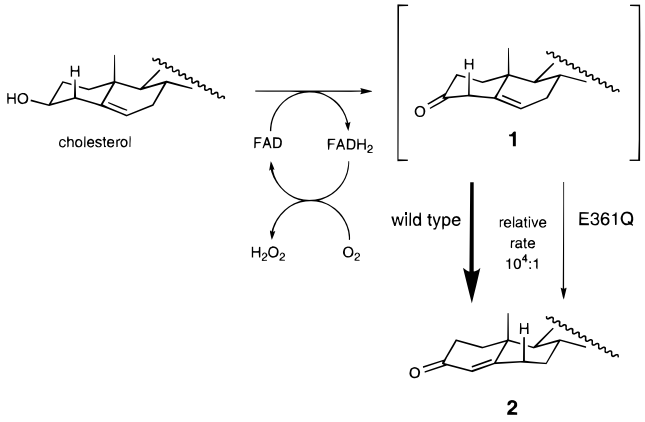
We describe here the results of experiments designed to elucidate the mechanism of cholesterol oxidase. Cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation and isomerization of cholesterol into cholest-4-ene-3-one, **2** (Scheme 1). It is produced by a variety of microorganisms, including *Brevibacterium sterolicum* (ATCC 81387) and *Streptomyces* sp. strain SA-COO, and is used to assay serum cholesterol levels. It is also used by cell biologists as a tool for studying cholesterol localization in cell membranes.[1] Recently, the insecticidal properties of cholesterol oxidase have been discovered.[2,3] Cholesterol oxidase causes complete lysis of mid-gut epithelial cells in boll weevil (*Anthonomus grandis grandis* Boheman) larvae. Information about the structure and mechanism of cholesterol oxidase will lead to improved analytical techniques and commercial products.

Cholesterol oxidase is part of the bacterial metabolic pathway for utilizing cholesterol as a carbon source and is secreted by gram-positive soil bacteria.[4] The cholesterol oxidases from *Streptomyces* (choA) and *B. sterolicum* (choB) are 58% and 64% identical in amino acid sequence and nucleotide sequence,

† e-mail: nicole.sampson@sunysb.edu.
⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.
(1) Lange, Y. *J. Lipid Res.* **1992**, *33*, 315−321.
(2) Purcell, J. P.; Greenplate, J. T.; Jennings, M. G.; Ryerse, J. S.; Pershing, J. C.; Sims, S. R.; Prinsen, M. J.; Corbin, D. R.; Tran, M.; Sammons, R. D.; Stonard, R. J. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 1406−13.
(3) Greenplate, J. T.; Duck, N. B.; Pershing, J. C.; Purcell, J. P. *Entomol. Exp. Appl.* **1995**, *74*, 253−258.
(4) Uwajima, T.; Yagi, H.; Nakamura, S.; Terada, O. *Agr. Biol. Chem.* **1973**, *37*, 2345−2350.

S0002-7863(96)02258-5 CCC: $14.00

Scheme 1

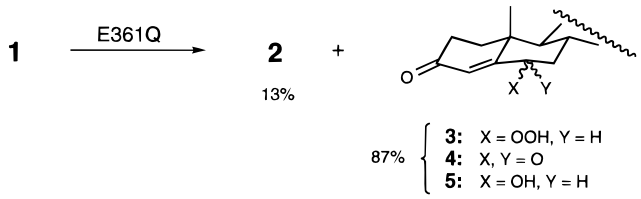


respectively.[5] Both the choA enzyme, used in this report, and the choB oxidase are monomers (57 kD) and require one flavin adenine dinucleotide (FAD).[6,7] Blow and co-workers[8,9] have solved the X-ray crystal structures of the choB oxidase without substrate bound and with epiandrosterone, a substrate analogue, bound in the active site. These structures show that there is a single active site for both oxidation and isomerization.

Previously, using deuterated and nondeuterated substrates, we demonstrated that the isomerization step proceeds via a stereospecific proton transfer from the 4β-position to the 6β-position to form cholest-4-ene-3-one.[10] This result implies that there is one active site base responsible for isomerization. This isomerization mechanism is analogous to those determined for the unrelated cholesterol oxidase from *Nocardia erythropolis*[11] and ketosteroid isomerases.[12−14] Furthermore, orbital symmetry requires that isomerization is a stepwise 1,3 shift.[15] Examination of the X-ray crystal structure of cholesterol oxidase reveals that there is one charged residue in the active site, glutamate-361,[16] that is positioned directly over the β-face of the bound sterol.[9] This residue is the most reasonable choice of base responsible for isomerization of the double bond into conjugation with the ketone of the intermediate, and we wanted to experimentally confirm its role in catalysis.

Site-directed mutagenesis of glutamate-361 to glutamine was the method by which we verified the role of this residue. We report here the mutation of the putative active site base of

Scheme 2



cholesterol oxidase. When glutamate-361 is mutated to a glutamine, the isomerization chemistry catalyzed by E361Q cholesterol oxidase is suppressed. Upon incubation of cholesterol with E361Q cholesterol oxidase, the intermediate cholest-5-ene-3-one, **1**, is isolated (Scheme 1). Furthermore, prolonged incubation of **1** with E361Q cholesterol oxidase results in the production of cholest-4-ene-6-hydroperoxy-3-one, **3**. In addition, cholest-4-ene-3,6-dione, **4**, and cholest-4-ene-6-ol-3-one, **5**, are formed (Scheme 2). We propose that **3** is the product of radical chain autoxidation and that **4** and **5** are hydroperoxy radical decomposition products.

Experimental Procedures

General Procedures. Cholest-4-ene-3-one, **2**, cholest-5-ene-3-one, **1**, and Triton X-100 were from Aldrich Chemical Co., Milwaukee, WI. Cholest-4-ene-6β-ol-3-one, **5**β, and cholest-4-ene-3,6-dione, **4**, were obtained from Steraloids, Wilton, NH. Cholesterol was from Sigma Chemical Co., St. Louis, MO, and [26-^{14}C]cholesterol from E. I. Dupont, Philadelphia, PA. pCO117 was a generous gift from Y. Murooka.[18] All chemicals and solvents, of reagent or HPLC grade, were supplied by Fisher Scientific, Pittsburgh, PA, unless otherwise specified. Water for assays and chromatography was distilled, followed by passage through a Barnstead NANOpure® filtration system to give a resistivity better than 18 MΩ. A Shimadzu UV2101 PC spectrophotometer was used for assays and recording spectra. CD spectra were acquired on an Aviv Model 62A circular dichroism spectrophotometer. Mass spectra were obtained on a VG 70-VSE mass spectrometer at the University of Illinois. ^{1}H NMR data are reported in the following manner: chemical shift in ppm with solvent as standard (multiplicity, integrated intensity, coupling constant in hertz, and steroid carbon to which proton is attached). Only the resolved steroid resonances are given. NMR spectra were recorded in $CDCl_3$. The following buffers were used: (A) 50 mM sodium phosphate, pH 7.0; (B) A + 0.025% Triton X-100; (C) A + 1.5 M $(NH_4)_2SO_4$. HPLC capacity factors (k′s) reported correspond to the conditions described below.

