Beilstein-Institut ESCEC, September 23rd – 26th, 2007, Rüdesheim/Rhein, Germany

Catalysis at the Membrane Interface: Cholesterol Oxidase as a Case Study

Nicole S. Sampson* **and Sungjong Kwak**

Department of Chemistry, Stony Brook University, Stony Brook, NY 11794 – 3400, U.S.A.

E-Mail: *nicole.sampson@stonybrook.edu

Received: 21st February 2008 / Published: 20th August 2008

Abstract

Interfacial enzymes present additional challenges in their study compared to enzymes with soluble substrates. Cholesterol oxidase is an interfacial enzyme that transiently associates with lipid membranes to convert cholesterol to cholest-4-en-3-one. As a case study to exemplify the issues that should be considered, we describe our structural and mechanistic understanding of cholesterol oxidase kinetic activity based on X-ray crystal structures and kinetic analysis.

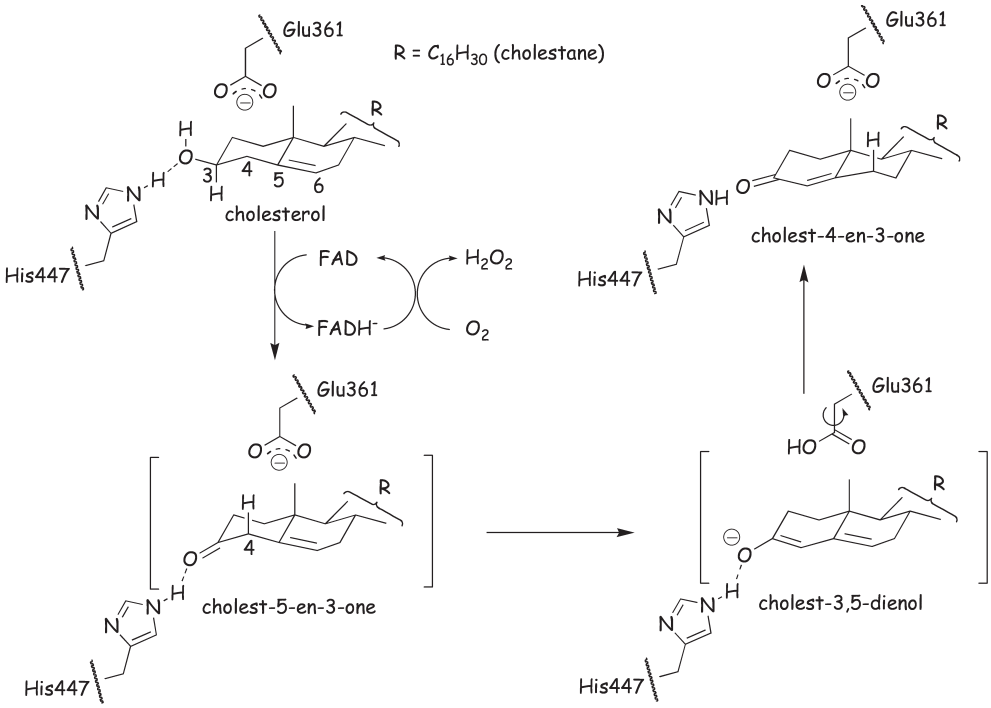
Introduction

Interfacial enzymes are water-soluble enzymes that catalyze reactions with membrane-soluble substrates. Kinetic characterization of these enzymes is made more complex by the necessity to consider the role of the interface and interactions with the interface in assessing the catalytic activity. Moreover, the interface can change during catalysis, further complicating the kinetic analysis. The interface used in assaying the enzyme influences the apparent substrate specificity measured. Thus, the assignment of a physiological role for an enzyme is dependent on the interface employed in enzymatic assays.

Cholesterol oxidase is one such water-soluble enzyme that is catalytically active at the membrane interface from which cholesterol, the substrate, is accessed. As a case study, we present work from our laboratory that characterizes what happens at the membrane interface. We delineate the kinetic issues in reporting the catalytic activity of such an enzyme.

