Investigation of Membrane Disruption in the Reaction Catalyzed by Cholesterol Oxidase†

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ABSTRACT: Dye leakage experiments were undertaken to investigate the membrane disruption properties of cholesterol oxidase. Inspection of the X-ray crystal structures of cholesterol oxidase suggested that an active-site "lid" opens in order to bind substrate [Li, J., Vrielink, A., Brick, P., & Blow, D. M. (1993) *Biochemistry 32*, 11507−11515]. We tested whether the interaction of the putative active-site lid with the membrane was sufficiently disruptive of the membrane structure to cause leakage or lysis of the cell membrane. Vesicles (100 nm) composed of egg phosphatidylcholine, 2-palmitoyl-3-oleoyl-1-*sn*-phosphatidylethanolamine, and 2-palmitoyl-3-oleoyl-1-*sn*-phosphatidylcholine were used in this study to mimic biomembranes. To separate the effects of membrane binding from conversion of cholesterol to cholest-4-en-3-one, the active-site mutant E361Q was utilized. In the reaction catalyzed by E361Q, isomerization of the cholest-5-en-3-one intermediate is suppressed and cholest-5-en-3-one is the major product isolated. Furthermore, E361Q produces cholest-5-en-3-one 20-fold more slowly than wild type produces cholest-4-en-3-one from cholesterol. Wild-type and E361Q cholesterol oxidases bind to vesicles with an apparent K_D of approximately 25 μM, as measured by quenching of intrinsic tryptophan fluorescence, irrespective of headgroup size and cholesterol content. Membrane disruption was measured by leakage of the encapsulated marker carboxyfluorescein. Leakage was observed with cholesterol-containing vesicles and wild-type enzyme only; the rate of leakage was dependent on the rate of cholest-4-en-3-one production. E361Q did not induce membrane disruption, regardless of vesicle type tested. Thus, binding of cholesterol oxidase to the membrane and partitioning of cholesterol into the active site does not sufficiently perturb the bilayer to cause leakage of vesicle contents. Formation of the product cholest-4-en-3-one, however, does increase membrane permeability. Expansion of the lipid bilayer upon conversion of cholesterol to cholest-4-en-3-one is the likely cause of this increased permeability.

We report here the results of experiments conducted to determine the degree of membrane disruption caused by cholesterol oxidase (EC 1.1.3.6). Cholesterol oxidase catalyzes the oxidation and isomerization of cholesterol to form cholest-4-en-3-one (Figure 1). This enzyme is part of a bacterial metabolic pathway for utilizing cholesterol as its carbon source and is secreted by Gram-positive soil bacteria, including *Brevibacterium sterolicum* (ATCC 81387) and *Streptomyces* sp. strain SA-COO. [*B. sterolicum* choB and *Streptomyces* choA are 58% and 64% identical in amino acid sequence and nucleotide sequence, respectively (Ohta et al., 1991)]. The enzyme is used to assay serum cholesterol levels, and recently, its larvicidal properties were discovered (Greenplate et al., 1995; Purcell et al., 1993).

The rate of cholesterol oxidation catalyzed by cholesterol oxidase is dependent on the concentration of enzyme and is orders of magnitude faster than the rate of sterol desorption from the membrane (Bar et al., 1989; Lund-Katz et al., 1988; Phillips et al., 1987). Thus, from a kinetic argument, cholesterol oxidase must interact with the lipid bilayer in order to bind its substrate. Furthermore, examination of the X-ray crystal structure of cholesterol oxidase solved by Blow and co-workers (Li et al., 1993; Vrielink et al., 1991) reveals that the enzyme must undergo a conformational change involving 10−20 amino acid residues in order to bind substrate. Although the conformation of the open enzyme is not known, it is postulated that an active-site lid, composed of some of these 20 residues, opens to form a hydrophobic channel between the membrane and the active site in order to bind sterol isoergonically. Upon binding, the four rings of the sterol are completely buried in the active site. As part of our ongoing investigation to determine the conformation of the open enzyme and the nature of its interactions with substrate and with lipid bilayer, we wanted to probe membrane disruption by cholesterol oxidase. We tested whether the interaction of the putative active-site lid with the membrane was sufficiently disruptive of the membrane structure to cause leakage or lysis of the cell membrane. These experiments are particularly relevant in light of the recent results of Greenplate et al. (1995) demonstrating that cholesterol oxidase causes complete lysis of mid-gut epithelial cells in boll weevil (*Anthonomus grandis grandis* Boheman) larvae.