Construction of pCO219 (E361Q Plasmid). The *Streptomyces* cholesterol oxidase gene was subcloned[19] from pCO117[18] into M13mp18 phage using EcoRI and HindIII restriction sites, and the E361Q mutant gene was constructed using the method of Eckstein.[20,21] To change glutamate-361 to glutamine, the mutagenic primer 5′-ggggCgAT**CT-g**CgCgAAgAC-3′ was used. The mutant gene was subcloned into pKK223-3 using EcoRI and HindIII restriction sites to generate the expression plasmid containing mutant cholesterol oxidase, pCO219. The mutation was verified by dideoxy termination sequencing[22] of the pCO219 construct.

Purification of Wild-Type Cholesterol Oxidase. Cell paste of *E. coli* BL21(DE3)plysS(pCO117) obtained from Luria broth containing ampicillin (0.5 mM) medium grown for 12 h after addition of IPTG (0.2 mM) at mid-log phase was resuspended in 50 mM Tris·HCl, 1 mM EDTA, pH 7.0, and lysed by French press at 18 000 psi. All

(5) Ohta, T.; Fujishiro, K.; Yamaguchi, K.; Tamura, Y.; Aisaka, K.; Uwajima, T.; Hasegawa, M. *Gene* **1991**, *103*, 93−96.
(6) Kamei, T.; Takiguchi, H.; Ishimaru, S.; Nakamura, S.; Nomi, T. *Chem. Pharm. Bull.* **1982**, *26*, 2799−2804.
(7) Uwajima, T.; Yagi, H.; Terada, O. *Agr. Biol. Chem.* **1974**, *38*, 1149−1156.
(8) Vrielink, A.; Lloyd, L. F.; Blow, D. M. *J. Mol. Biol.* **1991**, *219*, 533−554.
(9) Li, J.; Vrielink, A.; Brick, P.; Blow, D. M. *Biochemistry* **1993**, *32*, 11507−11515.
(10) Kass, I. J.; Sampson, N. S. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 688−693.
(11) Smith, A. G.; Brooks, C. J. W. *Biochem. J.* **1977**, *167*, 121−129.
(12) Batzold, F. H.; Benson, A. M.; Covey, D.; Robinson, C. H.; Talalay, P. In *Advances in Enzyme Regulation*; Weber, G., Ed.; Pergamon Press: New York, 1975; Vol. 14, pp, 243−267.
(13) Kuliopulos, A.; Shortle, D.; Talalay, P. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8894−8897.
(14) Schwab, J. M.; Henderson, B. S. *Chem. Rev.* **1990**, *90*, 1203−1245.
(15) Woodward, R. B.; Hoffman, R. *The Conservation of Orbital Symmetry*; Verlag Chemie: Weinheim, 1970; pp 114−132.
(16) This numbering refers to the X-ray crystal structure system for numbering amino acid residues.[8] Numbering begins at the N-terminus of the processed *Brevibacterium* enzyme. Glutamate-361 is encoded by codon 406 in the *Brevibacterium* gene[5] and codon 398 in the *Streptomyces* gene.[17]
(17) Ishizaki, T.; Hirayama, N.; Shinkawa, H.; Nimi, O.; Murooka, Y. *J. Bacteriol.* **1989**, *171*, 596−601.
(18) Nomura, N.; Choi, K.-P.; Murooka, Y. *J. Ferm. Bioeng.* **1995**, *79*, 410−416.
(19) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning*; 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989.
(20) Nakamaye, K.; Eckstein, F. *Nucleic Acids Res.* **1986**, *14*, 9679−9698.
(21) Sayers, J. R.; Schmidt, W.; Eckstein, F. *Nucleic Acids Res.* **1988**, *16*, 791−802.
(22) Sanger, F.; Niklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463−5467.
(23) Fasman, G. D. *Practical Handbook of Biochemistry and Molecular Biology*; Fasman, G. D., Ed.; CRC Press: Boca Raton, FL, 1992.

subsequent purification steps were performed at 4 °C. Cell debris was removed by centrifugation at 135 000g for 30 min. The supernatant was loaded onto a column of DEAE-cellulose (DE-52, Whatman) preequilibrated with buffer A and eluted with buffer A. Fractions containing cholesterol oxidase were concentrated by $(NH_4)_2SO_4$ precipitation, the pellet was redissolved in buffer A, and $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. The protein was further purified on a butyl-Sepharose column (Pharmacia) that had been equilibrated with buffer C and eluted with a linear gradient (0−100%) of buffer A. Fractions were collected and analyzed by SDS-PAGE. Fractions containing pure oxidase were pooled, concentrated, and desalted by ultrafiltration into buffer A. Typically, 20−30 mg of pure cholesterol oxidase was obtained per liter of culture. Protein concentrations were determined by UV absorbance using $\epsilon_{280} = 81\,924\ M^{-1}\ cm^{-1}$ (calculated from the molar absorptivities of tryptophan and tyrosine[23]).

Purification of E361Q Cholesterol Oxidase. Cell paste of *E. coli* BL21(DE3)plysS(pCO219) was purified as described above for pCO117, the wild-type cholesterol oxidase. Typically, 20−30 mg of pure E361Q cholesterol oxidase was obtained per liter of culture.

UV and CD Spectra of Cholesterol Oxidase. A solution of cholesterol oxidase was prepared in buffer A. A baseline spectrum of buffer A was subtracted from the sample spectrum. The concentration of cholesterol oxidase was 13−19 μM for UV spectra and CD spectra in the near UV. For CD spectra in the far UV, the protein concentration was 54−58 μM.

Specific Activity Assay of Cholesterol Oxidase. Cholesterol was added as a propan-2-ol solution (4 mM) to buffer B prewarmed to 37 °C. The final assay mixture was 1.26% propan-2-ol, 50 μM cholesterol. The rate of formation of cholest-4-ene-3-one, **2**, was followed at 240 nm at 37 °C. The slope of the first 10% of the reaction was determined by linear regression and converted to μmol/min·mg using $\epsilon_{240} = 12\,100\ M^{-1}\ cm^{-1}$ for **2**.[11] The rate of formation of H_2O_2 was determined using a horseradish peroxidase coupled assay. The standard assay conditions were the same as the cholest-4-ene-3-one assay with the addition of 1.13 mM phenol, 0.87 mM 4-aminoantipyrine (Aldrich, Milwaukee, WI), and 10 U of horseradish peroxidase (Sigma, St. Louis, MO). The formation of quinonimine at 510 nm was followed as a function of time. The slope of the first 10% of the reaction was determined and converted to μmol/min·mg using $\epsilon_{510} = 5780\ M^{-1}\ min^{-1}$. This ϵ was calculated by calibration of solutions using the above referenced ϵ_{240} of **2**.

The individual specific activities for the formation of **2**, **3**, **4**, and **5** were measured under the same conditions as the A_{240} assay using [26-^{14}C]cholesterol. The quantity of each product formed was analyzed by HPLC (*vide infra*).

Time Course of Cholesterol Oxidase Reaction. Cholesterol or cholest-5-ene-3-one, **1**, was added as a propan-2-ol solution (3 mM) to buffer B prewarmed to 37 °C, to a final concentration of 140 μM. Reactions were initiated by the addition of cholesterol oxidase (6 nM wild-type or 294 nM E361Q). Reaction mixtures were incubated under foil to protect them from ambient light in a screw-capped vial with a silicone seal at 37 °C. Aliquots (1 mL) were removed at various time points and analyzed by HPLC (*vide infra*).