Cholesterol Oxidase

The history of cholesterol oxidase derives from the discovery over 50 years ago that some actinomycetes can utilize cholesterol as a carbon source [8, 9]. They are believed to break down the side-chain and the ring of cholesterol to acetyl CoA and propionyl CoA through a multi-step process. The enzyme which catalyzes the first step is cholesterol oxidase. Cholesterol oxidase was isolated in bio-panning experiments when there was a search for an enzyme to use in clinical serum cholesterol assays [10 – 12].



Scheme 1: The reaction catalyzed by cholesterol oxidase. Active site residues are shown schematically.

The chemistry that is catalyzed by cholesterol oxidase occurs in one active site (Scheme 1). Cholesterol is oxidized to cholest-5-en-3-one by the flavin cofactor. The reduced cofactor is recycled by oxygen to form hydrogen peroxide. This product is the basis of the serum cholesterol assays, because hydrogen peroxide can be coupled to colorimetric assays using horseradish peroxidase. However, the cholest-5-en-3-one intermediate is not particularly stable. It is susceptible to radical oxygenation, and forms cholest-4-en-6-hydroperoxy-3-one that disproportionates to cholest-4-en-3,6-dione and cholest-4-en-6-hydroxy-3-one. Thus, the cholest-5-en-3-one is isomerized to cholest-4-en-3-one, the α,β-unsaturated ketone before being released from the enzyme [13].

The identity of general acids and bases to help catalyze the reaction may be surmised upon inspection of the active site model with a dehydroepiandrosterone bound [3]. Histidine 447 and asparagine 485 hydrogen bond to the alcohol of the substrate helping to position the steroid relative to the flavin cofactor (Scheme 1). Glutamate 361 is poised over the β-face of the steroid, to act as a base in the isomerization reaction.

Mutagenesis of glutamate 361 to glutamine turned the oxidase/isomerase into an oxidase-only enzyme [13]. The E361Q enzyme no longer isomerizes the intermediate, cholest-5-en-3-one. However, it is released from the mutant enzyme at a catalytically competent rate. The turnover of cholesterol is only 30 times slower than that of wild-type enzyme (Table).

Table. Catalytic parameters for wild-type and mutant cholesterol oxidases.

Enzyme	k_{cat} (s^{-1})	K_m^{app} (μM)[a]	Product formed	reference
Wild type	45 ± 3	3.2 ± 0.2	cholest-4-en-3-one	13
E361Q	1.4 ± 0.2	5.3 ± 1.5	cholest-5-en-3-one	13
H447E/E361Q	0.0015	n.d.[b]	n.d.	14

[a]Rates were assayed in triton X-micelles. K_m^{app} is the apparent Michaelis-Menten constant that includes a micelle binding term. [b]n.d.: not determined.

Mutation of histidine 447 in conjunction with glutamate 361 blocks oxidation as well as isomerization and provides a ‘‘dead’’ mutant that turns over cholesterol some 30,000-fold times slower than wild type [14]. The enzyme still folds correctly based on its behavior in solution [14] as well as its X-ray crystal structure (A. Vrielink, personal communication). This dead enzyme is an important tool for the study of an important aspect of catalysis by cholesterol oxidase: catalysis at the membrane interface.

In order to understand catalysis at the membrane interface, it is important to look at the three-dimensional crystal structures that have been solved by Prof Alice Vrielink and her laboratory. Several structures have been solved of wild-type and mutant enzymes. Some of the structures are at sub-Ångstrom resolution and allow hydrogen bonding within the enzyme to be visualized directly [3, 15]. However, those structures are not the focus for understanding interfacial catalysis. What is important is to examine how the steroid binds to the enzyme and to consider the changes that must occur upon binding to the membrane.

The unliganded structures reveal a deep, long pocket adjacent to the isoalloxazine ring of the flavin suitable for binding a steroid substrate. Dehydroepiandrosterone was used to obtain a substrate bound structure because the limited solubility of cholesterol precluded getting crystals in the presence of cholesterol. The steroid binds in the deep pocket as expected (Fig. 1A). The surprising observation is that the steroid is completely solvent inaccessible when bound (Fig. 1B). The protein encapsulates the A-D ring of the steroid. Dehydroepiandrosterone is of course lacking the 8-carbon tail of cholesterol.

