

Cholesterol Oxidases: A Study of Nature's Approach to Protein Design

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ABSTRACT

Cholesterol oxidases are important as clinical reagents, potential larvicides, and tools in cell biology, and they are implicated in bacterial pathogenesis. Here we review chemical aspects of their function. We describe our current structural and mechanistic understanding of the type I and II cholesterol oxidases, our identification of an NH- π hydrogen bond motif for stabilization of reduced flavins, our structural hypothesis of how O₂ gains access to the flavin, and our present understanding of type I cholesterol oxidase–lipid bilayer interactions.

Bacterial cholesterol oxidases have a long and varied history of use. Originally isolated to use in serum cholesterol assays, cholesterol oxidases catalyze the formation of cholest-4-en-3-one from cholesterol (Figure 1). The oxidation of the sterol requires an FAD cofactor that is concomitantly reduced. Regeneration of the oxidized cofactor is effected by the reduction of O₂ to hydrogen peroxide. The colorimetric detection of hydrogen peroxide forms the basis of clinical assays. Bacteria, however, have their own reasons for producing cholesterol oxidases. The types of bacteria that produce them can be classified into two types: nonpathogenic and pathogenic. Nonpathogenic bacteria, e.g., *Streptomyces* and fast-growing *Mycobacteria*, can utilize cholesterol as their carbon source and up-regulate expression of cholesterol oxidase in the presence of cholesterol.^{1,2} Pathogenic bacteria, e.g., *Rhodococcus equi* and slow-growing *Mycobacteria*, require cho-

lesterol oxidase for infection of the host macrophage; cholesterol also regulates expression of the enzyme in these organisms.^{2–4} The role of cholesterol oxidase in pathogenesis appears to be due to its ability to alter the physical structure of the lipid membrane by converting cholesterol into cholest-4-en-3-one, but it is not yet clear exactly where this alteration fits into the pathway of infection.

Cholesterol oxidase is a water-soluble, interfacial enzyme that binds transiently to the membrane surface during catalysis. Interestingly, it was one of the early tools used to probe the heterogeneity of cell membranes and the localization of cholesterol in the lipid bilayer.⁵ Although originally discounted by many, these early observations have proven to be correct and provide some of the first evidence that lipid rafts are present in cell membranes.⁶ In addition, cholesterol oxidase has insecticidal properties against *Coeloptera* larvae, agricultural pests,⁷ and is being developed for use in agricultural crop treatment. We have begun to unravel how bacteria utilize cholesterol oxidase to alter the physical properties of lipid membranes. Understanding both the chemistry and the physics of the enzyme's action may lead to novel anti-infective and pesticidal agents.

The Vrieling laboratory has solved the three-dimensional structures of three cholesterol oxidases. Two of these oxidases from *Streptomyces sp. SA-COO*⁸ and *Brevibacterium sterolicum*^{9,10} are nearly identical in sequence and structure and are classed as type I oxidases (Figure 2a). The sequence and structure of the third oxidase, a type II oxidase, obtained from a different *Brevibacterium* strain¹¹ are completely different (Figure 2b). These structures provide an opportunity to analyze the catalytic mechanism in terms of structure. Moreover, the recent acquisition of very high-resolution structural data in combination with kinetic data has provided an opportunity to understand at a very fundamental level how proteins modulate oxidation chemistry.¹² In this Account, we will highlight four areas of investigation that detail (a) our current structural and mechanistic understanding of the type I and II cholesterol oxidases, (b) our identification of a novel protein motif for flavin reduction potential modulation in the type I oxidase, (c) our structural hypothesis of how O₂ gains access to the flavin in both types, and (d) our present understanding of type I cholesterol oxidase–lipid bilayer interactions.

Structure and Mechanism of Type I Cholesterol Oxidase

Cholesterol oxidases are bifunctional flavoenzymes that catalyze two reactions in one active site. The first is oxidation of cholesterol to cholest-5-en-3-one, and the second is isomerization to cholest-4-en-3-one (Figure 1). *Streptomyces sp. SA-COO* and *B. sterolicum* type I choles-

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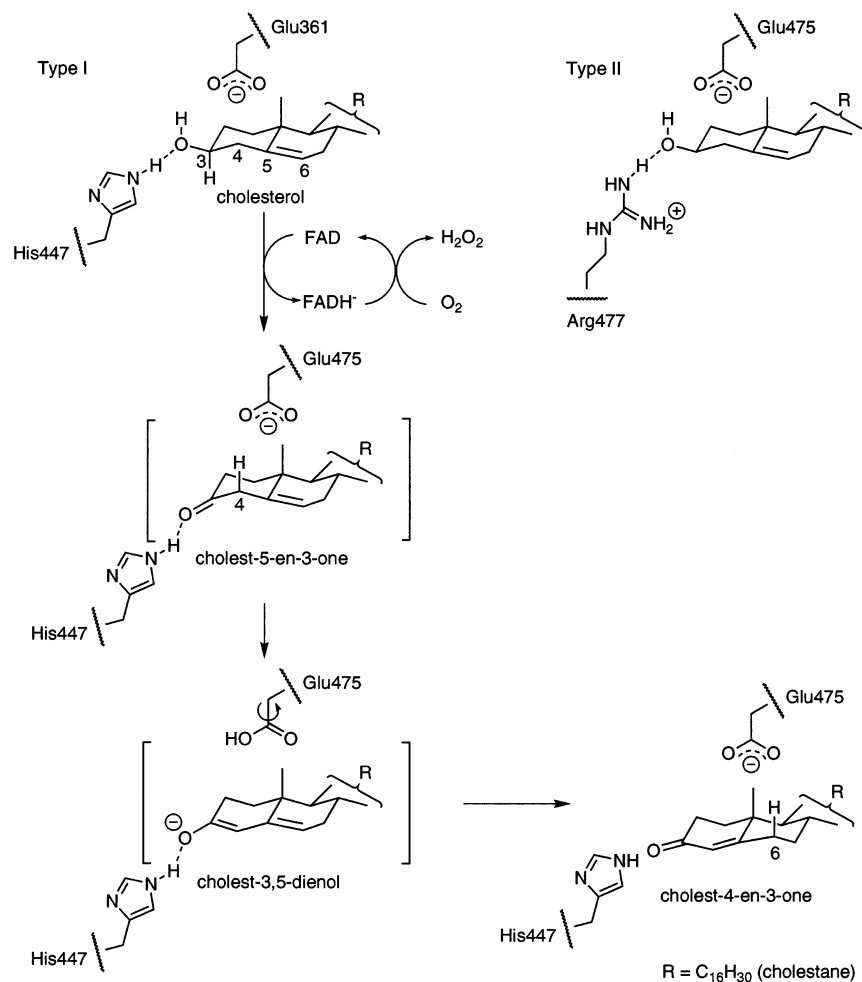


