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Forum Review

Dissection of a Flavoenzyme Active Site: The Reaction Catalyzed by Cholesterol Oxidase

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ABSTRACT

Cholesterol oxidase is a member of the glucose–methanol–choline (GMC) oxidoreductase family that is characterized by a conserved topology. We review our investigations into the reactivity of the *Streptomyces* cholesterol oxidase cofactor, flavin adenine dinucleotide (FAD), and the role of active-site residues. All of our mutagenesis, enzyme inhibition, and kinetic data demonstrate that the cofactor catalyzes oxidation of alcohols to ketones, but not oxygenation of carbon. Cholesterol oxidase catalyzes two reactions, oxidation and isomerization, in one active site, presumably because of the susceptibility of the reaction intermediate cholest-5-en-3-one to radical oxidation. This bifunctionality is not a shared characteristic with other GMC oxidoreductase family members. Furthermore, we have characterized the unusual inactivation of FAD by electrophilic substitution at C6 of the isoalloxazine ring upon ring opening of a cyclopropyl steroid. Another member of the GMC oxidoreductase family, methanol oxidase, is also inactivated by a cyclopropanol suggesting that inhibition by cyclopropanol inhibitors may be diagnostic of membership in this family. Antioxid. Redox Signal. 3, 839–846.

INTRODUCTION

In animals and insects, cholesterol is essential for the maintenance of cell membrane structure and phase. Moreover, improper maintenance of cholesterol concentrations and locales can have severe effects on the physiologic function of an organism. Cholesterol oxidase has proven to be very useful for human applications that require detection or disruption of cholesterol-containing membranes. It is a bacterial enzyme that enables the bacteria to utilize cholesterol as a carbon source in primary metabolism. However, one of the reaction products, hydrogen peroxide (H_2O_2), is easily detected. Thus, cholesterol oxidase has been widely used to measure serum cholesterol levels as part of human disease monitoring and treatment. Furthermore, the second product of the reaction, cholest-4-en-3-one, alters the structure of lipid membranes, and intentional disruption of cholesterol membrane homeostasis with cholesterol oxidase is a possible mechanism for pesticidal action. The biotechnology uses for the bacterial cholesterol oxidase have arisen because of the products that are formed in the catalytic reaction. Although there is not a mammalian homolog of cholesterol oxidase, there are mammalian enzymes that catalyze the same chemistry as part of steroid biosynthesis.

Cholesterol oxidase catalyzes the oxidation and isomerization of cholesterol into cholest-4-en-3-one (Fig. 1). It is a monomeric flavoenzyme that contains 1 mole of tightly bound flavin adenine dinucleotide (FAD) per mole of protein (29). Upon oxidation of cholesterol, the FAD is reduced to $FADH^-$. Oxygen is the second substrate that recycles the flavin to its ox-

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This paper is dedicated to the memory of Robert H. Abeles (1926–2000).