In order to assess membrane leakage, we required a method to measure the affinity of the enzyme for the membrane surface. We utilized the change in intrinsic tryptophan fluorescence of cholesterol oxidase that occurs upon interaction with phospholipid vesicles. This assay allowed us to measure apparent K_Ds of wild-type and mutant

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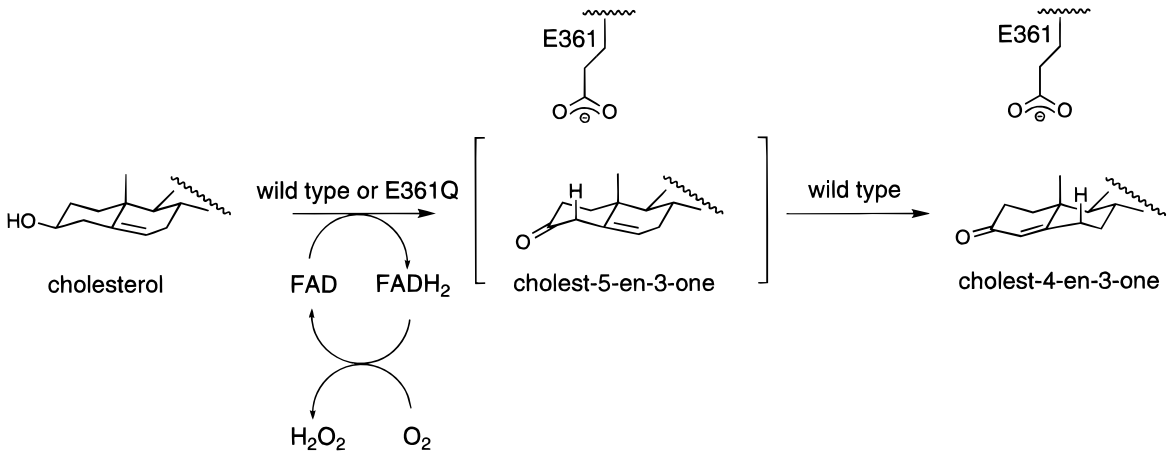

FIGURE 1: Reaction catalyzed by cholesterol oxidase. Glutamate 361 is the general base that catalyzes isomerization of the intermediate into product. The product of the wild-type reaction is cholest-4-en-3-one. The product of the E361Q mutant reaction is cholest-5-en-3-one.

cholesterol oxidases regardless of their kinetic properties.

We measured membrane permeability by monitoring release of encapsulated carboxyfluorescein from 100 nm unilamellar vesicles. Potentially, both cholesterol oxidation and cholesterol oxidase binding to the vesicle could perturb membrane permeability. We separated these effects on permeability by utilizing an enzyme with the active-site mutation E361Q.[1] This mutation suppresses isomerization of cholest-5-en-3-one, the reaction intermediate, to cholest-4-en-3-one (Figure 1). Furthermore, conversion of cholesterol by wild type is 20-fold faster than conversion by E361Q (Sampson & Kass, 1997). We report here the results of cholesterol oxidase binding to ePC,[2] POPC, and POPE/POPC vesicles with varying amounts of cholesterol. In order to assess the implications for insecticides, vesicles composed of the lipids predominantly found in the order Coleoptera, of which *A. grandis* is a member, were studied. The microvillar membranes of Coleoptera are predominantly phospholipids (Jordao et al., 1995). POPC and POPE were chosen because they are the predominant lipids present in *A. grandis* (Lambremont & Blum, 1963; Lambremont et al., 1964, 1965; Stanley-Samuelson et al., 1988).

EXPERIMENTAL PROCEDURES

Materials. Cholest-4-en-3-one and cholesterol were purchased from Sigma Chemical Co., St. Louis, MO. POPE, ePC and POPC were purchased from Avanti Polar Lipids, Alabaster, AL. CF (mixture of 5- and 6- isomers) was obtained from Molecular Probes, Eugene, OR. Sephadex G-75 was from Pharmacia-LKB, Uppsala, Sweden. The plasmid for heterologous expression of *Streptomyces* cholesterol oxidase, pCO117, was a generous gift from Y. Murooka (Nomura et al., 1995). Unless otherwise specified, all chemicals and solvents, of reagent or HPLC grade, were supplied by Fisher Scientific, Pittsburgh, PA. 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 and a Perkin-Elmer LS-5B fluorometer were used for assays. Vesicles were prepared with an extruder from Lipex Biomembranes Inc., Vancouver, BC, Canada.