FIGURE 1. Reaction catalyzed by cholesterol oxidases. The proposed role of key active site residues is shown for the type I cholesterol oxidase. The analogous residues for the type II cholesterol oxidase are illustrated for the Michaelis substrate complex.

terol oxidases each contain the FAD cofactor noncovalently bound to the enzyme. Their crystallographic models reveal structurally conserved active sites (Figure 2a). The noncovalent FAD forms of the enzyme are members of the GMC (glucose–methanol–choline) oxidoreductase family of flavoenzymes in which two residues, His447 and Asn485, thought to be involved in substrate oxidation, are semiconserved.¹³

A structure of the complex of the reduced enzyme with dehydroepiandrosterone showed the steroid bound in a deeply buried active site with the hydroxyl group near to a bound water molecule (Wat541)¹⁰ (Figure 3a). The position of this water molecule in the active site originally suggested that deprotonation of the steroid hydroxyl proton was mediated by His447 through the bound water molecule.¹⁰ We recently refined the X-ray structure of the *Streptomyces* enzyme to sub-Ångstrom resolution (0.95 Å).¹² This much more detailed view of the active site has led us to propose a new model for the Michaelis complex of the enzyme (Figure 3b) and to reinterpret the mechanism of general base catalysis.¹¹ In order for efficient hydride transfer to occur, the C–H bond of the substrate must be aligned with the lowest unoccupied molecular orbital (LUMO) of the FAD, a π -type orbital on N5 of the cofactor. Positioning the substrate so as to maximize

orbital overlap places the hydroxyl group at the position of the bound water molecule (Wat541) in both the native structure^{8,9,12} and the substrate/reduced enzyme structure.¹⁰ This new model for the Michaelis complex suggests, therefore, that the bound water molecule in the native enzyme structure mimics the substrate hydroxyl group.

Sequence and structure alignments of the active site revealed that His447 is completely conserved within the GMC oxidoreductase family,¹⁰ and mutagenesis and kinetic studies implied its importance in cholesterol oxidation.^{8,14} The homologous histidine residues in glucose oxidase¹⁵ and cellobiose dehydrogenase¹⁶ are also critical for oxidation of their respective substrates. In addition, in the case of glucose oxidase, the protonated histidine provides the optimal protein dielectric for electron transfer to O₂.¹⁷ In cholesterol oxidase, this residue was proposed to act as the base for abstraction of the hydroxyl hydrogen atom of the steroid substrate. However, the atomic resolution crystal structure of cholesterol oxidase revealed that His447 is present as the neutral imidazole with a hydrogen atom on NE2 of His447, the nitrogen atom oriented toward the substrate hydroxyl.¹² Two neighboring protein residues (Asn341 and Asn343) are donating hydrogen bonds to the ND1 of His447, precluding its tautomeriza-