If the larger steroid were to be bound it is not clear exactly how the protein would accommodate the steroid. What is proposed from inspection of the structure is that one or more loops of the protein must open at the membrane surface to allow sterol exit from the membrane and entry into the enzyme (Fig. 1C). The 8-carbon isoprenyl tail of cholesterol would pack with the loops and prevent them closing completely. The amphipathic nature of the loops would allow them to pack with the hydrophobic sterol on their inside face, and more polar headgroups of the lipid bilayer on their outside face. Our model of how the enzyme works is that it sits on the surface of the membrane and the loops provide a hydrophobic pathway for the substrate to partition from the membrane into the active site of the enzyme.

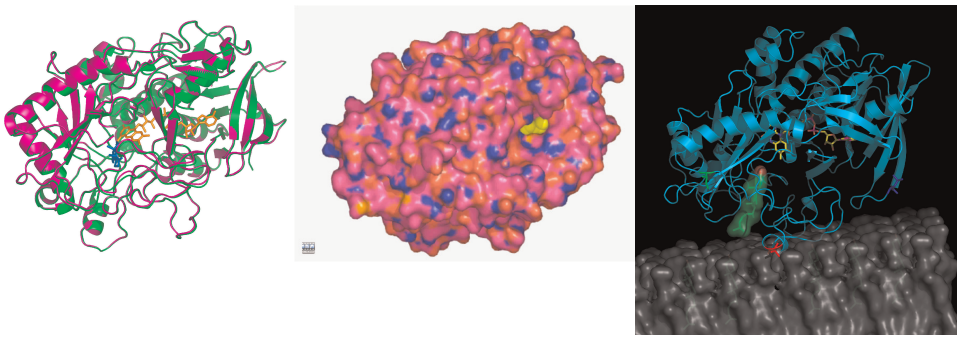


Figure 1. Cholesterol oxidase structure. (A) Ribbon diagram with steroid bound in active site (green) overlaid with unbound structure (magenta) [1 – 3]. (B) Solvent accessible surface on steroid-bound structure shown in A. Residues are colored by polarity: red, acidic; blue, basic; magenta, all other residues; yellow, flavin. (C) Model for how enzyme binds to the membrane interface. The loops that cover the active site have been modeled into an open conformation. The coordinates for the bilayer were obtained from Heller *et al.* [7].

We asked the question whether the formation of an enzyme-membrane complex results in perturbation of the membrane. This question was inspired by the Monsanto discovery that cholesterol oxidase is the biologically active component of bacterial fermentation broths that lyses boll weevil larval gut endothelial cells [16].

Addition of 10 µg/mL cholesterol oxidase to the larval feed results in disruption of the endothelial cell membranes. Using the active site mutants described above and vesicles that have a self-quenching dye encapsulated, we determined that leakage of vesicle contents only occurs if the cholesterol in the membrane is converted to cholest-4-en-3-one. That is, the chemical changes in the membrane catalyzed by cholesterol oxidase cause membrane structural changes rather than the physical interaction of the enzyme with the membrane. Our observation is consistent with what is known about cholesterol and the fluid phases of membranes. Cholesterol mixed with liquid-disordered phase phospholipids promotes order-

ing of the membrane to form a liquid-ordered phase. In contrast, mixing of cholest-4-en-3-one with liquid phase phospholipids maintains the liquid-disordered state [17]. This order-disorder effect occurs in both model membranes and in cell membranes.

Kinetics at the Membrane Interface

We used model membranes to establish how sensitive cholesterol oxidase activity is to membrane structure and lipid phase. We asked the question what are the relative catalytic activities with different membranes. Ultimately, the answer to this question is important for understanding the identity of the physiological substrate.

To address these questions, we used the binary phase diagram of dipalmitoylphosphatidylcholine (DPPC) and cholesterol as a starting point (Fig. 2) [4]. In the case of DPPC, the melting temperature for the gel (solid) phase to liquid-disordered phase transition is 41 °C in the absence of cholesterol. Above 30 mol% cholesterol, the phase transition is lost and the lipid phase is liquid-ordered above and below the DPPC melting temperature. In between 5 and 30 mol% cholesterol the gel phase is in coexistence with the liquid-ordered phase below the T_m, and the liquid-ordered and liquid-disordered phase coexist above the T_m.