HPLC Analysis. Samples were analyzed with a Model 680 gradient controller, three M510 solvent pumps, and a Model 490 multiwavelength detector (Waters Corp., Milford, MA) or a Model PDA-1 photodiode array detector (Rainin Instrument, Woburn, MA). The following conditions were used: stationary phase, Microsorb-MV® C-18 column (Rainin Instrument Corp., Woburn, MA, 5 μm, 10 Å, 4.6 × 250 mm); gradient elution at 1.25 mL/min; solvent A, CH_3CN; solvent B, propan-2-ol; solvent C, CH_3CN/H_2O (1:1, v:v); detection at 212 and 240 nm and by scintillation counting of fractions. A 25-min isocratic elution with 80% A and 20% C followed by 10-min linear gradient to 85% A and 15% B followed by 25-min isocratic elution at the same conditions yielded the separation shown in Figure 1. Samples were injected directly from assay solutions. Product ratios were determined by liquid scintillation counting of collected fractions and, when necessary, integration of peak area as detected at 212 nm. Isolated peaks were characterized as described below.

Product Characterization. Cholest-4-ene-6*β*-hydroperoxy-3-one (3*β*): ^{1}H-NMR δ 4.42 (dd, 1, J = 3.6, 2.4 Hz, 6-H), 5.87 (s, 1, 4-H), 7.75 (s, 1, OOH); $k' = 14.3$; $\lambda_{max} = 240$ nm. **Cholest-4-ene-6α-hydroperoxy-3-one (3α):** ^{1}H-NMR δ 4.60 (ddd, 1, $J = 12.6, 5.4, 1.8$ Hz, 6-H), 6.09 (s, 1, 4-H), 8.05 (bs , 1, OOH); $k' = 11.5$; $\lambda_{max} = 240$ nm. **Cholest-4-ene-6α-ol-3-one (5α):** *m/z* (EI) = 400; ^{1}H-NMR δ 4.31 (m, 1, 6-H), 6.15 (s, 1, 4-H); $k' = 11.8$; $\lambda_{max} = 240$ nm. **Cholest-4-ene-6*β*-ol-3-one (5*β*):** *m/z* (EI) = 400; ^{1}H-NMR δ 4.33 (dd, 1, J = 2.7, 2.7 Hz, 6-H), 5.80 (s, 1, 4-H); $k' = 16.5$; $\lambda_{max} = 240$ nm. **Cholest-4-ene-3,6-dione (4):** *m/z* (EI) = 398; ^{1}H-NMR δ 2.66 (dd, 1, J = 15.5, 3.6, 2- or 7-H), 6.15 (s, 1, 4-H); $k' = 15.7$; $\lambda_{max} = 251$ nm.

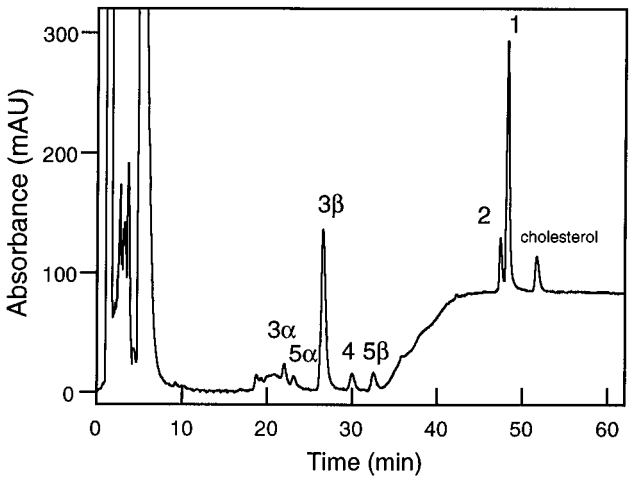


Figure 1. A typical HPLC chromatogram of E361Q reaction mixture after 3 h. HPLC conditions are given in Experimental Procedures: **3**α, cholest-4-ene-6α-hydroperoxy-3-one, $k' = 11.5$; **5**α, cholest-4-ene-6α-ol-3-one, $k' = 11.8$; **3***β*, cholest-4-ene-6*β*-hydroperoxy-3-one, $k' = 14.3$; **4**, cholest-4-ene-3,6-dione, $k' = 15.7$; **5***β*, cholest-4-ene-6*β*-ol-3-one, $k' = 16.5$; **2**, cholest-4-ene-3-one, $k' = 23.3$; **1**, cholest-5-ene-3-one, $k' = 23.8$; cholesterol, $k' = 25.7$.

Attempted Inhibition of 6-Oxygenation Activity. The specific activities of E361Q and wild-type cholesterol oxidase were measured in buffer B with cholesterol (50 μM, 1.6% propan-2-ol) in the presence of NaCN (1 mM), dithiothreitol (1 mM), or tris(carboethoxy)phosphine (3 mM) by following the increase in absorbance at 240 nm at 37 °C. The tris(carboethoxy)phosphine reaction mixture was analyzed by HPLC (*vide supra*). Catalase (21 U) and E361Q cholesterol oxidase (16 μg) were added to a 1 mL solution of cholesterol (140 μM, 3.3% propan-2-ol) in buffer B. The products formed were analyzed by HPLC (*vide supra*).

Anaerobic Titration of E361Q Cholesterol Oxidase. A quartz cuvette, modified as described by Williams et al.,[24] was used for anaerobic titrations. A 32 μM solution of E361Q cholesterol oxidase was prepared in buffer B. Protein solutions were deoxygenated by lyophilization and resuspended in degassed water under an Ar atmosphere. Residual O_2 (<5 μM) was removed from the solution by titration with cholesterol. The solution was judged to be O_2 free when titration of the FAD spectrum with 0.5 equiv of cholesterol showed a 50% reduction in absorption and remained constant, i.e., the $FADH_2$ formed was not reoxidized. This titration served to generate 0.5 equiv of **1** *in situ* in the absence of O_2. Thus, oxidized E361Q cholesterol oxidase was incubated with **1** in the absence of O_2. Flavin spectra were recorded every 20 min. After 1 h, the FAD was completely reduced by the addition of a second 0.5 equiv of cholesterol. Thus, reduced E361Q was incubated with **1**. Spectra were recorded every 20 min over 1 h. The $FADH_2$ was reoxidized by O_2 to FAD.

Initiation of 1 Oxidation by E361Q Cholesterol Oxidase. A solution of E361Q cholesterol oxidase (877 nM) in buffer B was ultrafiltered (NMWCO = 30 000) to remove cholesterol oxidase and the filtrate added to a solution of **1** (50 μM, 1.2% propan-2-ol) in buffer B. The rate of product formation was followed by UV assay at 240 nm. A portion of the filtrate was tested for residual cholesterol oxidase activity by measuring the specific activity with cholesterol as substrate in the absence of **1**. In a second experiment, E361Q cholesterol oxidase

(24) Williams, C. H., Jr.; Arscott, L. D.; Matthews, R. G.; Thorpe, C.; Wilkinson, K. D. *Meth. Enzymol.* **1979**, *62*, 185−197.