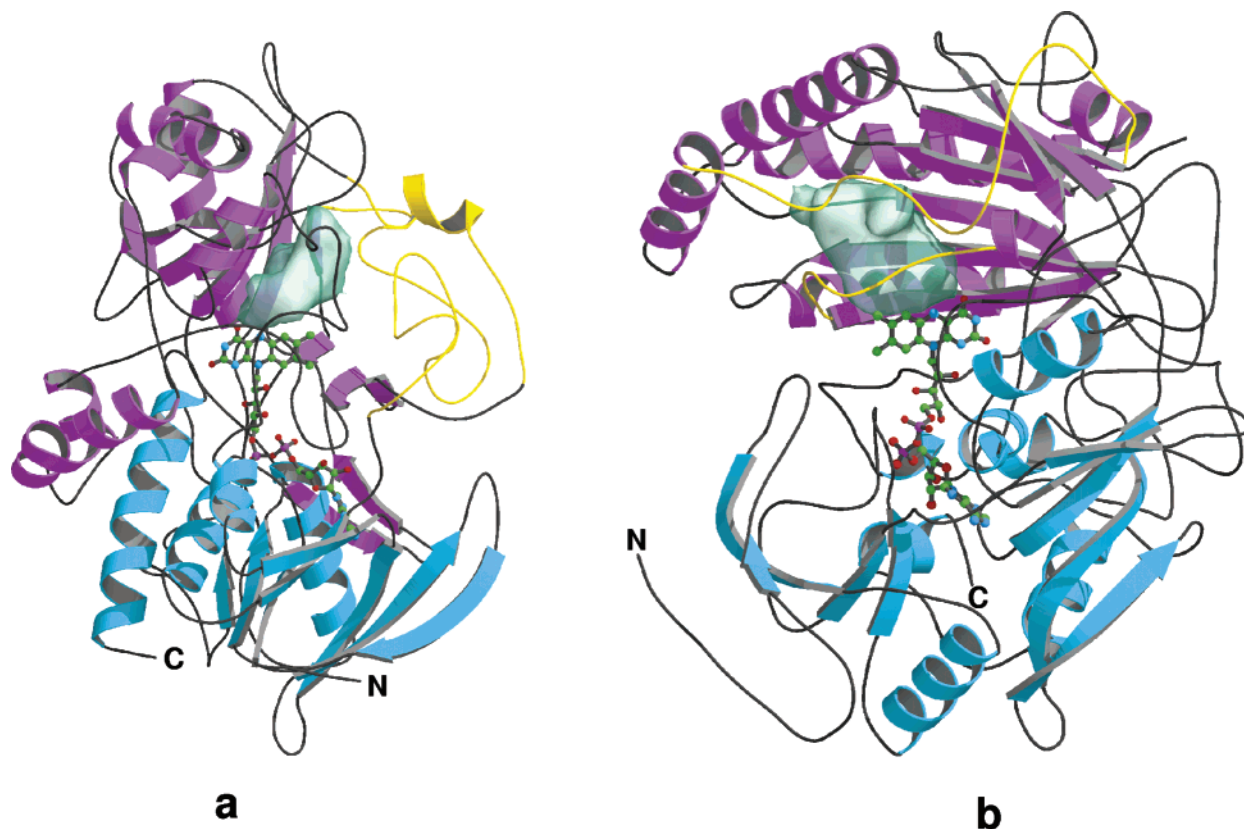


FIGURE 2. Secondary structure representations of (a) type I cholesterol oxidase (PDB entry 1B4V) and (b) type II cholesterol oxidase (PDB entry 1I19) showing the buried substrate cavity. The substrate binding and FAD binding domains are colored in magenta and blue, respectively. The substrate binding loops are shown in yellow. The FAD cofactor is represented as a ball-and-stick model. The surfaces, shown in green, were computed as molecular surfaces using the program SPOCK⁴¹ using a probe radius of 1.4Å. The figures were made with MOLSCRIPT⁴² and rendered with RASTER3D.⁴³

tion upon substrate binding to the other neutral form of imidazole. Additional atomic resolution structural studies on the enzyme over a broad pH range showed that the NE2 atom of His447 remains protonated up to at least pH 7.5 (P. Lario, personal communication, 2003). This hydrogen bond network suggests that the role of His447 is as a hydrogen bond donor to the hydroxyl oxygen atom of the substrate and that it serves to position the substrate with respect to the flavin. In this arrangement, the hydrogen atom of the steroid hydroxyl group is directed toward the side chain of Glu361 and the lone pair of electrons points toward the protonated NE2 of His447. This antiperiplanar configuration supports a concerted trans-elimination reaction of proton and hydride. Thus, the substrate O–H bonding electrons can delocalize into the antibonding orbital of the C–H group to promote hydride transfer. It is not clear that complete proton transfer from the hydroxyl group to Glu361 occurs, since the following isomerization reaction would be enhanced by general acid catalysis at the oxygen. Moreover, mutation of Glu361 to glutamine in combination with mutation H447Q only reduced k_{cat} and k_{cat}/K_m 3-fold relative to the single-point mutant H447Q.¹⁸

Uniquely among GMC family members, cholesterol oxidase catalyzes a second nonredox reaction in the active site, isomerization of a β,γ -unsaturated ketone to an α,β -unsaturated ketone. This isomerization is a base-catalyzed process, as demonstrated by deuterium transfer studies

and mutagenesis.^{19–21} The 4 β hydrogen is transferred to the 6 β position on the steroid.^{19,21} Glu361 is strategically positioned over the β -face to catalyze this isomerization.^{8,10} Indeed, mutagenesis to a glutamine shut down isomerization completely. The reason for this bifunctionality may be the instability of the cholest-5-en-3-one initially formed upon oxidation of cholesterol. The cholest-5-en-3-one is particularly susceptible to auto-catalyzed radical peroxidation and forms cholest-4-en-6-hydroperoxy-3-one that disproportionates to cholest-4-en-3,6-dione and cholest-4-en-6-ol-3-one.²¹ The various reports that different sources of cholesterol oxidase produce more or less cholest-4-en-6-ol-3-one^{22,23} probably reflect the varying degrees to which different cholesterol oxidases release cholest-5-en-3-one before isomerization occurs. For example, in the case of wild-type *Streptomyces* cholesterol oxidase, we observed by HPLC that 2% of the product initially formed is cholest-5-en-3-one, which is subsequently degraded to the hydroperoxide.²¹

An N–H $\cdots\pi$ Electrostatic Interaction Stabilizes the Hydroquinone Form of the Flavin

During the course of our study of general acid/base catalysis, Asn485 was mutated to leucine in order to determine whether it was contributing to either the oxidation or isomerization reactions. Our kinetic results indicated that Asn485 plays a crucial role in the oxidation