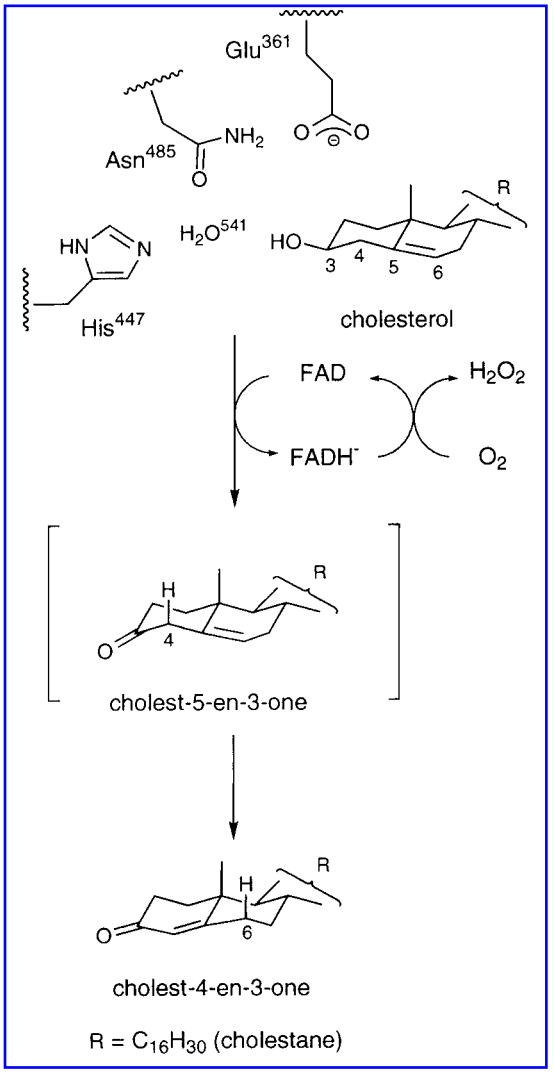


FIG. 1. Reaction catalyzed by cholesterol oxidase and the hydrogen bond network in the active site. [Reproduced with permission from 18; Copyright 2000 American Chemical Society]

idized form, FAD, with the production of 1 mole of H_2O_2. Thus, the two products of the enzymatic reaction are cholest-4-en-3-one, a lipid, and H_2O_2, a gas.

The substrate specificity of cholesterol oxidase and the stoichiometric production of H_2O_2 upon oxidation of cholesterol led to its implementation in clinical serum cholesterol assays. The cholesterol oxidase reaction is coupled to the peroxidation of aromatic dyes, catalyzed by horseradish peroxidase, to form an easily detected and quantified colored or fluorescent product. This coupled assay has been in clinical use for over >20 years, and is also one way in which the activity of the enzyme is followed in *in vitro* experiments.

The production of cholest-4-en-3-one from cellular cholesterol is the basis for its insecticidal activity against *Coleoptera*, *i.e.*, boll weevils (4, 10, 20, 21, 24). The conversion of cholesterol to cholest-4-en-3-one significantly alters the structural integrity of a membrane (9, 33). The membrane becomes more porous and more susceptible to internal osmotic pressure. After ingestion of cholesterol oxidase by boll weevil larvae, their gut endothelia lyse. Through genetic engineering, this potent insecticide may be introduced into crops. The cholesterol oxidase can be expressed by the plants that are susceptible to attack (2), or by bacteria that colonize the plant (3). Cholesterol oxidase has, therefore, a second industrial use, *i.e.*, as a larvicide.

It is the same conversion of cholesterol in the membrane to cholest-4-en-3-one and H_2O_2 that has led to its use in cell biology as a probe of membrane structure (15), and of cholesterol localization in the membrane. Thus, cholesterol oxidase has been and will continue to be employed in many practical uses. Understanding the importance of its active-site residues for the function of the flavin, and consequently for its catalytic activity, is of extreme importance for interpreting and modifying cholesterol oxidase activity. Below, we review the work from our laboratory that has been directed at understanding the role of active-site residues and the reactivity of the flavin.

STRUCTURE

The design of our experiments began with inspection of the atomic structures of cholesterol oxidase obtained by x-ray crystallography. The native *Brevibacterium sterolicum* structure and a *B. sterolicum* structure with dehydroepiandrosterone in the active site have been solved by David Blow, Alice Vrielink, and co-workers (16, 30–32). In addition, during the course of our studies, our collaborator Alice Vrielink and her research team have solved the structures of native *Streptomyces* and several *Streptomyces* site-directed mutants (34). The *B. sterolicum* and *Streptomyces* enzymes are 58%

identical in amino acid sequence and have nearly identical tertiary structures. The crystallographic models of cholesterol oxidase suggested that active-site residues histidine-447, glutamate-361, asparagine-485, and H_2O-541 are important. Interestingly, these residues are highly conserved in the active sites of the glucose–methanol–choline (GMC) oxidoreductase family (1, 14). Aside from these residues and the FAD-binding domain, cholesterol oxidase has no sequence similarity (<10% overall) with members of the GMC oxidoreductase family, although its tertiary structure is very similar. On the basis of this similarity, cholesterol oxidase has been classed in this family that includes glucose oxidase, methanol oxidase, and choline dehydrogenase. The conserved active-site residues suggest that the catalytic mechanisms may be conserved as well. It should be noted that *B. sterolicum* has a second cholesterol oxidase that has a covalently bound FAD with an altered redox potential (6). This altered potential suggests that the protein environment around the flavin is different and that this cholesterol oxidase may be structurally distinct from the GMC oxidoreductase family.