Purification of Cholesterol Oxidase. Cell paste of *Escherichia coli* BL21(DE3) plysS(pCO117) obtained from LB-ampicillin (200 μg/mL) medium grown for 12 h after addition of IPTG (0.2 mM) at mid-log phase was resuspended in 50 mM Tris·HCl and 1 mM EDTA, pH 7, and lysed by French press at 18 000 psi. All subsequent purification steps were performed at 4 °C. Cell debris was removed by centrifugation at 135000*g* for 30 min. The supernatant was loaded onto a column of DEAE-cellulose (DE-52, Whatman) preequilibrated with 50 mM sodium phosphate, pH 7.0, and eluted with the same buffer. Fractions containing cholesterol oxidase were concentrated by $(NH_4)_2SO_4$ precipitation, the pellet was redissolved in 50 mM sodium phosphate, pH 7.0, 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 50 mM sodium phosphate and 1.5 M $(NH_4)_2SO_4$, pH 7.0, and eluted with a linear gradient [1.5−0 M $(NH_4)_2SO_4$] in the same buffer. Fractions were collected and analyzed by SDS−PAGE. Fractions containing pure oxidase were pooled, concentrated, and desalted by ultrafiltration into 50 mM sodium phosphate, pH 7.0 buffer. Typically, 20−30 mg of pure cholesterol oxidase were 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 (Fasman, 1992)].

Preparation of E361Q Mutant (Sampson & Kass, 1997). The *Streptomyces* cholesterol oxidase gene was subcloned (Sambrook et al., 1989) from pCO117 into M13mp18 phage and the E361Q mutant gene was constructed using the method of Eckstein (Nakamaye & Eckstein, 1986; Sayers et al., 1988). To change glutamate 361 to glutamine, the mutagenic primer 5′-ggggCgAT**CTg**CgCgAAgAC-3′ was used. The mutant gene was subcloned into pKK223-3 to generate the expression plasmid containing mutant cholesterol oxidase, pCO219. The mutation was verified by dideoxy chain termination sequencing (Sanger et al., 1977) of the pCO219 construct. The E361Q mutant protein was purified as described above for wild type to yield ap-

[1] The residue numbering corresponds to the convention established in the X-ray crystal structure (Vrielink et al., 1991).

[2] Abbreviations: ePC, egg phosphatidylcholine; POPE, 2-palmitoyl-3-oleoyl-1-*sn*-1-phosphatidylethanolamine; POPC, 2-palmitoyl-3-oleoyl-1-*sn*-phosphatidylcholine; DMPC, 2,3-dimyristoyl-1-*sn*-phosphatidylcholine; CF, mixture of 5- and 6-carboxyfluorescein; LB, Luria broth; IPTG, isopropyl β−D-thiogalactoside; Tris, tris(hydroxymethyl)aminomethane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; TEA, triethanolamine; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography.

proximately 20−30 mg of mutant cholesterol oxidase/L of culture that was a single band as judged by SDS−PAGE.

Synthesis of Lipid Vesicles. Medium, 100 nm unilamellar vesicles were made from mixtures of steroids and lipids by extrusion (Hope et al., 1985). Lipid (20 nmol) was mixed with varying amounts of steroid as a $CHCl_3$ solution in a round-bottomed flask, dried as a thin film under reduced pressure in a rotary evaporator for 20 min, and evacuated under high vacuum for 2 h. Thin films of cholest-5-en-3-one and lipid were prepared in propan-2-ol because cholest-5-en-3-one rapidly decomposes in $CHCl_3$ solutions. The lipid was resuspended in 4 mL of assay buffer (50 mM sodium phosphate, pH 7.0) with vortexing. Five freeze−thaw cycles, at −80 and 37 °C, followed by 10 extrusion cycles through two stacked 100 nm filters (Costar) using a nitrogen gas pressure of 350−400 psi, provided a homogeneous batch of vesicles. Phospholipid concentrations of vesicle solutions were measured using the Stewart assay (Stewart, 1959). Cholesterol concentrations were measured by lysing vesicles with 0.1% Triton X-100 and using cholesterol oxidase to quantitate total cholesterol concentration as described below.