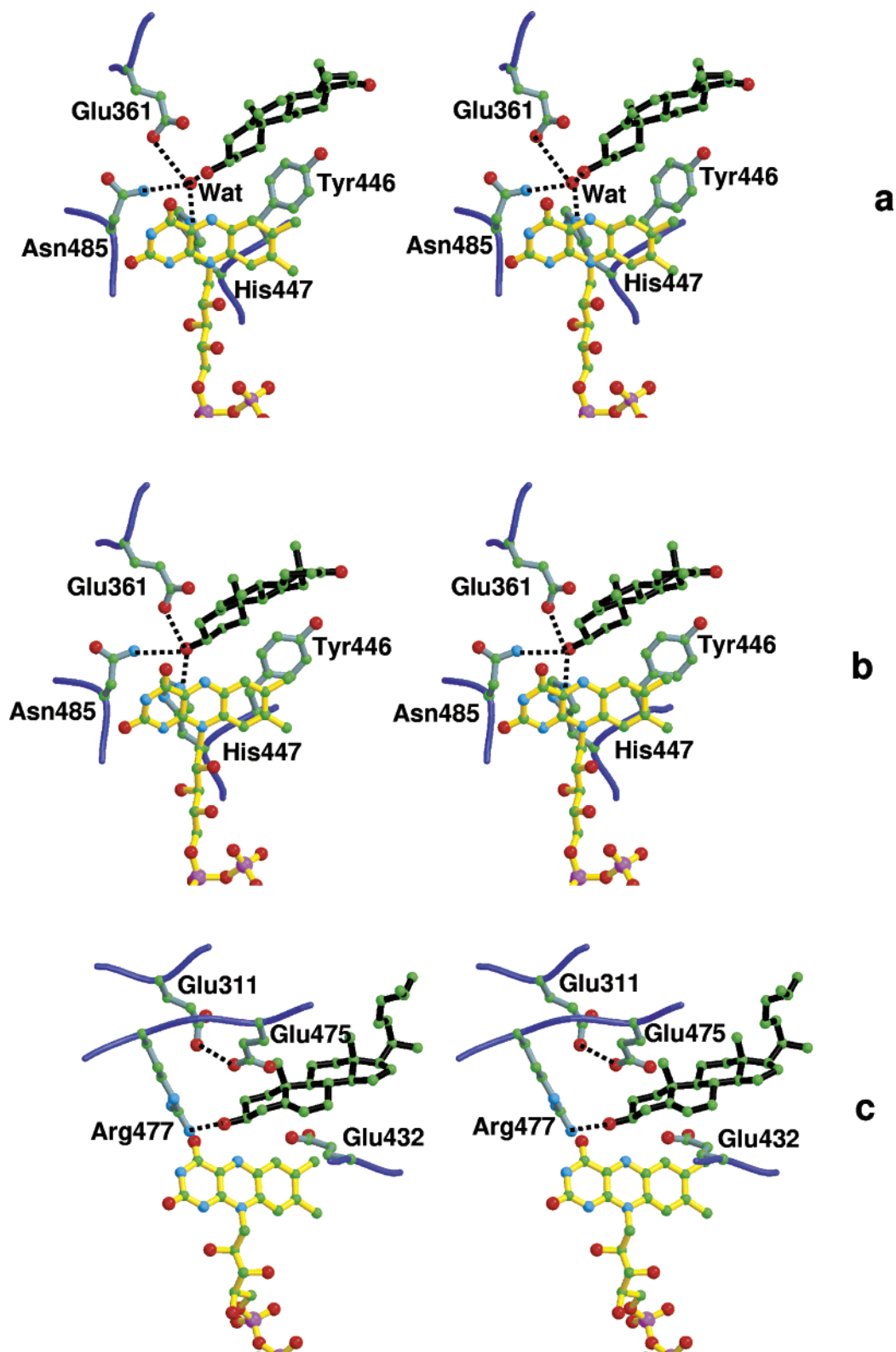


FIGURE 3. (a) Stereo representation of the active site region of the complex of type I *Brevibacterium* cholesterol oxidase with dehydroepiandrosterone (pdbentry 1COY). Stereo representations of the *in silico* models of the Michaelis complexes for (b) type I cholesterol oxidase (pdbentry 1MXT) with bound dehydroepiandrosterone as proposed by Lario et al.¹² and (c) type II cholesterol oxidase (pdbentry 1119) with bound cholesterol. The FAD is shown in yellow bonds, the protein side chains in gray bonds, and the steroid molecules in black bonds. Water molecules are depicted as red spheres.

activity of the enzyme. When Asn485 was substituted with a leucine, the oxidation activity was greatly impaired. Steady-state kinetic analysis showed that k_{cat} for oxidation

is 1000-fold slower in N485L than in wild type, but is only 20-fold slower for isomerization. We examined the X-ray crystal structure of the N485L mutant in order to deter-

Table 1. Michaelis–Menten Constants for Wild-type and Mutant Cholesterol Oxidases

substrate	cholesterol ^a			cholest-5-en-3-one ^b		
	k_{cat} (s ⁻¹)	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}^{\text{mut}}/$ ($k_{\text{cat}}/K_{\text{m}}^{\text{wt}}$)	k_{cat} (s ⁻¹)	K_{m} (μM)	$(k_{\text{cat}}/K_{\text{m}})^{\text{mut}}/$ ($k_{\text{cat}}/K_{\text{m}}^{\text{wt}}$)
wild type	44 ± 2	3.0 ± 0.4		64 ± 3	6.2 ± 0.7	
E361Q	1.4 ± 0.1	5.4 ± 0.6	0.018	n.a. ^c	n.a.	n.a.
H447Q	0.32 ± 0.01	3.0 ± 0.3	0.0036	81 ± 4	7.1 ± 0.8	1.1
H447E	0.017 ± 0.003 ^d	n.m. ^e	n.a.	7 ± 2 × 10 ⁻³	n.m.	n.m.
H447Q/E361Q	0.093 ± 0.003	5.0 ± 0.1	0.0015	n.a.	n.a.	n.a.
H447E/E361Q	0.0014 ± 0.0001 ^d	n.m.	n.a.	n.a.	n.a.	n.a.
N485L	0.046 ± 0.004	4.2 ± 0.1	0.00075	3.2 ± 0.3	18 ± 0.6	0.017

^a Measured by H₂O₂ formation with HRP coupling. ^b Measured by cholest-4-en-3-one formation at 240 nm. ^c Not applicable as discussed in.²¹ ^d Measured at 32 μM cholesterol that was assumed to be saturating. ^e Not measured.