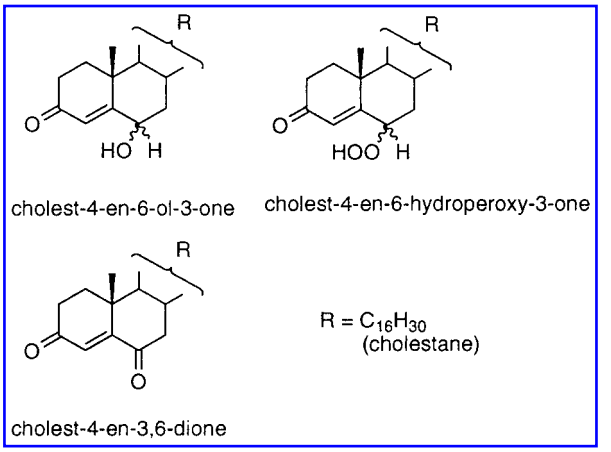
In cholesterol oxidase, a single water molecule, H_2O-541, acts as the keystone for the active site. H_2O-541 is the only water molecule that remains in the substrate-binding cavity when substrate is bound. It is at the center of a network of hydrogen bonds between residues histidine-447, glutamate-361, and asparagine-485 (Fig. 1). H_2O-541 bridges histidine-447 and the C3-hydroxyl of the substrate, suggesting that general base catalysis by histidine-447 is mediated by this water molecule. Kinetic analysis of histidine-447 mutants showed that the imidazole is required for efficient oxidation, but not isomerization (13). That is, the histidine is essential for oxidation of cholesterol or $FADH^-$; however, the imidazolium conjugate acid is not required for isomerization of cholest-5-en-3-one to cholest-4-en-3-one.

In the *B. sterolicum* enzyme complexed with dehydroepiandrosterone, glutamate-361 is positioned directly over the β-face of the steroid, and is close enough to the 4β proton to abstract it from the cholest-5-en-3-one intermediate (Fig. 1). Moreover, its diffuse electron density suggested that it is mobile enough to protonate the dienolate intermediate at the 6β position. Our isotopic labeling experiments confirmed that one base is responsible for both deprotonation at the 4β carbon and reprotonation at the 6β carbon (12, 23). In addition, site-directed mutagenesis of glutamate-361 to glutamine suppresses the isomerization reaction, and the reaction intermediate, cholest-5-en-3-one, is produced catalytically (22).

With the site-directed mutants constructed for the studies described above, we were able to explore the reactivity of the flavin. We investigated a previous report that cholesterol oxidase could catalyze hydroxylation at the 6 position of the steroid (19). We also explored the use of 2,3- and 3,4-cyclopropyl steroids as irreversible inhibitors of cholesterol oxidase.

WHAT TYPE OF OXIDATION CAN THE FAD OF CHOLESTEROL OXIDASE PERFORM?

During our characterization of glutamate-361 mutants, we discovered that a major product of the reaction catalyzed by some of these mutants was cholest-4-en-6-ol-3-one (Scheme 1). Murooka and co-workers had previously reported that the *Streptomyces* wild-type enzyme produced 3% cholest-4-en-6-ol-3-one (19). They suggested that this product was a result of the FAD cofactor catalyzing a monooxygenase reaction. If the C4a-hydroperoxy flavin adduct, formed during oxidation of the reduced $FADH^-$ by O_2 (7), were formed in the presence



Scheme 1.

of cholest-5-en-3-one, it might act as a steroid hydroxylation agent. Alternatively, H_2O_2 sequestered on the protein might hydroxylate the cholest-5-en-3-one intermediate. Because of the small amount of cholest-4-en-6-ol-3-one produced by wild type, it was difficult to investigate the mechanism of its formation. The discovery that E361Q cholesterol oxidase (as well as other glutamate-361 mutants) produced a significant amount of cholest-4-en-6-ol-3-one allowed us to characterize the mechanism of its formation (22).