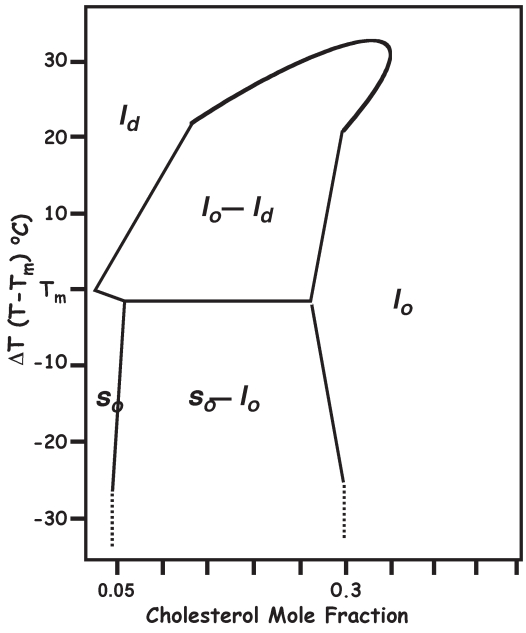
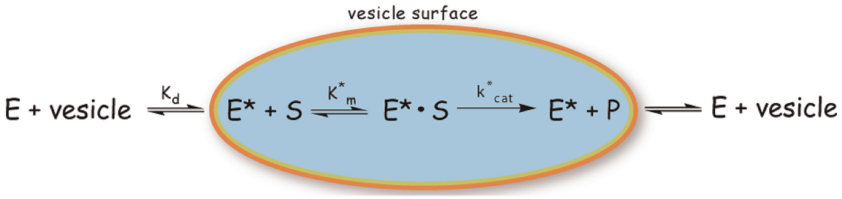


Figure 2. Binary phase diagram of DPPC:cholesterol adapted from Sankaram and Thompson [4]. The phase transition between s_o and l_d corresponds to the T_m of a lipid. For DPPC, the s_o to s_o-l_o transition is at 5 mol% cholesterol, and the s_o-l_o to l_o transition at 30 mol% cholesterol.

How does one measure the kinetics for an interfacial enzyme? Remember that the enzyme is soluble, but the substrate is a component of the membrane. The first step that must occur is association of the enzyme with the membrane surface (Scheme 2). The alternative is for the enzyme to wait for the substrate to dissociate from the membrane and then to bind the substrate from solution. The rate of cholesterol desorption has been measured for many different types of lipid bilayers. This rate is approximately 10^5 times slower than the turnover rate of the enzyme. Therefore, we conclude from a kinetic argument, that the enzyme must associate with the membrane in order for catalysis to occur. Moreover, measuring the change in intrinsic tryptophan fluorescence can follow the binding of the enzyme to the membrane surface [5]. Use of the catalytically inactive mutant H447E/E361Q enables binding to a substrate-containing vesicle to be measured [14]. Cholesterol oxidase binding to liquid-phase membranes shows little dependence on lipid composition [5, 18].



Scheme 2: Paradigm for determining interfacial steady-state rate constants. E: free enzyme; E*: membrane-bound enzyme; S: substrate in the membrane; P: product in the membrane; k_{cat}*: interfacial first-order rate constant; K_m*: interfacial Michaelis constant in units of mole fraction.

The next step is to measure the interfacial Michaelis-Menten constants. One common way of doing this is to measure the entire reaction progress curve and fit the integrated Michaelis-Menten equation. Recall that cholesterol orders the membrane and cholest-4-en-3-one disorders the membrane. Therefore, as you form more and more product, the structure of the membrane changes, and in fact the initial velocity of the catalyzed-reaction gets faster despite the decrease in mole fraction of substrate [5]. The net consequence is that we have to use initial velocities in order that we measure the rate corresponding to the initial, known, structure of the membrane.