Table 2. Reduction Potentials for Wild-Type and N485L Cholesterol Oxidases^a

species	type	maximal amount of semiquinone formed	ΔE (mV)	E_1 (mV) _p	E_2 (mV)	E_{m} (mV)	
<i>Strep</i>	I	wild type ^{18b}	98%	-112	-222 ± 14	-334 ± 11	-278
<i>Strep</i>	I	N485L ^{18b}	95%	-108	-300 ± 5	-408 ± 8	-354
<i>Brevi</i>	II	wild type ^{26c}	65%	-67	-74.1 ± 0.7	127.3 ± 4.3	-101

^a Determined spectroscopically using the xanthine and xanthine oxidase system.⁴⁰ ^b In 50 mM sodium phosphate, pH 7.0 at 15 °C. Safranin T was used as the redox standard ($E_{\text{m}} = -276$ mV). ^c In 100 mM potassium phosphate, pH 7.5 at 15 °C. Indigo disulfonate and cresyl violet were used as the redox standards ($E_{\text{m}} = -74$ and -176 mV, respectively).

mine the effect of the mutation on the active site structure. We observed a small shift in the position of the water structure in the active site. By analogy, the substrate position would be similarly shifted, and the substrate may no longer be ideally aligned with Glu361 and the FAD. However, the markedly lower oxidation activity of N485L relative to isomerization implied that the major effect of the mutation is not primarily due to the misalignment of the substrate in the active site.

Although the N485L reaction is 3 orders of magnitude slower than that of the wild type, primary deuterium kinetic isotope effects showed that 3 α -H transfer is still rate-determining in the mutant reaction. This means that mutation of Asn485 reduces the rate of 3 β -hydroxy oxidation, rather than decreasing the rate of another kinetic step, and that Asn485 is important for FAD reduction. In contrast, when His447 is mutated, 3 α -H transfer is no longer rate determining.¹⁴

The structural and kinetic data for N485L suggested that Asn485 is important for creating an electrostatic potential around the FAD that is favorable for oxidation of alcohol substrates. Indeed, the UV/vis spectrum of the FAD region of N485L is red-shifted relative to wild-type, indicating that the electronic environment around the FAD has been altered. In addition, the mutation of Asn485 to leucine and its associated structural changes resulted in a 76 mV decrease in the midpoint reduction potential of the FAD (Table 2). In other words, the N485L mutant is a much poorer oxidizing agent, and the reduction of the N485L-bound FAD is not as thermodynamically favorable as that of wild-type. This is consistent with the kinetic properties that we observed, i.e., a higher activation barrier to FAD reduction.

The change in reduction potential observed corresponds to approximately 3 kcal/mol transition state stabilization energy after correcting for other changes that occur in the active site. The interaction is an amide- π interaction between the asparagine side chain and the

π -system of the pyrimidine of the FAD. Furthermore, the helix 14 dipole amplifies the strength of this interaction (Figure 4). Empirical potential energy calculations have suggested that an N-H $\cdots\pi$ interaction has a stabilization energy of approximately 3 kcal/mol.²⁴ QM/MM calculations on the cholesterol oxidase system estimate that 2 kcal/mol stabilization energy may be derived from the interaction.²⁵ This magnitude range is qualitatively consistent with the rate reduction that we observed. Although many protein-flavin interactions have been studied in different systems, this is the first example, to our knowledge, of a π -cation-like interaction between the isoalloxazine ring and the protein.

Structure and Mechanism of Type II Cholesterol Oxidase

The structurally distinct type II cholesterol oxidase from *Brevibacterium* has a covalently bound FAD (Figure 2c).^{11,26} Despite the identical reactions carried out by the covalent and noncovalent FAD enzymes, comparisons reveal large differences in redox potential and kinetic properties, suggesting very different oxidation mechanisms. The midpoint reduction potential for the type II enzyme is -101 mV,²⁷ and for the type I enzyme it is -278 mV²⁸ (Table 2), making the type I cholesterol oxidase a poorer oxidizing agent (the reduction of the cofactor is thermodynamically less favorable) than the type II enzyme. These differences in both kinetic and redox properties as well as the structural diversity for these two enzyme forms provide insight into the specific roles of amino acid residues in oxidation and isomerization and the role that the microenvironment around the flavin cofactor plays in modulating the redox potential for oxidation.

The type II enzyme does not contain the characteristic nucleotide-binding fold commonly seen in the most FAD containing proteins. Rather the fold is similar to that seen in other flavoenzymes with covalently bound cofactor