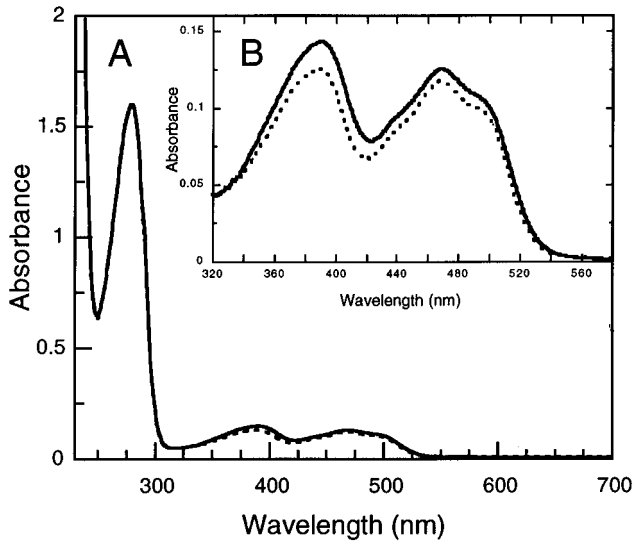


Figure 2. UV spectra of 19 μM wild-type (—) and 17 μM E361Q (- - -) cholesterol oxidase in sodium phosphate buffer, pH 7.0. Spectra were normalized at 280 nm. (A) Spectra from 225 nm to 700 nm. (B, inset) Closeup of FAD region, from 320 nm to 580 nm.

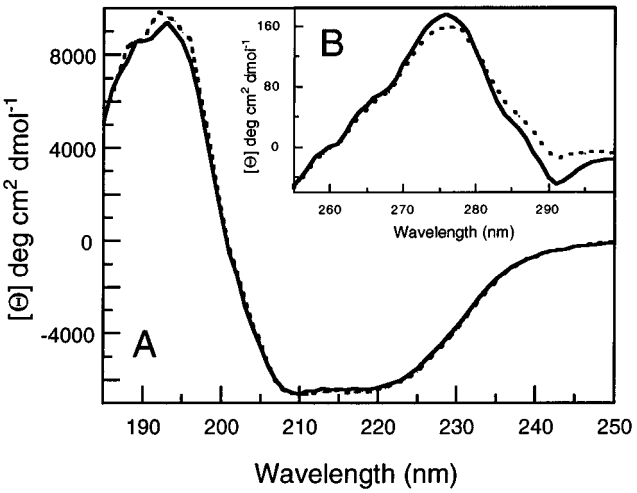


Figure 3. CD spectra of wild-type (—) and E361Q (- - -) cholesterol oxidase in sodium phosphate buffer, pH 7.0. (A) Spectra from 180 nm to 250 nm of 58 μM wild-type and 54 μM E361Q cholesterol oxidase. (B, inset) Spectra from 250 nm to 350 nm of 13 μM wild-type and 13 μM E361Q cholesterol oxidase.

(280 nM) was incubated with **1** until 10% of the cholesterol was consumed, as measured by HPLC assay. This solution was ultrafiltered and the filtrate added to a solution of **1**. The rate of product formation was followed by UV assay at 240 nm. The product content of both mixtures was analyzed by HPLC.

Initiation of 1 Oxidation by 3*β*. A solution of **1** (50 μM, 1.2% propan-2-ol) was prepared in buffer B. **3***β* (50 μM) was added and the rate of product formation followed by UV at 240 nm over 6 h. A control solution of **1** in buffer B was analyzed at the same time. In a second experiment, **1** (50 μM, 1.2% propan-2-ol) and **3***β* (50 μM) in buffer B were incubated with E361Q oxidase (294 nM), and the rate of product formation was followed at 240 nm. All three incubations were also analyzed by HPLC.

Attempted Initiation of 1 Oxidation by Riboflavin. Solutions of **1** and **2** (140 μM, 3.3% propan-2-ol) were prepared separately in buffer B. Riboflavin (1 mM) was added to each solution, and the solutions were incubated at 37 °C. Aliquots (500 μL) were analyzed at 0 min and 10 h by HPLC.

Rates of 1, 2, 3, 4, 5 and Cholesterol Decomposition. Solutions of **1, 2, 3, 4, 5** and **cholesterol** (50 μM, 1.2% propan-2-ol) were prepared separately in buffer B. After incubation at 37 °C for 24 h, the solution compositions were analyzed by HPLC.

Determination of Reaction Stereospecificity. Deuterium labeling experiments were conducted as previously described.[10] [4*β*-^{2}H]Cholesterol (140 μM) was incubated with E361Q cholesterol oxidase (984 nM) or recombinant wild-type oxidase (18 nM). The products were isolated by HPLC and analyzed by EI mass spectrometry. Only **2**, **4**, and **5***β* were analyzed by mass spectrometry. The mass spectra of the hydroperoxides **3**α and **3***β* do not contain a parent ion. The position of deuterium incorporation in **2**, **4**, **5**α, and **5***β* was determined by integration of ^{1}H resonances in their respective NMR spectra.

Results

Preparation of E361Q Cholesterol Oxidase Mutant. The E361Q point mutation was prepared by standard methods.[19−21] The mutant cholesterol oxidase was heterologously expressed in *E. coli* at the same levels as wild-type cholesterol oxidase (30 mg/L of culture). The mutant cholesterol oxidase was purified in an identical manner to wild-type and had the same elution characteristics on a hydrophobic affinity chromatography column. Further evidence that the mutant cholesterol oxidase is properly folded is presented in Figures 2 and 3. The UV spectra of the bound FAD cofactors have identical λ_{max}'s at 391 nm and 468 nm. The ratios of A_{280}/A_{391} and A_{280}/A_{468} are 11.1 and 12.7 for wild-type and 12.7 and 13.5 for E361Q, respectively. The CD spectra show that the enzyme is a mixture of α-helix and *β*-sheet, as expected from the X-ray crystal structure. The molar ellipticities are the same for wild-type and E361Q throughout the far- and near-UV.

HPLC Analysis of Product Mixtures. The products of the wild-type and E361Q cholesterol oxidase reactions were analyzed by reversed-phase C-18 chromatography using a ternary gradient. A typical analytical chromatogram is shown in Figure 1. Peaks corresponding to products of cholesterol turnover were distinguished from Triton X-100, protein and buffer peaks by the use of ^{14}C-labeled cholesterol. The identity of the peaks was established by coinjection with commercially-available and synthesized authentic compounds. Authentic samples of the hydroperoxy steroids **3**α and **3***β* were synthesized according to Cox.[25] The purity of peaks was confirmed by detecting the ratio of A_{240}/A_{212} in ratio-recording mode. Observation of a square peak confirmed peak purity. Ketones **1** and **2** were not baseline resolved, but the relative amount of each was determined using ratios of $A_{240}/A_{212} = 0.154$ and 3.52 for **1** and **2**, respectively. Standard solutions were used to determine the A_{240}/A_{212}. Steroids **3**α and **5**α also were not baseline resolved and values reported are for the combined products.

After 24 h, incubation of cholesterol or **1** with wild-type cholesterol oxidase yielded 98% **2** and 2% **3**, **4,** and **5**. After 24 h, incubation of cholesterol or **1** with E361Q cholesterol oxidase yielded 13% **2**, 6% **3***β*, 32% **4**, 13% **3**α and **5**α, and 36% **5***β*.

Measurement of Specific Activity. The solubility of the substrates precluded determining Michaelis−Menten rate constants, and, therefore, specific activities with 50 μM cholesterol were used for comparison of wild-type and E361Q cholesterol oxidase activities. The specific activity for the overall rate of reaction was determined by following the formation of conjugated enone at 240 nm and measuring the initial velocity. In the wild-type reaction, this rate corresponds to the rate of **2** formation. In the E361Q reaction, this rate corresponds to the rate of **2**, **3**, **4**, and **5** formation combined. The specific activity of the recombinant wild-type oxidase is 35 U/mg; the E361Q cholesterol oxidase is 1000-fold lower in activity with a specific activity of 0.035 U/mg. Note the A_{240} specific activity is only approximate because of the variety of products formed: a more

(25) Cox, A. J. *J. Org. Chem.* **1965**, *30*, 2052−2053.