The initial velocity measured depends on the fraction of enzyme that is actually bound to the vesicle surface. There are two substrate variables; the total concentration of lipid added which is proportional to number of vesicles, and the mole fraction of cholesterol in the vesicles. Increasing the concentration of vesicles pushes the first equilibrium to the right until all enzyme is bound to the vesicle surface. The initial velocity when all enzyme is bound (V_i^{sat}) is measured for a series of unilamellar vesicles of fixed size (prepared by extrusion) with varying mole fractions of cholesterol. The V_i^{sat} is then plotted versus mole

fraction of cholesterol and fit to the Michaelis-Menten equation in units of mole fraction. The rate dependence on mole fraction of cholesterol is expected to be hyperbolic, like an "ordinary" enzyme, as long as the substrate phase is not changing with mole fraction.

We first measured the initial velocities for cholesterol mixed with dioleoylphosphatidylcholine (DOPC), a lipid that is in the liquid-disordered phase regardless of mole fraction cholesterol. However, a hyperbolic dependence on cholesterol mole fraction was not observed (Fig. 3). That is, within the range of experimentally achievable mole fractions of cholesterol, the enzyme could not be saturated with substrate. The mole fraction dependence of V_i^{sat} was essentially linear. However, at 30 mol% cholesterol, the linear dependence of the rate has a discontinuity and above 30 mol% the slope is doubled. This increase in enzymatic activity above 30 mol% is consistent with the increase in chemical activity or potential that is observed in model membranes [19].

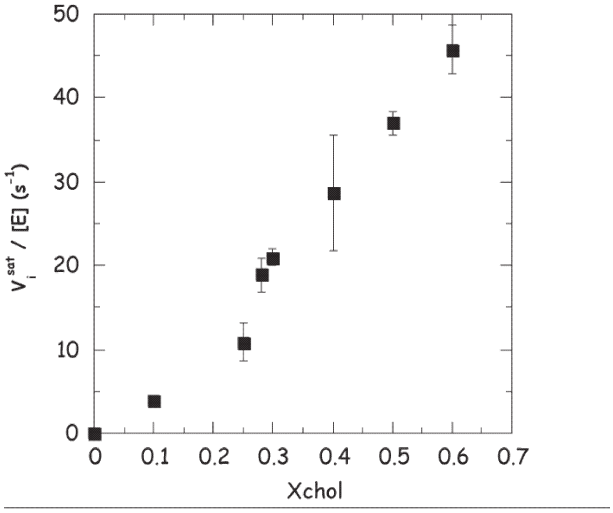


Figure 3. Detection of liquid-disordered cholesterol using cholesterol oxidase in a single phase vesicle composed of DOPC and varying mole fractions of cholesterol. The V_i^{sat} is the initial velocity of cholesterol oxidase turnover when all the enzyme is bound. The initial velocities were measured using 100 nm unilamellar vesicles at 31 °C. The error bars are standard deviations of three independent measurements. Adapted from Ahn and Sampson with a correction for concentration of active enzyme [5].

A similar lack of saturation was observed for vesicles composed of cholesterol mixed with dipalmitoylphosphatidylcholine (DPPC). Thus, we can only report k_{cat}^*/K_m^*. At 30 mol% cholesterol in the DPPC/cholesterol vesicles, the phase transitions to liquid-ordered. If we compare the k_{cat}^*/K_m^* for liquid-disordered membranes to liquid-ordered membranes in the same chemical potential regime, we observed that k_{cat}^*/K_m^* is two-fold slower with liquid-ordered membranes. However, if the DPPC is replaced with sphingomyelin (SM), the rate

drops more than 40-fold. Although DPPC/cholesterol is considered to be in the same phase as SM/cholesterol, the enzyme is sensitive to the precise packing of cholesterol with the lipids in the membrane. We surmise that the free energy of cholesterol in a sphingomyelin membrane is lower than in a DPPC membrane. It should be noted that cholesterol is predominantly localized with sphingomyelin in a cell membrane, and cholesterol in this environment is the worst substrate for the enzyme so far studied. Thus, the enzyme is specific for cholesterol that is in a low abundance form: liquid-disordered and high chemical potential.