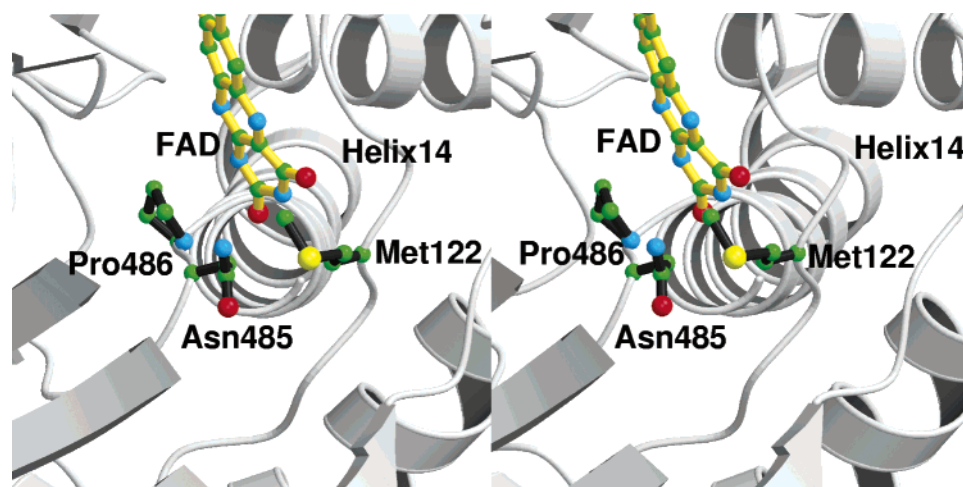


FIGURE 4. Stereoview of helix-14 in the type I *Streptomyces* native structure (PDB entry 1B4V). The bonds for the pyrimidine ring of the FAD cofactor are colored yellow, those for the side chains of Asn485, Pro486, and Met122 are in black, and the secondary structure elements are shown in gray. The side chains and the pyrimidine ring are located at the N-terminus of the helix.

such as vanillyl alcohol oxidase.²⁹ The cofactor is linked to the protein through a covalent bond between ND1 of the imidazole side chain of His121 and the 8-methyl group of the isoalloxazine ring. The active site of the enzyme is located on the *re* face of the isoalloxazine ring and consists of a large internal cavity sufficient in size to house an entire cholesterol molecule including the terpene moiety of the substrate (Figure 2b). The entrance to this cavity is provided by two mobile loops in the substrate-binding domain. The position and size of the cavity is in contrast to the type I enzyme, where it is located on the *si* face of the cofactor and is only large enough to accommodate the 4-ring system of the steroid substrate (Figure 2a). In order for the terpene moiety at C17 of the steroid nucleus to bind, a repositioning of a series of loops at the entrance to the cavity is required.

The active site of the type II enzyme is much more hydrophilic than that seen for the type I enzyme. The pyrimidine region of the active site cavity contains four glutamate residues (Glu475, Glu551, Glu432 and Glu311), an arginine (Arg477), a lysine (Lys554), and an asparagine (Asn516). In comparison, the only polar residues in the active site of type I cholesterol oxidases are Glu361, His447, and Asn485. Two distinct conformations are seen in the structure for the side chains of Glu475, Arg477, and Ile423 and these movements are correlated with each other.

A model for the cholesterol-bound complex shows the possible interactions between amino acid side chains and the steroid substrate (Figure 3c). The steroid hydroxyl group is positioned within hydrogen bonding distance of the guanidinium group of one side chain conformation of Arg477, the carboxylate group of one side chain conformation of Glu475 and O4 of the flavin. Thus, the Arg477 takes the place of His447 in the type I reaction. Furthermore, the C3-H of the substrate is well positioned for transfer to N5 of the cofactor. On the basis of this model, Vrieling and co-workers proposed that Glu475 acts as the general base for proton abstraction of the substrate C3-OH group and that the guanidinium group of Arg477

stabilizes the reduced FADH cofactor.¹¹ This suggests therefore that Arg477 plays two roles in the oxidation reaction of type II enzyme: (1) to orient the substrate hydroxyl group in an analogous fashion to what we proposed for His447 in the type I enzyme; and (2) to stabilize the reduced cofactor through a π -cation interaction, analogous to the N-H $\cdots\pi$ electrostatic interaction proposed for Asn485 in the type I enzyme. Glu475 has also been proposed as the base needed for transfer of the C4 β -H to the C6 β position of the steroid in the isomerization reaction, analogous to Glu361 in the type I reaction. Prior to isomerization however, the proton on Glu475 from the oxidation step is most likely transferred to Glu311, positioned near the oxygen entrance tunnel (described below), from which it is transferred to the incoming oxygen molecule during the oxidative half-reaction.

Oxygen Accessibility to the Active Site

A structural comparison of the two types of cholesterol oxidase shows that despite distinct differences in the overall fold for the enzyme, they both contain a narrow tunnel that extends from the exterior surface of the molecule to the buried active site cavity. In the type II enzyme, this tunnel was immediately evident from the 1.7 Å crystal structure. The tunnel is situated between secondary structure elements of the FAD-binding domain and the substrate-binding domain. Furthermore, the multiple conformations of side chains at the active site revealed that in one conformational arrangement the tunnel was blocked from direct access to the active site (by the side chain of Arg477), and in the second conformational arrangement the tunnel is accessible to the active site (Figure 5a,b). Thus the structure indicates that the tunnel is gated, primarily by Arg477.

In the type I structure, the tunnel only becomes evident at 0.95 Å resolution. This is due primarily to the ability to visualize many more multiple conformations in the higher resolution structure. The tunnel in this structure is also narrow in width, essentially only wide enough to accom-