We utilized an HPLC assay to follow the course of the E361Q-catalyzed reaction with cholesterol as substrate. This assay revealed that, in addition to cholest-4-en-6β-ol-3-one, four other 6-oxygenated products were formed: cholest-4-en-6α-ol-3-one, cholest-4-en-6α-hydroperoxy-3-one, cholest-4-en-6β-hydroperoxy-3-one, and cholest-4-en-3,6-dione (Scheme 1). However, cholest-5-en-3-one was formed first, and the 6-oxy products appeared subsequent to its formation, concomitant with cholest-5-en-3-one disappearance. Based on the kinetics of product formation, it seemed that we were observing radical $^3O_2$ oxidation of cholest-5-en-3-one. Although $^1O_2$ oxidation also produces the 6-oxy steroids observed, 3α,5α-epidioxycholest-3β,6-ol is the major product (5). We never observed any of this product, and thus $^3O_2$ seemed a more likely reactant.

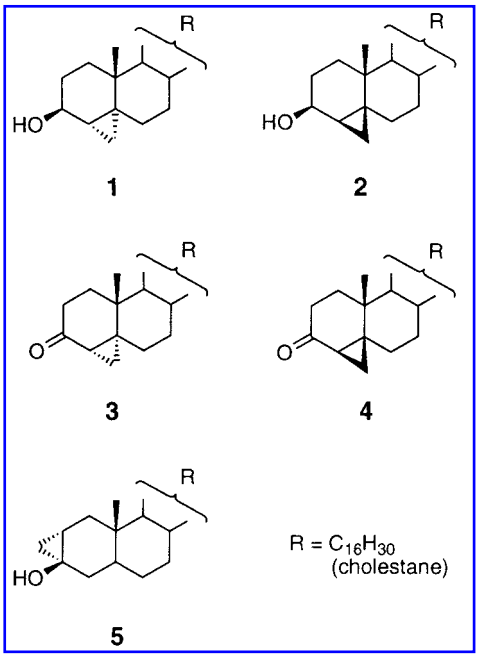
We performed further experiments to demonstrate that cholesterol oxidase was not acting as a monooxygenase. If either the C4a-adduct or H_2O_2 were the hydroxylating agent, then oxidation of cholesterol to form the agent would be a prerequisite for 6-hydroxylation. However, incubation of E361Q with cholest-5-en-3-one produced the same products at the same rates. That is, flavin redox cycling was not necessary for 6-hydroxylation. Moreover, when cholesterol was used as the substrate, E361Q cholesterol oxidase produced H_2O_2 in stoichiometric amounts. None of the H_2O_2 was funneled into the hydroxylation pathway. Furthermore, hydrogen abstraction was not stereospecific. When 4β-deutero cholesterol was incubated with E361Q, not all of the deuterium label was removed upon formation of the 6-oxy products. In the wild-type conversion of 4β-deutero cholesterol to cholest-4-en-3-one, all of the 4β-deuterium was abstracted and 4α-deuterium was not (13, 23).

All of these results taken together suggested that the 6-oxygenation observed was a result of radical chain decomposition of the cholest-5-en-3-one that is formed upon oxidation of cholesterol by E361Q cholesterol oxidase. In other words, cholesterol oxidase does not have an intrinsic monooxygenase activity, rather it has an unstable intermediate. The instability of cholest-5-en-3-one suggests that cholesterol oxidase evolved as a bifunctional enzyme with one active site to avoid the formation of radical and hydroperoxy steroids. This bifunctionality is a consequence of the substrate being a steroid, and is in contrast to the functionality of the other GMC oxidoreductases that only catalyze oxidation and not isomerization of their substrates.

WHAT TYPE OF CYCLOPROPYL STEROIDS CAN INHIBIT CHOLESTEROL OXIDASE?