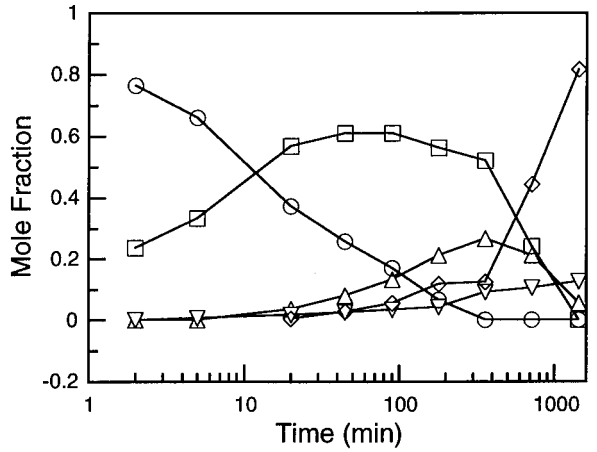


Figure 4. Plot of product mole fraction formed in E361Q (294 nM) reaction with 140 μM cholesterol as a function of time (log scale). The mole fractions of products were monitored by HPLC as described under Experimental Procedures: Cholesterol (○); cholest-5-ene-3-one, **1** (□); cholest-4-ene-3-one, **2** (▽); cholest-4-ene-6β-hydroperoxy-3-one, **3**β (△); cholest-4-ene-6-ol-3-one, **5**, cholest-4-ene-3,6-dione, **4**, and cholest-4-ene-6α-hydroperoxy-3-one, **3**α (◇). Lines are drawn to clarify the plot and do not represent curve-fitting to any kinetic equation.

Table 1. Specific Activities of Wild-Type and E361Q Cholesterol Oxidases

product	wild-type (μmol/min·mg)	E361Q (μmol/min·mg)
2	35[a,d]	0.003[b]
1	n.o.[c]	1.8[d]
3, 4, 5	2×10^{-6} [b]	0.015[b]

[a] Measured by UV at 240 nm. [b] Measured by HPLC assay. [c] None observed. [d] Measured as formation of H_2O_2.

accurate measurement of activities was made by HPLC assay (*vide infra*) and these activities are presented in Table 1.

The rate of H_2O_2 formation was determined by following the formation of quinonimine using a horseradish peroxidase coupled assay. In the wild-type reaction, this rate corresponds to the rate of **2** formation that was measured independently by following the appearance of **2** at 240 nm. In the mutant E361Q reaction, the rate of H_2O_2 formation corresponds to the rate of **1** formation; this was confirmed by HPLC analysis. The specific activity of E361Q for **1** formation is 1.8 U/mg, only 20-fold reduced from wild-type (see Table 1).

The variety of conjugated steroid products that were formed in the E361Q reaction reduced the utility of initial velocity measurements at 240 nm to follow the formation of specific products. In order to follow the E361Q reactions and the very slow noncatalyzed reactions, assay mixtures were analyzed by HPLC. Use of [26-^{14}C]cholesterol allowed quantitation of the concentration of each steroid with good precision (±5%). The time course of E361Q reaction is presented in Figure 4, and the specific activities for E361Q catalyzed formation of various products are in Table 1. In the case of the wild-type oxidase, accumulation of **1** in solution was not detected. Cholestenone **2** is the first product observed; **3**, **4**, and **5** are detected after 95% of the substrate is consumed.

Characterization of 6-Oxygenation Activity. The addition of catalase to incubation mixtures of E361Q had no effect on the quantities of 6-oxygenated products obtained. The addition of 1 mM NaCN or 1 mM DTT to assay mixtures of E361Q and cholesterol did not affect the rate of conjugated cholestenone formation as observed at 240 nm. The specific activity of E361Q at 240 nm was 0.031 μmol/min·mg with 3 mM tris-(carboethoxy)phosphine in the assay mixture.

The stability of the products in assay buffer over 24 h, without added wild-type or E361Q cholesterol oxidase, was analyzed by HPLC (Table 2). Cholest-5-ene-3-one, **1**, slowly decomposes to **3**, **4**, and **5** (18% after 24 h). The hydroperoxy steroid **3**, the alcohol **5,** the dione **4**, enone **2**, and cholesterol were stable over 24 h. Moreover, cholest-5-ene-3-one, **1**, is stable in the presence of 10 mM riboflavin for at least 10 h.

Table 2. Determination of Stability of Steroids in Assay Buffer

steroid	% remaining after 24 h[a]	decomposition products observed by HPLC
cholesterol	100	none
1	82	1% **2,** 10% **3,** 2% **4,** 5% **5**
2	100	none
3β	100	none
4	100	none
5β	100	none

[a] A 140 μM solution was incubated in buffer B at 37 °C. *No E361Q cholesterol oxidase was added.* After 24 h, the steroid composition of each solution was analyzed by HPLC as described in Experimental Procedures.

An E361Q cholesterol oxidase solution (877 nM) was ultrafiltered to remove the protein, and the filtrate added to a solution of **1** (50 μM) in assay buffer. The rate of **1** turnover as measured by ΔA_{240} was 0.02 μM min^{-1} and unchanged compared to a buffer-only control (using $\epsilon = 12\ 100$ M^{-1}cm^{-1} for all species). In a separate experiment, an E361Q cholesterol oxidase solution (280 nM) was incubated with cholesterol until 10% had been consumed. The rate of **1** turnover as measured by ΔA_{240} was 0.3 μM min^{-1}. The solution was ultrafiltered to remove the protein and the filtrate added to a solution of **1** (50 μM) in assay buffer. The rate of conjugated enone formation was 0.08 μM min^{-1}, this rate was faster (4-fold) than in the previous experiment, although slower (4-fold) than the turnover rate observed in the presence of E361Q.

The rate of oxidation of **1** in assay buffer containing 1 equiv of cholest-4-ene-6β-hydroperoxy-3-one, **3**β was followed by HPLC for 6 h. This rate was no faster than a buffer-only control. Incubation of **1** and **3**β in the presence of E361Q cholesterol oxidase had no effect on the rate of **1** autoxidation (data not shown).

The stoichiometric reaction of E361Q with **1** was followed by UV spectroscopy. The absorption spectrum of the flavin cofactor between 300 nm and 500 nm was used as an indicator of the oxidation state of the cofactor. By careful titration with cholesterol, **1** was formed in the presence of the oxidized flavin, FAD, under anaerobic conditions. No change in the FAD spectrum was observed over 1 h (Figure 5). Upon further titration with cholesterol, **1** was formed in the presence of $FADH_2$ under anaerobic conditions. No change in the $FADH_2$ spectrum was observed over 1 h. Furthermore, in the absence of oxygen, **1** was completely stable. Thus, no radical flavin species were detected.

Measurement of ^{2}H Transfer. [4β-^{2}H]Cholesterol was incubated with recombinant *Streptomyces* wild-type cholesterol oxidase and completely converted to **2**; 30% of the deuterium label remained in the product **2** as determined by mass spectrometry. ^{1}H-NMR spectral analysis of **2** revealed that the deuterium label transferred to the product was incorporated at carbon-6. No deuterium label remained at carbon-4. [4β-^{2}H]-Cholesterol was incubated with E361Q cholesterol oxidase and completely converted to **2**, **3**, **4**, and **5**. Product **2** had 27% deuterium, products **4** and **5**β had 23% deuterium label. ^{1}H-NMR spectral analysis of the products revealed that all of the deuterium label remaining in the product was on carbon-4. No deuterium label had been transferred to carbon-6.