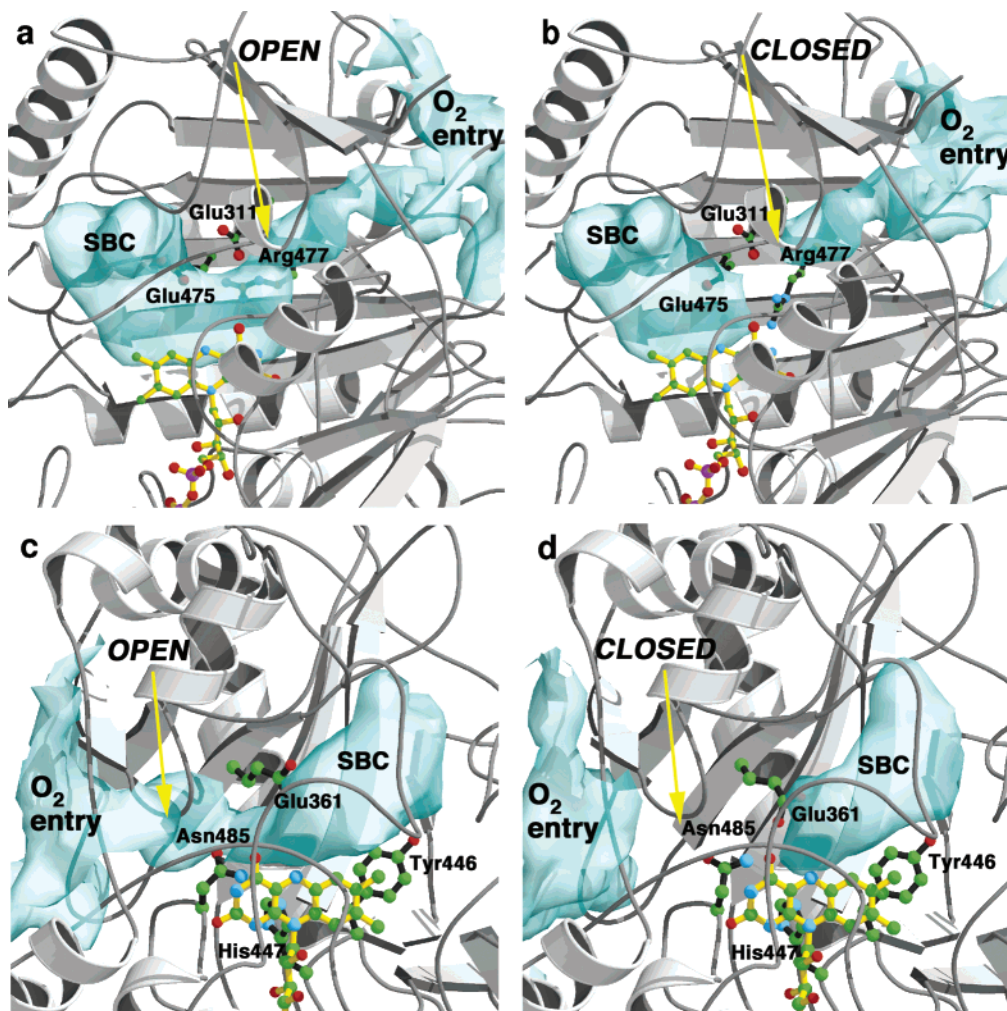


FIGURE 5. Surface representation of the two forms of cholesterol oxidase showing the proposed oxygen tunnels in the open and closed positions. The oxygen entry points and the steroid binding cavities (SBC) are labeled. The yellow arrows identify the gating residue whose conformation restricts access from the oxygen tunnel to the steroid binding cavity. (a) The type II enzyme (pdbentry 1119) in the tunnel open conformation and (b) the tunnel closed conformation. (c) The type I enzyme (PDB entry 1MX1) in the open and (d) closed conformations. The surfaces, colored turquoise, were computed as described in Figure 2.

modate a single water molecule. In addition, it is gated by the side chain of Asn485, which stabilizes the reduced cofactor through an $N-H \cdots \pi$ interaction. Upon reduction of the cofactor, the asparagine side chain moves closer to stabilize the extra electron density on the FAD; this movement results in a series of other side chain movements in the region of the tunnel, thereby forming the tunnel and providing access to the active site for molecular oxygen (Figure 5c and d).

Both tunnels are wide enough to house only a single string of water molecules linked to each other by a hydrogen bond network. Furthermore, the residues lining the tunnel are all hydrophobic in nature; this results in a very unreactive environment. The size of these tunnels, the nature of the residues that line them, the gating aspects of the tunnels, and the location of their endpoints at the pyrimidine ring of the FAD cofactor suggest that they may function as entry points for molecular oxygen during the oxidative half-reaction of the enzymes. The timing of oxygen access may be required to prevent radical peroxidation of the cholest-5-en-3-one in the active site.

Interestingly, the tunnel gates operate in opposite fashions: in type II cholesterol oxidase, the tunnel is closed when Arg477 is positioned within hydrogen bonding distance of the flavin ring system, whereas in the type I oxidase, the tunnel is open when Asn485 lies nearer to the flavin ring system. These structural differences may contribute to the differences in reactivity of the flavin cofactor and in the different kinetic mechanisms observed for the two enzyme forms. Indeed, the presence of a positive charge from the guanidinium group of Arg477 within hydrogen bond distance of the flavin may explain the higher redox potential observed for the covalent enzyme form. An interaction between a positively charged side chain and the reduced cofactor can be considered more stabilizing than that of an amide side chain and the reduced cofactor. The caveat to this interpretation is that the FAD reduction potentials in the presence of substrate or substrate analogue have not yet been obtained. Replacement of active site water with a hydrophobic steroid may substantially shift the reduction potential. Whether the reduction potentials of the type I and type II cofactors

will shift to the same degree is difficult to predict because their active site architectures are so different.

Cholesterol Oxidase Association with the Lipid Bilayer

One of the intriguing aspects of cholesterol oxidase function is its association with the lipid bilayer and its importance in bacterial metabolism and pathogenesis. To understand these roles, it is necessary to first understand how substrate is bound and what physical forms of substrate are preferred. Being very hydrophobic, cholesterol partitions preferentially into the lipid bilayer, although it does have a measurable rate of spontaneous transfer via the aqueous milieu between membranes.³⁰ The spontaneous rate of cholesterol leaving the lipid bilayer is insufficient to account for the rate of cholesterol oxidation catalyzed by the enzyme. Thus, the enzyme must form a complex with the lipid bilayer that allows cholesterol to move directly from the membrane into the active site, without aqueous solvation of cholesterol. Examination of all the X-ray crystal structures revealed active sites that are deep cavities and sequestered from bulk solvent by protein loops that cover the entrance to the cavity. We postulate that in the lipid bilayer–protein complex, the loops open to allow active site access to the cholesterol.