The flavin-catalyzed oxidation of cholesterol may proceed in one step via hydride transfer or two steps via a radical intermediate. Moreover, mutagenesis of histidine-447 had suggested that the imidazole was important for general base catalysis during oxidation, *i.e.*, for deprotonation of the 3-hydroxyl. To probe the nature of the FAD-mediated oxidation, we synthesized a series of cyclopropyl steroids to be tested as mechanism-based inhibitors (Scheme 2). We explored the reactivity of irreversible inhibitors rather than competitive, reversible inhibitors because they have a second purpose. We plan to use inactivated steroid–cholesterol oxidase complexes for x-ray structural studies. The available structure with dehydroepiandrosterone complexed to cholesterol oxidase does not provide insight into the enzyme specificity for different C17 steroids, because the keto group at C17 of dehydroepiandrosterone is sterically small. Inactivation with cholesterol analogs can provide complexes suitable for studying C17 specificity by x-ray crystallography.

With steroids **1** and **2,** if a radical intermediate is formed, cyclopropyl ring opening might occur. Depending upon the structure and re-



Scheme 2.

activity of the ring-opened intermediate formed, it could undergo further reaction to form a flavin semiquinone steroid adduct, or the radical could potentially react with the enzyme or solvent. Cyclopropyl steroids **3** and **4** were synthesized as controls for experiments with steroids **1** and **2,** because the ketones **3** and **4** are the products of "normal" oxidation of **1** and **2,** and could possibly inactivate cholesterol oxidase via nucleophilic ring opening. As discussed earlier, glutamate-361 is positioned directly over the steroid β-face and the anionic carboxylate could act as a nucleophile (17).

When either **1** or **2** is incubated with cholesterol oxidase, the ring-intact ketone, **3** or **4,** respectively, is formed. That is, no ring-opened products were observed. This could be because a radical is not formed, or because the steric constraints of the active site prevent rearrangement to the ring-opened conformation. Moreover, the ketone products do not inactivate cholesterol oxidase. Lack of nucleophilic reaction with glutamate-361 is consistent with the absence of electrophilic catalysis at the C3 oxygen during isomerization. In other words, the conjugate acid of histidine-447 is not involved in catalysis of cholest-5-en-3-one isomerization to cholest-4-en-3-one, and is not available for catalysis of nucleophilic addition to the cyclopropyl ketones. Thus, our studies with cyclopropyl steroids **1, 2, 3,** and **4** did not offer much insight into the FAD reactivity of cholesterol oxidase. However, reaction of **5** with cholesterol oxidase did.

A cyclopropyl steroid such as **5** can undergo radical or anionic ring opening in the presence of an electrophile and/or a base. FAD is an excellent electrophile and, in the cholesterol oxidase active site, is positioned under the α-face of the sterol (16). Thus, the FAD is appropriately positioned to aid anionic ring opening of **5.** Alternatively, the ring-opened anion can transfer an electron to the FAD to form the semiquinone. The methylene and semiquinone radicals could then collapse to form an adduct. In addition to an active-site electrophile, this reaction can be catalyzed by a base via deprotonation of the alcohol (28). If histidine-447 were involved in 3-hydroxyl deprotonation, then it could possibly facilitate the ring-opening reaction. Sherry and Abeles had previously studied the inactivation of methanol oxidase by cyclopropanol, an inhibitor analogous to **5** (25). Their work suggested that inactivation was a radical process because the rate of inactivation observed was significantly faster than cyclopropoxide ring-opening (28). As methanol oxidase and cholesterol oxidase are both members of the GMC oxidoreductase family, we felt the reactivity of **5** with cholesterol oxidase worth investigation.

We first tested **5** as a substrate for cholesterol oxidase. We were unable to detect formation of H_2O_2, that is, there was no redox cycling of the flavin. However, incubation of **5** did result in a pseudo-first order loss of enzyme activity over time. The inactivation kinetics were saturable, with a K_i of 36 μM and a k_{inact} of 0.010 min^{-1}, and competitive with substrate. Moreover, the inhibition of cholesterol oxidase was irreversible by either dialysis or gel filtration. Thus, it appeared that **5** was indeed an irreversible inactivator of cholesterol oxidase.