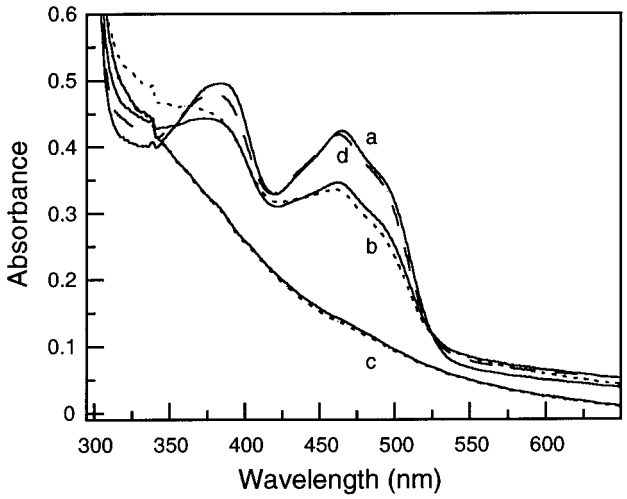


Figure 5. Spectral changes observed during titration of E361Q cholesterol oxidase (32 μM) with cholesterol under an Ar atmosphere in sodium phosphate buffer, 0.025% Triton X-100, pH 7.0: (a) 0 μM cholesterol (—); (b) 16 μM cholesterol (—), and after 1 h (- - -); (c) 32 μM cholesterol (—), and after 1 h (- - -); (d) 32 μM cholesterol and air (— —).

Discussion

In our effort to elucidate the mechanism of cholesterol oxidase, we sought to identify the active site base responsible for isomerization of the intermediate, cholest-5-ene-3-one, **1**, to cholest-4-ene-3-one, **2**. Inspection of the X-ray crystal structure of the *B. sterolicum* oxidase (choB)[8,9] suggested that glutamate-361 was positioned over the β-face of the steroid and could act as the base. The position of glutamate-361 was consistent with the stereochemistry results we reported earlier,[10] that the 4β-H is transferred to the 6β-position. We mutagenized glutamate-361 to glutamine, thus substituting the putative carboxylate base isosterically with a neutral moiety. We performed our experiments with the *Streptomyces* oxidase (choA) because the heterologous expression plasmid pCO117 constructed by Murooka and co-workers[18] yields approximately 30 mg of pure protein per liter of growth culture. The choA oxidase is 58% identical in amino acid sequence with choB, and E361 is conserved. The high quantities of protein obtained have allowed us to perform biophysical characterization of the mutant E361Q protein.

The UV and CD spectra of both the wild-type and the E361Q cholesterol oxidase protein are presented in Figures 1 and 2. The λ_{max}'s of the FAD cofactor remain unchanged upon replacement of glutamine for glutamate-361. Furthermore, the ratios of A_{280}/A_{391} and A_{280}/A_{468} are within 13% and 6% of the ratio for wild-type, respectively. The CD spectra show that the secondary structure of the mutant enzyme is unchanged from that of wild-type. Taken together, these spectroscopic data strongly suggest that the E361Q protein has folded into the same structure as wild-type.

Among the variety of continuous assays that may be used to analyze cholesterol oxidase activity, we have chosen two to study the E361Q mutant. The first assay follows directly the formation of conjugated enone product by UV absorbance at 240 nm. In the wild-type reaction, the conjugated products are formed as a result of two chemical reactions, oxidation and isomerization. The second assay follows indirectly the production of H_2O_2 by coupling the reaction to horse radish peroxidase and observing the formation of quinonimine dye at 510 nm. This assay serves as an indicator of the rate of turnover of the flavin cofactor, and thus of the 3β-hydroxy oxidation chemistry catalyzed by cholesterol oxidase.

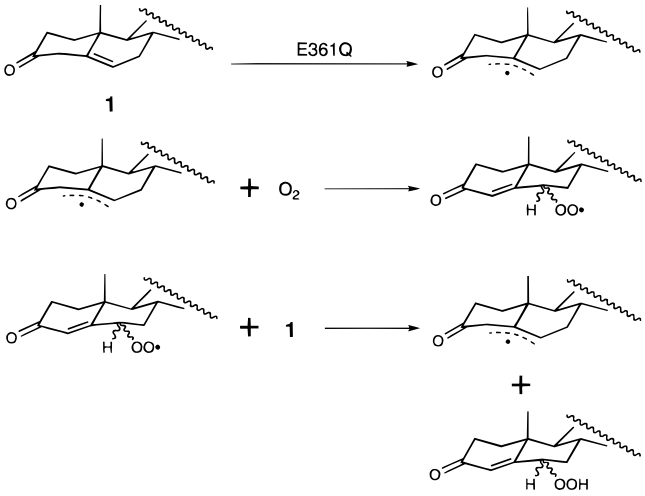
With the wild-type enzyme, the specific activity measured by either assay is the same, 35 μmol/min·mg. In addition, analysis of the reaction products by HPLC revealed that 98% of the product formed in the reaction is cholest-4-ene-3-one, **2**. The remainder of the products are **3**, **4**, and **5**. None of the intermediate, cholest-5-ene-3-one, **1**, was detected during the turnover of cholesterol, even at low concentrations of wild-type cholesterol oxidase. Thus, we cannot say whether the formation of **3**, **4**, and **5** is due to a second catalytic activity of the wild-type enzyme or to the release of the intermediate, **1**, from the enzyme and its subsequent decomposition. The close retention times of **1** and **2**, however, prohibit detecting small amounts of **1** (<5%) in the presence of **2**.

When the E361Q specific activity was measured by following conjugated enone formation, the activity is 1000-fold reduced from that of wild-type (Table 1). Moreover, reversed-phase HPLC analysis of the assay mixture at the completion of the reaction revealed that four steroid products were formed. The products were identified as **2**, **3**, **4**, and **5** by mass spectral and ^{1}H-NMR analysis. The HPLC retention times (*k*'s) and ^{1}H-NMR spectra are identical with authentic **4** and **5** obtained from Steraloids, Inc., and **3** synthesized as described by Cox.[25] The specific activity of E361Q cholesterol oxidase measured by following H_2O_2 production was 1.8 μmol/min·mg, only 20-fold reduced from wild-type and much faster than the formation of conjugated enones. When the reaction was followed by HPLC, we observed that **1** was the initial product formed, and that upon prolonged incubation with the enzyme, **1** was isomerized to **2,** and **1** was oxidized further to the 6-oxygenated products, **3**, **4**, and **5** (Figure 4). When **2** was incubated with E361Q cholesterol oxidase, no 6-oxygenated products were formed. However, when **1** was incubated with E361Q cholesterol oxidase, the same products **2**, **3**, **4**, and **5** were formed as observed upon extended incubation of cholesterol with E361Q. Individual rates of formation were measured by HPLC. The specific activity for **2** formation is 0.003 μmol/min·mg, 10 000-fold slower than wild-type. Thus, mutagenesis of glutamate-361 separated 3β-hydroxy oxidation from isomerization and confirmed that it is the base responsible for isomerization. However, increased amounts of 6-oxygenated products were obtained in the mutant catalyzed reaction, albeit, slowly.