We have specifically labeled the type I protein with an acrylodan fluorophore on one of these loops to probe explicitly the loop–lipid bilayer interactions.³¹ Using the fluorescence parallax method of London,³² we determined that the probe is located on average 8.1 Å from the membrane center. This was the first direct evidence that cholesterol oxidase physically associates with the lipid bilayer. Although we do not know the orientation of the protein to the fluorophore, i.e., whether the linker is extended or kinked, the depth measured suggests that the protein sits on the surface of the membrane, with only minimal insertion. This position is consistent with the weak binding affinities (approximately 100–200 μM) of cholesterol oxidase measured for 100 nm unilamellar vesicles that are independent of the presence of acrylodan. Moreover, the binding constants varied little with membrane surface charge, or ionic strength, suggesting that binding of cholesterol oxidase to the membrane is primarily driven by hydrophobic interactions.

In light of the observations that cholesterol oxidase is larvicidal against *Coeloptera* and its putative role in microbial pathogenesis, we investigated whether its physical interaction with the membrane or its chemical conversion of cholesterol in the membrane is responsible for its toxic nature. We used a model membrane lysis vesicle system with carboxyfluorescein encapsulated in the interior of unilamellar vesicles.³³ We found that control vesicles containing 10–50 mol % of the cholest-4-en-3-one product of the enzymatic reaction rapidly leaked fluorescent dye. Unlike cholesterol, cholest-4-en-3-one does not have a condensing effect, and cholest-4-en-3-one membranes are more permeable.^{34–36} Thus, any

cholesterol-containing membrane susceptible to wild-type cholesterol oxidase-catalyzed conversion will become leaky and, under sufficient osmotic pressure, lyse. We employed an isomerization inactive mutant, E361Q, to test whether binding of protein was sufficient to cause membrane leakage. This mutant produces cholest-5-en-3-one not cholest-4-en-3-one. Cholest-5-en-3-one packs such as cholesterol in a lipid bilayer and does not inherently cause leakage. Carboxyfluorescein-containing cholesterol:phosphatidylcholine vesicles treated with the E361Q mutant did not leak, even at ratios of 1000 enzymes per vesicle. We concluded that physical interaction of the enzyme with the membrane does not perturb the membrane structure sufficiently to create even temporary pores and that the chemical oxidation and isomerization of cholesterol is required for lytic activity.

Cholesterol oxidase has long been used to deplete cellular membranes of cholesterol. This treatment is used to implicate cholesterol-rich lipid rafts or detergent-resistant membranes in a cellular process such as receptor-mediated signaling. For example, Anderson and co-workers have examined the importance of cholesterol in caveolae for platelet-derived growth factor β (PDGFβ) mediated signaling. Incubation of caveolae with wild-type cholesterol oxidase during PDGFβ treatment prevents tyrosine phosphorylation of several caveolar proteins, but not the PDGFβ receptor itself.^{18,37} Thus, in a very different experiment with cellular membranes, we arrived at the same conclusion. The membrane-perturbing effects of cholesterol oxidase require the chemical conversion of cholesterol to cholest-4-en-3-one.

What types of membranes are good substrates for cholesterol oxidase? We have found that the catalytic activity ($k_{\text{cat}}^*/K_{\text{m}}^*$, an interfacial rate constant in units of mol fraction⁻¹ s⁻¹) is dependent on the lipid phase and is even sensitive to changes in cholesterol chemical activity within one lipid phase (K. Ahn, personal communication, 2003). The most efficient turnover is observed with lipid bilayers that have the lowest affinity for cholesterol. Thus, any comparisons of substrate specificity must take into account the changes substrate structure may effect on lipid phase or detergent micelle structure. All of these results are consistent with our model that cholesterol oxidase is loosely associated with the membrane and that the physical association does not severely perturb the membrane structure.

Envoi

The observation of multiple conformations and gated oxygen tunnels suggests that the many chemical steps of cholesterol oxidases are controlled by correlated side-chain motions within the protein. Moreover, the opposite gating mechanisms of the proposed oxygen tunnels in the two types of cholesterol oxidase suggest that the timing of redox and isomerization chemistry may be very different for the two forms. Fully understanding these mechanisms, as well as redox potential modulation, will require further structural information, as well as thermodynamic

measurements, with bound substrate or substrate analogues, in combination with kinetic measurements directed at elucidating the order of events. Moreover, we have noted that the type I cholesterol oxidase crystals are bleached by the X-ray beam used during crystallographic studies. This color change most likely reflects differences in the redox state of the cofactor. We are currently utilizing this phenomenon to obtain structures of various redox forms of the enzyme at atomic resolution. Such structural studies provide us with a unique opportunity to establish changes that occur to the protein as a function of the flavin redox state and to generate a "map" of the energetics associated with specific interactions that modulate flavin reactivity.

In contrast, investigations with membrane substrates suggest that larger conformational changes occur during substrate binding. Structural studies of the enzyme in the presence of detergents, simulating a membrane-like environment may reveal specific changes that occur as the protein interacts with the membrane. We are particularly interested to know whether the loops assume a different conformation as has been observed in the structures of lipases.^{38,39} Elucidation of the role of cholesterol oxidase in microbial infection and pathogenesis will require a combination of these types of biophysical studies with genetic studies to connect our understanding of lipid metabolism with that of host demise. Thus, the future promises to reveal even more of the secrets of nature's approach to protein design.

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