Synthesis of CF-Encapsulated Lipid Vesicles. CF-encapsulated vesicles were prepared as described above for lipid vesicles, except that after preparation of the thin film, the lipid was resuspended in 2 mL of CF solution (50 mM CF and 100 mM TEA·HCl, pH 7.0) with vortexing. Five freeze−thaw cycles, at −80 and 37 °C, followed by 10 extrusion cycles through two stacked 100 nm filters (Costar) using a nitrogen gas pressure of 350−400 psi, provided a homogeneous batch of vesicles. Nonencapsulated CF was removed by size-exclusion chromatography with Sephadex G-75. Typically, columns were run in 10 mL syringes by spinning at 470*g* in a swinging-bucket rotor for 3 min. The vesicles were eluted with a solution of 100 mM TEA·HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.0.

Activity Assay of Cholesterol Oxidase. The activity of wild-type cholesterol oxidase was measured by following the appearance of cholest-4-en-3-one at 240 nm [ϵ_{240} = 12 100 M^{-1} cm^{-1} (Smith & Brooks, 1977)]. The standard assay conditions were in 50 mM sodium phosphate buffer, pH 7.0, at 37 °C. Cholesterol was added as phospholipid vesicles or as a propan-2-ol solution, depending on the experiment. The final assay mixture was never more than 1.6% propan-2-ol. The activity of the mutant E361Q oxidase was determined using a horseradish peroxidase coupled assay to quantitate the rate of formation of H_2O_2, and thus indirectly, cholest-5-en-3-one. The formation of quinonimine at 510 nm was followed as a function of time. The standard assay conditions were the same as the UV cholest-4-en-3-one assay with the addition of 1.13 mM phenol, 0.87 mM 4-aminoantipyrine (Aldrich, Milwaukee, WI), and 10 units of horseradish peroxidase (Sigma, St. Louis, MO).

Fluorescence Binding Measurements. Binding of cholesterol oxidase to vesicles was assayed through the quenching of intrinsic tryptophan fluorescence. All binding assays were conducted in 50 mM sodium phosphate buffer, pH 7.05, ambient temperature, using 1 cm quartz cuvettes. Tryptophan was excited at 280 nm and emission measured at 333 nm, with slit widths of 10 nm. Protein (10 μg/mL) was titrated with increasing amounts of lipid vesicles (0−100 μL of 3.3 mM) to a final concentration of 330 μM. Titrations with wild-type oxidase were performed rapidly in order to measure binding to substrate vesicles. A standard curve for light scattering was determined by performing a titration in the absence of protein. This data was used to linearly correct the fluorescence titration data. Corrected relative fluorescence was plotted versus increasing lipid concentration, and apparent K_Ds were calculated using KaleidaGraph software (Synergy Software, Reading, PA) by fitting data to (Fersht, 1985)

$$\Delta F = (F_{max}[L])/([L] + K_D) \quad (1)$$

where [L] is lipid concentration, K_D is the apparent dissociation constant, ΔF is the change in fluorescence intensity, and F_{max} is the maximum change in fluorescence intensity.

Carboxyfluorescein Leakage Assays. Leakage assays were conducted at 37 °C in 100 mM TEA·HCl buffer and 100 mM NaCl, pH 7.0, to maintain isotonicity of the vesicles. Leakage of CF was monitored as a function of time with excitation at 430 nm and emission at 535 nm and slit widths of 3 nm. In a typical experiment, 100 μM phospholipid and 10 μg/mL of cholesterol oxidase were used. To verify the concentration of encapsulated CF, complete release was achieved by adding 0.1% Triton X-100 to lyse the vesicles. Fluorescence imaging microscopy (Jovin & Arndt-Jovin, 1989) of CF-encapsulated vesicles was used to determine homogeneity and stability over 24 h at 4 °C. CF-loaded vesicle images were acquired and analyzed using MetaMorph v2.0 software (Universal Imaging Corp., Westchester, PA) and ODYSSEY control software on an Odyssey laser scanning confocal microscope (Noran Instruments, Middleton, WI). CF was excited with the 488 nm line of an argon ion laser and the emission was spectrally separated using a 510 nm long-pass barrier filter in front of the photomultiplier detector. The average diameter of the vesicles was calculated to be approximately 100 nm.

HPLC Analysis. Samples were analyzed as previously described (Sampson & Kass, 1997) 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 Corp., 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. Isocratic elution (25 min) 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 was used to separate the steroids. Samples were injected directly from assay solutions. The identity of the peaks was established by coinjection with authentic standards. Product ratios were determined by integration of peak areas as detected at 212 and 240 nm.