In biphasic vesicles prepared with DOPC, SM and cholesterol, this rate difference elucidates the partitioning of cholesterol between the two phases (Fig. 4). At mole fractions less than 40 – 45% cholesterol, the phase is a liquid-ordered/disordered coexistence region. Above 40 – 45% cholesterol, the phase is liquid disordered [6]. We observed very little enzymatic activity below 45 mol% cholesterol, consistent with cholesterol portioning preferentially into the liquid-ordered phase. As the mole fraction increases, a steep increase in catalytic activity is observed. This increase correlates with the phase change to liquid-disordered. The shape of the activity dependence on mole fraction cholesterol indicates that despite the coexistence of liquid-ordered and liquid-disordered phases, the cholesterol resides primarily in the liquid-ordered region.

This experiment demonstrates the utility of cholesterol oxidase for this type of measurement. Many methods rely on microscopic observation of membranes that does not reveal which molecules are in which phase, or fluorescence spectroscopy that introduces reporter molecules. Cholesterol oxidase directly reports on the partitioning of cholesterol. However, it is not straightforward to quantitate precisely how much cholesterol is in the liquid-disordered region of coexisting phases at low mole fractions.

Substrate Specificity

What is substrate specificity for an interfacial enzyme? If one looks at the original work of Uwajima, who first isolated and characterized the *Brevibacterium sterolicum* (now *Rhodococcus equi*) cholesterol oxidase, the initial rate using cholesterol as a substrate is 10-fold higher than for sitosterol or stigmasterol, plant sterols [20, 21]. Consequently, the enzyme was named a cholesterol oxidase. However, these assays were run in triton X-100 detergent micelles. When we measured k_{cat}^{*}/K_{m}^{*} in liquid-disordered vesicles with 25 mol% cholesterol/palmitoyl, oleoylphosphatidylcholine, the specificity constant is the same for all three steroids [22]. In model membranes, cholesterol oxidase is not more specific for animal sterols over plant sterols.

In general, we know that detergent micelles are not physiologically relevant. Relative activities measured in micellar systems may not reflect relative substrate specificities under cellular conditions. The difficulty is identifying the correct physical state of potential sub-

strates to be utilized in assays of substrate specificity. If the activity of cholesterol oxidase had first been measured in vesicles of cholesterol and sphingomyelin, the predominant form of cellular cholesterol, turnover may never have been detected!

Conclusion

In comparison to characterizing enzymes with water-soluble substrates, interfacial enzymes require consideration of additional parameters. Cholesterol oxidase is just one example of an interfacial enzyme. Other classes are the well-known and studied phospholipases and lipases, as well as lipid-transforming enzymes and steroid biosynthetic enzymes. Characterization of integral membrane proteins also requires similar considerations.

The kinetics of an interfacial enzyme can be reliably determined if a measurement of membrane binding affinity (e.g., K_d for a standard state of model membrane) is included in the reported rate parameters. One way to access this binding parameter is use of a catalytically inactive mutant that still folds and associates with the membrane.

Rate constants must be reported in units of mole fraction as the use of bulk concentrations does not describe what the enzyme encounters at the membrane interface.

The structure and components of the membrane containing substrate can alter the kinetic activity, as well as the binding constant for the membrane surface. Moreover, the physical state of the substrate can be very temperature sensitive.

The kinetic characterization of interfacial enzymes is complicated by the uncertainties of knowing the physical state of the substrate that is relevant under physiological or *in vivo* conditions. In the case of integral membrane proteins, identification of the substrate structure may be simplified by identifying the location of the enzyme.

Acknowledgments

This work was supported by the National Institutes of Health (HL 53306, N.S.S) and the American Heart Association (0725861T, S.K.).

References

[1] Vrielink, A., Lloyd, L.F., Blow, D.M. (1991) Crystal structure of cholesterol oxidase from *Brevibacterium sterolicum* refinded at 1.8 Å resolution. *J. Mol. Biol*. **219**:533 – 554.

[2] Li, J., Vrielink, A., Brick, P., Blow, D.M. (1993) Crystal structure of cholesterol oxidase complexed with a steroid substrate: implication for flavin adenine dinucleotide dependent alcohol oxidases. *Biochemistry* **32**:11507 – 11515.