The report of Murooka and co-workers that wild-type cholesterol oxidase has an unusual monooxygenase activity aided us in our identification of the E361Q oxidation products. They had previously reported that 3% of the product produced by wild-type cholesterol oxidase is **5**, as well as some other minor unidentified products.[26] They proposed that perhaps the FAD could act as both an oxidase and a monooxygenase. Potentially, the C4a-hydroperoxy flavin adduct, formed upon reaction of oxygen and the reduced flavin, $FADH_2$, in the reoxidation pathway,[27] could be formed in the presence of **1**. This C4a adduct might act directly as a hydroxylation agent, or upon elimination of H_2O_2 provide a source of sequestered hydroxylating agent. The reduced flavin is formed upon oxidation of the 3-hydroxyl moiety of cholesterol. Our results suggested that mutation of E361 to glutamine had unmasked this reactivity and further experiments were undertaken to characterize it.

(26) Molnar, I.; Hayashi, N.; Choi, K. P.; Yamamoto, H.; Yamashita, M.; Murooka, Y. *Mol. Microbiol.* **1993**, *7*, 419−28.
(27) Ghisla, S.; Massey, V. *Eur. J. Biochem.* **1989**, *181*, 1−17.
(28) Nickon, A.; Mendelson, W. L. *J. Org. Chem.* **1965**, *30*, 2087−2089.
(29) Furutachi, N.; Nakadaira, Y.; Nakanishi, K. *J. Chem. Soc., Chem. Commun.* **1968**, 1625−1626.
(30) Dang, H.-S.; Davies, A. G.; Schiesser, C. H. *J. Chem. Soc., Perkin Trans. 1* **1990**, 789−794.

Scheme 3



If the C4a-flavin or H_2O_2 were the hydroxylating agent, incubation of **1** with E361Q cholesterol oxidase should not yield any 6-oxygenated products. Wild-type cholesterol oxidase will catalyze isomerization of **1** to **2** without any redox cycling of the flavin. As stated above, when **1** was incubated with E361Q cholesterol oxidase, the 6-oxygenated products were still produced in the same ratios that were obtained when cholesterol was incubated with E361Q cholesterol oxidase. Moreover, when cholesterol was incubated with E361Q cholesterol oxidase, H_2O_2 production was stoichiometric with cholesterol consumption, i.e., H_2O_2 was not consumed in the hydroperoxylation reaction. This stoichiometry was inconsistent with hydroxylation by the C4a adduct or H_2O_2 to form **3**, **4**, or **5**. If the C4a adduct were a hydroxylating agent, H_2O would be formed instead of H_2O_2. Removal of H_2O_2 by catalase, a very efficient enzyme, also had no effect. In addition, the difference in rate between **1** formation and **3**, **4**, and **5** formation implied that flavin redox cycling was not involved in the 6-oxygenation reaction.

It seemed that we were observing the well-known air oxidation of cholest-5-ene-3-one, **1**. Both the benzoyl peroxide initiated radical reaction and the photosensitized singlet oxygenation reaction produce **3**, **4**, and **5**.[25,28−30] In addition, singlet oxygenation of **1** produces 3α,5α-epidioxycholest-6−3β-ol.[30] 3,5-Cholesten-3-ol ethyl ether is converted to **5** in 60% yield after exposure to direct sunlight.[31] The oxidation of **1**, however, proceeded faster in the presence of E361Q cholesterol oxidase than in buffer-only incubation mixtures (see Figure 4 and Table 2). Only 18% of **1** was oxidized in air-saturated buffer over 24 h; however, the reaction was 15 times faster in the presence of 280 nM E361Q cholesterol oxidase. Furthermore, the absence or presence of light had no effect on the rate or ratio of product formation. Incubation of riboflavin with **1** in buffer did not initiate autoxidation. The production of **3** suggested to us that **1** was undergoing radical oxidation, and that **4** and **5** were the induced decomposition products of the hydroperoxy radical.[30,32] Extended conjugation would favor the formation of an allylic cholestenyl radical. We hypothesized that the oxidation reaction was initiated by E361Q cholesterol oxidase or a co-purified contaminant to form the cholest-4-enyl-3-one radical (Scheme 3). Reaction of the cholestenyl radical with $^3O_2$ forms the 6-hydroperoxy radical that propagates the chain reaction by abstraction of the 6-H from **1** to regenerate the cholestenyl radical. To distinguish between E361Q cholesterol oxidase and another species acting as the initiator, for example metal ions, the following experiments were performed.

(31) Gardi, R.; Lusignani, A. *J. Org. Chem.* **1967**, *32*, 2647−2649.

Inhibitors of heme iron were added; NaCN and DTT had no effect on the oxidation of **1** to **3**, **4**, and **5**. Addition of tris-(carboethoxy)phosphine gave a 10% reduction in specific activity. HPLC analysis of the reaction products showed that, after 24 h, the predominant product was **5**, cholest-4-ene-6-ol-3-one. This slight difference in rate is most likely due to the small variations in ϵ_{240} between hydroxy and hydroperoxy steroids. Thus, we were not able to inhibit the reaction with additives.

A solution of E361Q cholesterol oxidase was ultrafiltered (NMWCO = 30 000) and the filtrate added to a solution of **1**. The rate of autoxidation observed was the same as a buffer control. In a second experiment, E361Q cholesterol oxidase was incubated with **1** until 10% had been converted to 6-oxygenated products. This solution was ultrafiltered and the filtrate added to a solution of **1**. The initial rate of oxidation observed was faster than the rate observed in buffer alone, although not as fast as the initial reaction. We conclude that the initiator has a molecular weight greater than 30 kD, and it is presumed to be E361Q cholesterol oxidase. Furthermore, this initiator can be removed from solution and the reaction continues, this behavior is indicative of a radical chain reaction. One might expect that removal of the initiator (by ultrafiltration) would lead to termination of the chain process, because in most autoxidation sequences, the steady state oxidation of substrate depends on the rate of chain initiation.[32] In fact, once intermediate peroxide species are formed, their breakdown products may serve as initiators.[33] Although our experiments demonstrated that **3** alone does not initiate the autoxidation of **1**, there may be other species formed in the reaction, e.g., dicholestenyl peroxide,[34] that will initiate the reaction. Furthermore, we do observe that the reaction is 4-fold slower after removal of E361Q cholesterol oxidase. Analysis of the product mixture by HPLC shows that the reaction initiated with ultrafiltration goes to completion. Thus the rate of termination must be very slow compared to the rate of propagation. This relative rate is consistent with the observation that the primary pathway of termination appears to be the reaction of two hydroperoxy radical species to form **4** and **5**. As long as the concentration of **1** remains higher than the concentration of hydroperoxy radical, propagation will dominate over termination, i.e., the kinetic chain length is long.

We attempted to detect intermediate radical species that might be formed using the flavin absorption spectrum as an indicator. Under anaerobic conditions, **1** was generated in the presence of FAD-E361Q oxidase and $FADH_2$-E361Q oxidase (Figure 5). No flavin semiquinone species were detected in either incubation; only the spectra of FAD and $FADH_2$ were observed. Upon addition of O_2, the $FADH_2$-oxidase was restored to the fully oxidized form without loss of protein signal. The variability in absorption at 325 nm is due to the formation of product and the high absorbance of Triton X-100. We conclude that, if any radical flavin species is formed during the 6-oxygenation reaction, it is a very small percentage of the total flavin. That is, there is no stoichiometric formation of flavin semiquinone.