RESULTS

Binding of Cholesterol Oxidase to Lipid Bilayer. We measured the affinity of cholesterol oxidase for the lipid bilayer using quenching of the intrinsic tryptophan fluorescence of the protein as a reporter signal. After application of a linear correction for vesicle light scattering, the data were fit to a hyperbolic isotherm. These results are summarized in Table 1.

We tested the activity of cholesterol oxidase with ePC/cholesterol 1:1 as substrate using ePC vesicles as inhibitor. Upon addition of increasing concentrations of ePC vesicles,

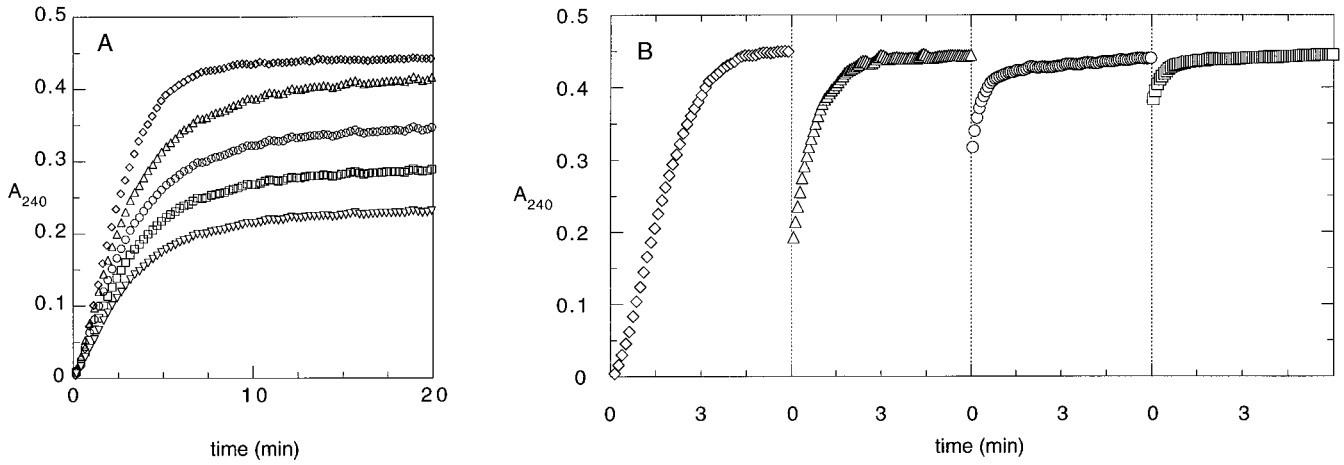

FIGURE 2: Reaction progress curves observed for the turnover of substrate in ePC/cholesterol vesicles. The same assay conditions as described in Experimental Procedures were used. (A) In the presence of 1.29 μg/mL wild-type cholesterol oxidase and 32 μM ePC/cholesterol 1:1 vesicles, the turnover was inhibited by adding different amounts of ePC vesicles: [ePC] = 0 μM, ◇; [ePC] = 16 μM, △; [ePC] = 32 μM, ○; [ePC] = 64 μM, □; [ePC] = 128 μM, ▽. (B) Turnover of 1.29 μg/mL wild-type cholesterol oxidase and 32 μM ePC/cholesterol/cholest-4-en-3-one vesicles with varying mole fractions: 1:1:0, ◇; 1:0.75:0.25, △; 1:0.5:0.5, ○; 1:0.25:0.75, □.

Table 1: Apparent K_Ds of Cholesterol Oxidase for Vesicle Surface[a]

lipid	wild type (μM)	E361Q (μM)
ePC	20 ± 9	26 ± 3
ePC/cholesterol 1:1	36 ± 6	26 ± 3
ePC/cholest-4-en-3-one 1:1	19 ± 2	25 ± 6
POPC	20 ± 6	19 ± 6
POPC/cholesterol 1:1	22 ± 4	41 ± 4
POPC/POPE 1:1	25 ± 12	46 ± 7
POPC/POPE/cholesterol 1:1:2	36 ± 4	290 ± 39

[a] K_Ds are represented as means ± standard errors, $n = 2-4$. See Experimental Procedures for preparation of vesicles, assays conditions, and curve-fitting method.