[3] Lario, P., Sampson, N.S., Vrielink, A. (2003) Sub-atomic resolution crystal structure of cholesterol oxidase: What atomic resolution crystallography reveals about enzyme mechanism and the role of the FAD cofactor in redox activity. *J. Mol. Biol*. **326**:1635 – 1650.

[4] Sankaram, M.B., Thompson, T.E. (1991) Cholesterol-induced fluid-phase immiscibility in membranes. *Proc. Natl. Acad. Sci. U.S.A*. **88**:8686 – 8690.

[5] Ahn, K.W., Sampson, N.S. (2004) Cholesterol oxidase senses subtle changes in lipid bilayer structure. *Biochemistry* **43**:827 – 836.

[6] Veatch, S.L., Keller, S.L. (2005) Miscibility phase diagrams of giant vesicles containing sphingomyelin. *Phys. Rev. Lett*. **94**:148101.

[7] Heller, H., Schaefer, M., Schulten, K. (1993) Molecular-Dynamics Simulation of a Bilayer of 200 Lipids in the Gel and in the Liquid-Crystal Phases. *J. Phys. Chem. B*. **97**:8343 – 8360.

[8] Tak, J. (1942) On bacteria decomposing cholesterol. *Antonie Leeuwenhoek* **8**:32 – 40.

[9] Turfitt, G.E. (1944) The microbiological degradation of steroids. 2. Oxidation of cholesterol by *Proactinomyces* spp. *Biochem. J*. **38**:492 – 496.

[10] Richmond, W. (1973) Preparation and properties of a cholesterol oxidase from *Nocardia* sp. and its application to the enzyme assays of total cholesterol in serum. *Clin. Chem*. **19**:1350 – 1356.

[11] Fukuda, H., Kawakami, Y., Nakamura, S. (1973) A method to screen anticholesterol substances produced by microbes and a new cholesterol oxidase produced by *Streptomyces violascens*. *Chem. Pharm- Bull. (Tokyo)* **21**:2057 – 2060.

[12] Smith, A.G., Brooks, C.J.W. (1974) Application of cholesterol oxidase in the analysis of steroids. *J. Chromat.* **101**:373 – 378.

[13] Sampson, N.S., Kass, I.J. (1997) Isomerization, but not oxidation, is suppressed by a single point mutation, E361Q, in the reaction catalyzed by cholesterol oxidase. *J. Am. Chem. Soc*. **119**-855 – 862.

[14] Ye, Y., Liu, P., Anderson, R.G.W., Sampson, N.S. (2002) Construction of a catalytically inactive cholesterol oxidase mutant: investigation of the interplay between active site residues glutamate 361 and histidine 447. *Arch. Biochem. Biophys*. **402**:235 – 242.

[15] Sampson, N.S., Vrielink, A. (2003) Cholesterol oxidase: A study of nature's approach to protein design. *Acc. Chem. Res*. **36**:713 – 722.

[16] 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. (1993) Cholesterol oxidase: a potent insecticidal protein active aganst boll weevil larvae. *Biochem. Biophys. Res. Commun*. **196**:1406 – 1413.

[17] Xu, X., London, E. (2000) The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry* **39**:843 – 849.

[18] Chen, X., Wolfgang, D., Sampson, N.S. (2000) Use of the parallax-quench method to determine the position of the active-site loop of cholesterol oxidase in lipid bilayers. *Biochemistry* **39**:13383 – 13389.

[19] Radhakrishnan, A., McConnell, H.M. (2000) Chemical activity of cholesterol in membranes. *Biochemistry* **39**:8114 – 8124.

[20] Uwajima, T., Yagi, H., Nakamura, S., Terada, O. (1973) Isolation and crystallization of extracellular 3β-hydroxysteroid oxidase of *Brevibacterium sterolicum* nov. sp. *Agr. Biol. Chem*. **37**:2345 – 2350.

[21] Xiang, J., Sampson, N.S. (2004) Library screening studies to investigate substrate specificity in the reaction catalyzed by cholesterol oxidase. *Prot. Eng. Design & Select*. **17**:341 – 348.