To determine the structure(s) of the inhibition product(s), we denatured the inactivated protein and separated the protein and cofactor fractions by chromatography. We found that the protein was unaltered. However, there were two new species in the cofactor fraction that appeared to be steroid–FAD adducts. They were present in a 2:1 ratio. We characterized these adducts by MALDI-TOF (matrix-assisted

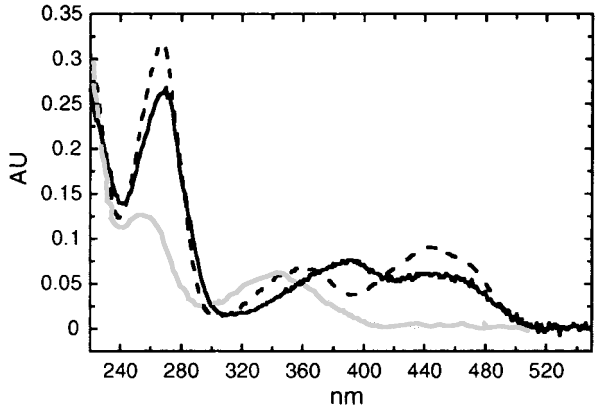


FIG. 2. UV/visible spectra of FAD (– –), 6 (——), and 7/8 (——).

laser desorption/ionization–time of flight) mass spectrometry and UV/visible and fluorescence spectroscopy. The masses of both adducts were consistent with steroid–FAD adducts. The UV/visible spectrum of the major adduct indicated that it contained an oxidized isoalloxazine ring; however, the spectrum was red-shifted compared with that of FAD (Fig. 2). These spectra are consistent with alkyl substitution at C6 of the isoalloxazine ring to form **6** (Fig. 3). In contrast, the minor adduct has a single absorbance maximum in the visible region at 340 nm (Fig. 2), and a fluorescence emission maximum at 480 nm. These spectra are consistent with alkyl substitution at N5 to form a reduced flavin, **7,** or a cyclic N5-C4a adduct, **8** (Fig. 3). In fact, a cyclic adduct similar to **8** was the product isolated upon inactivation of methanol oxidase with cyclopropanol (25).

Although formation of the C6 adduct was unexpected, reaction at C6 has been observed with at least two other flavoenzymes, D-lactate dehydrogenase (8), and general acyl-CoA dehydrogenase (35). Moreover, diethylamine and triethylamine dehydrogenases contain flavin mononucleotide covalently linked to the protein via a cysteine thioether bridge (26). Work with model systems has shown the flavin C6 to undergo electrophilic substitution (11). The predominant reactivity at C6 observed with cholesterol oxidase probably reflects the orientation of FAD with respect to cyclopropyl steroid **5** that is constrained by the enzyme. Further characterization of the adducts, for example by NMR spectroscopy or x-ray crystallography, have been hindered by the limited solubility of the steroid and the aggregation characteristics of the inhibited enzyme. Recently, however, we have identified new detergent conditions that may allow us to crystallize the inactivated complex (Y. Yin, unpublished observations).

We have proposed a mechanism in which cyclopropoxide is formed in the active site, and subsequently undergoes ring opening to form the ketone and a methylene anion equivalent (Fig. 3) (18). As discussed above, the FAD electrophile can participate directly in the ring opening or serve as an electron acceptor to form the flavin semiquinone. After reaction of the steroid methylene with FAD at the C6 position, the flavin is effectively reduced. Oxygen can