We tested the stereospecificity of hydrogen abstraction in the E361Q catalyzed isomerization and oxygenation reactions by following deuterium transfer from the 4β-carbon to the 6β-carbon as previously described.[10] The cholest-4-ene-3-one product, **2**, has 27% of the deuterium label remaining as determined by mass spectrometry. However, ^{1}H-NMR analysis revealed that none of the deuterium label had been transferred

(32) Kochi, J. S. *Autooxidation*; Kochi, J. S., Ed., John Wiley and Sons: New York, 1967; Vol. 2, pp 3−62.
(33) Betts, J. *Quart. Rev.* **1971**, *25*, 265−288.
(34) Ingold, K. U. *Acc. Chem. Res.* **1969**, *2*, 1−9.

to the 6-carbon. The 27% deuterium remaining after conversion of cholesterol to product is located on the 4-carbon. In the wild-type reaction, 30% of the label is transferred to the 6β-position. Clearly, the base responsible for transfer of deuterium is glutamate-361, and after removal, the isomerization reaction is no longer stereospecific. The 6-oxygenated products also have little deuterium label remaining. The 23% of the label that remains is at the 4-carbon. This experiment demonstrated the lack of stereospecificity of hydrogen abstraction in the hydroperoxylation reaction.

It is clear the 6-oxidation chemistry is faster in the presence of E361Q. A modest rate enhancement of 15-fold with 280 nM E361Q cholesterol oxidase is observed. The 6-oxidation chemistry is not stereospecific; both the 6α- and 6β-hydroperoxycholest-4-ene-3-ones are formed. Furthermore, abstraction of the C4-hydrogen is not stereospecific; both the 4α- and the 4β-hydrogens are removed. The lack of stereospecificity in the reaction, as well as the kinetic results, suggests that 6-oxygenation is a result of radical chain chemistry. The role of E361Q cholesterol oxidase is limited. Perhaps the active site of E361Q simply stabilizes the cholestenyl radical and simultaneously lowers the activation barrier for its formation.

Our experiments clearly show that the oxidase and isomerase activities of cholesterol oxidase can be separated by a single mutation, E361Q. The susceptibility of the intermediate, cholest-5-ene-3-one, **1**, to radical chain oxidation, however, complicates the analysis of the reaction. This reactivity points to another example of one important function of enzymes, that is, to sequester reactive intermediates. Possibly, these strains of bacteria have evolved a bifunctional enzyme in order to avoid the formation of reactive radical and hydroperoxy steroids. In the wild-type reaction, within the limits of our detection methods, accumulation of **1** in solution is not observed. This sequestration of intermediate highlights the balance in affinities required for an efficient enzyme. The enzyme must have an affinity for the intermediate sufficient to prevent its release, yet not bind the product so tightly that turnover of catalyst is prevented. Note that the difference between intermediate and product is a subtle difference in their conformations. The A- and B-rings of **2** are more planar and more chair-like, respectively, than those of **1**.

In view of the sequestration of intermediate by wild-type, one easily could envision that E361Q might release the intermediate very slowly.[35] Slow release of cholest-5-ene-3-one, **1**, would lead to product inhibition and limited turnover of cholesterol. We see, however, a very small reduction in turnover upon mutation, indicating that **1** is not bound by the mutant much more tightly than **2** is by wild-type. Thus, with a single mutation, we have constructed a catalytically active enzyme with altered function. If we assume that the rate of 3β-hydroxy oxidation is unaffected by the E361Q mutation, release of **1** is only 20-fold slower than isomerization of **1** to **2** and release of **2**. These relative rates suggest that, in the wild-type reaction, one molecule of intermediate should be released into solution for every 20 turnovers of **1** to product. We observed that 2% of the total product of the wild-type reaction was autoxidation products derived from **1**. This percentage is quite close to the 5% predicted from the relative specific activities of wild-type and E361Q cholesterol oxidase and suggests that the formation of **3**, **4**, and **5** in the wild-type reaction is a result of loss of intermediate and its subsequent decomposition, not a second intrinsic catalytic activity. Furthermore, the very slow appearance of **3**, **4**, and **5** (2×10^{-6} μmol/min·mg) relative to **2** formation (35 μmol/min·mg) also suggests that approximately 2% of enzyme-bound **1** is released and slowly autoxidizes in solution to the 6-oxygenated products.

Besides characterization of the mechanism of cholesterol oxidase, the E361Q mutant is potentially useful as a tool in cell biology. Cholesterol oxidase is used to quantitate cholesterol in cell membranes and to characterize the lipid structure and phase of the membranes.[1,36−38] One of the experimental difficulties commonly encountered is increased permeability and even lysis of cell membranes upon their treatment with cholesterol oxidase. This problem is due to the formation of cholest-4-ene-3-one, **2**.[36,39−41] The employment of E361Q cholesterol oxidase as a tool would reduce the permeability problems, because cholest-5-ene-3-one, **1**, does not increase cell membrane permeability.[36,41] Quantitation of cholesterol would still be facile using horse radish peroxidase coupled assays. E361Q binds to lipid bilayers, i.e., unilamellar vesicles, with the same affinity as wild-type.[42] With an understanding of the kinetics of cholesterol and cholest-5-ene-3-one, **1**, oxidation, the mutant E361Q could be applied readily to membrane structure problems.

In conclusion, replacement of glutamate-361 by glutamine suppresses isomerization but not oxidation in the reaction catalyzed by cholesterol oxidase. The rate of oxidation is 20-fold slower than the wild-type reaction, and the rate of isomerization is 10 000-fold slower. Isolation of **1**, the cholest-5-ene-3-one product of the mutant catalyzed reaction, is complicated by its susceptibility to radical chain autoxidation. This E361Q mutant cholesterol oxidase will be used to obtain information about the structure of the enzyme in the presence of the reaction intermediate and thus to further characterize its catalytic mechanism. This improved understanding of the mechanism of action of cholesterol oxidase will facilitate its use in biotechnology and cell biology.

Acknowledgments. Elizabeth Anderson prepared the M13mp18 subclone of cholesterol oxidase and initiated mutagenesis experiments. Professor Y. Murooka kindly provided the pCO117 clone of cholesterol oxidase. The 70-VSE mass spectrometer in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, was purchased in part with a grant from the Division of Research Resources, National Institutes of Health. Funding for this work was provided by a grant-in-aid from the American Heart Association, National Center (N.S.), a Camille and Henry Dreyfus New Faculty Award (N.S.), National Institutes of Health Grant HL-53306 (N.S.), and a DOE/GAANN fellowship (I.K.).

JA962258O

(35) Cleland, W. W. *Biochemistry* **1990**, *29*, 3194−3197.

(36) Grönberg, L.; Slotte, J. P. *Biochemistry* **1990**, *29*, 3173−3178.

(37) Slotte, J. P. *Biochim. Biophys. Acta* **1992**, *1124*, 23−28.

(38) Mattjus, P.; Slotte, J. P. *Chem. Phys. Lipids* **1996**, *81*, 69−80.

(39) Brasaemle, D. L.; Attie, A. D. *J. Lipid Res.* **1990**, *31*, 103−112.

(40) Demel, R. A.; Bruckdorfer, K. R.; Van Deenen, L. L. M. *Biochim. Biophys. Acta* **1972**, *255*, 321−330.

(41) Ben-Yashar, V.; Barenholz, Y. *Biochim. Biophys. Acta* **1989**, *985*, 271−278.

(42) Ghoshroy, K. B.; Zhu, W.; Sampson, N. S. Personal communication.