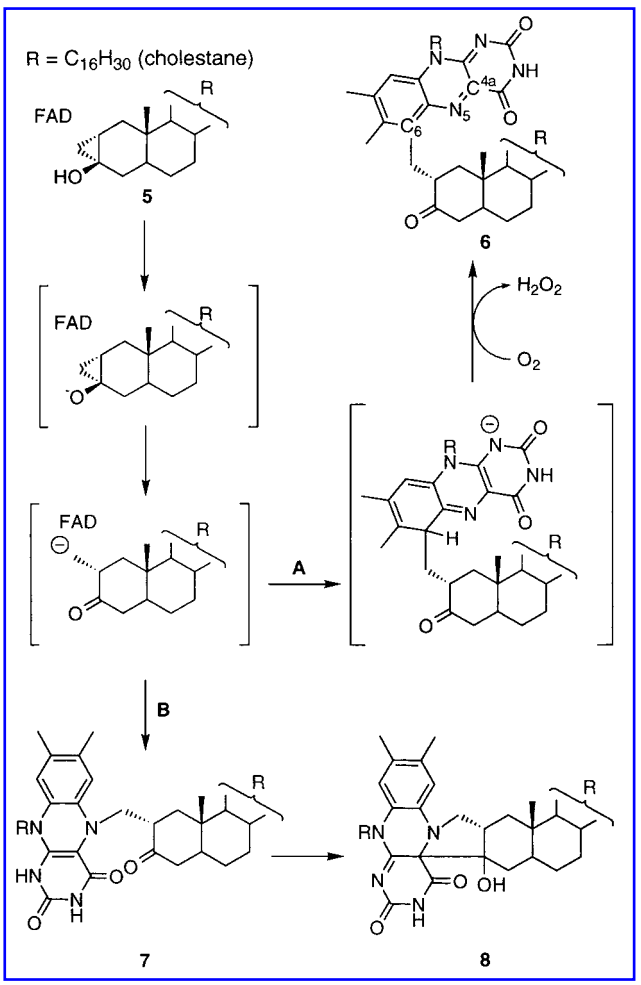


FIG. 3. Proposed mechanism for inactivation of cholesterol oxidase by 2α,3α-cyclopropano-5α-cholestan-3β-ol, 5, and structures of adducts 6, 7, and 8. [Reproduced with permission from 18; Copyright 2000 American Chemical Society]

then oxidize the isoalloxazine, just as in the native reaction. We measured the rates of inactivation with histidine-447 mutants to test whether the imidazole participated in the process. As expected, the mutants are inactivated more slowly or not at all. For example, H447Q is inactivated by **5** 10 times more slowly than wild-type enzyme (k_{cat} is 100-fold slower). In contrast, E361Q (the mutant that no longer catalyzes cholest-5-en-3-one to cholest-4-en-3-one isomerization) is inactivated at the same rate as wild-type enzyme. Clearly, formation of **6–8** is mechanism-based. The role of histidine-447 is most likely twofold: (a) formation of the cyclopropoxide, and (b) electrostatic stabilization of superoxide during O_2 oxidation of the reduced flavin. Klinman and co-workers have elegantly shown that in the glucose oxidase-catalyzed reaction, protonated histidine plays an important role in the oxidation of $FADH^-$ by O_2 (27). The kinetic rate constants and isotope effects observed with our histidine-447 mutants are consistent with this being the case for cholesterol oxidase as well (13). These results further highlight the mechanistic similarities between members of the GMC oxidoreductase family.

CONCLUSION

We have investigated the reactivity of the FAD cofactor in *Streptomyces* cholesterol oxidase. All of our mutagenesis, enzyme inhibition, and kinetic data demonstrate that the cofactor catalyzes oxidation of alcohols to ketones, but not oxygenation of carbon. Cholesterol oxidase catalyzes two reactions, oxidation and isomerization, in one active site, presumably because of the susceptibility of the reaction intermediate cholest-5-en-3-one to radical oxidation. This bifunctionality is not a shared characteristic with other GMC oxidoreductase family members. Furthermore, we have characterized the unusual, but not unprecedented, inactivation of FAD by electrophilic substitution at C6 of the isoalloxazine ring upon ring opening of a cyclopropyl steroid. Another member of the GMC oxidoreductase family, methanol oxidase, is also inactivated by cyclopropanol, suggesting that inhibition by cyclopropanol inhibitors may be diagnostic of membership in this family.

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ABBREVIATIONS

FAD, flavin adenine dinucleotide; GMC, glucose–methanol–choline; H_2O_2, hydrogen peroxide.

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