the catalytic reaction was inhibited, indicating that cholesterol oxidase binds to non-sterol containing vesicles (Figure 2A). Relative initial velocities were plotted versus inhibitor concentration and fit to eq 2 to obtain the apparent K_D and K_i for ePC:cholesterol (1:1) and ePC.

$$v_i/v_{max} = [L]K_i/\{K_i[L] + K_D[I] + K_iK_D\} \qquad (2)$$

where [L] is the lipid concentration of ePC/cholesterol 1:1 vesicles, [I] is the lipid concentration of ePC vesicles, K_D is the apparent dissociation constant for ePC/cholesterol 1:1 vesicles and cholesterol oxidase, K_i is the apparent dissociation constant for ePC vesicles and cholesterol oxidase, v_i is the initial velocity of product formation, and v_{max} is the maximum initial velocity of product formation. K_i and K_D were determined to be 43 ± 9 μM and 39 ± 3 μM, respectively.

Separation of Binding Effects from Catalytic Effects. We utilized the active-site mutant E361Q to separate the effects of binding from the effects of product formation. In the reaction catalyzed by E361Q, the isomerization of the cholest-5-en-3-one intermediate is suppressed and cholest-5-en-3-one is the major product isolated (Figure 1). E361Q converts cholesterol to cholest-5-en-3-one 20-fold more slowly than wild type converts cholesterol to cholest-4-en-3-one (Sampson & Kass, 1997). This active-site mutant, however, still binds to the vesicle membrane with the same affinity as wild-type with the exception of POPC:POPE:cholesterol vesicles (Table 1). Thus, with the mutant enzyme, we could study the effects of binding to a phospholipid/cholesterol vesicle during the initial phase of the reaction. As the reaction continued, we could observe the effects of binding to a phospholipid vesicle containing increasing amounts of cholest-5-en-3-one and decreasing amounts of cholesterol.

Measurement of Catalytic Activity. Comparisons of wild-type and E361Q activity were made by measuring the rate of H_2O_2 formation using the horseradish peroxidase coupled assay. In the wild-type reaction, this rate corresponds to the rate of cholest-4-en-3-one formation that was measured independently by following the appearance of cholest-4-en-3-one at 240 nm. In the mutant E361Q reaction, the rate of H_2O_2 formation corresponds to the rate of cholest-5-en-3-one formation; cholest-4-en-3-one formation is suppressed by the mutation (Sampson & Kass, 1997). The identity of the reaction products was confirmed by HPLC. Typically, assays were performed in 50 mM sodium phosphate buffer, pH 7.0, at 37 °C with 100 μM phospholipid vesicles containing cholesterol. Addition of 100 mM NaCl to the assay buffer, or the use of 100 mM TEA·HCl buffer, pH 7.0, had no effect on the reaction rates.

The sigmoidicity of the reaction progress curves precluded determining first-order rate constants, and therefore, the time for 50% conversion was reported. The total concentration of cholesterol in the vesicles was measured by converting it to cholest-4-en-3-one with wild-type cholesterol oxidase after the vesicles were lysed with Triton X-100. In the case of the wild-type oxidase, the reaction went to 100% completion. With E361Q oxidase, the reaction was sufficiently slow that it was necessary to use the independently measured infinity point for calculations of 50% conversion of substrate. These data are presented in Table 2.

Measurement of Vesicle Leakage. Vesicles were prepared with encapsulated CF. The high concentration (50 mM) of encapsulated dye led to self-quenching of its fluorescence, and the background fluorescence of the vesicle solution was quite low. Release of dye into the assay mixture resulted in dilution of the CF and leakage could be monitored by following the increase in fluorescence intensity. All vesicles prepared, except those containing cholest-4-en-3-one, had a very slow rate of background leakage in the absence of cholesterol oxidase (Figure 3H,I and Table 2). Vesicles containing only phospholipid and cholest-4-en-3-one lost most of their encapsulated CF during purification. ePC/

Table 2: Comparison of Vesicle Leakage and Substrate Conversion[a]

		time required for 50% CF leakage (min)			time required for 50% conversion of substrate (min)	
entry	lipid	wild type	E361Q	no enzyme	wild type[b]	E361Q[c]
1	ePC	>10 000	>10 000	>10 000	na[d]	na
2	ePC/cholesterol 1:1	1.5 ± 0.1	>10 000	>10 000	0.5 ± 0.1	17 ± 1
3	ePC/cholesterol 1:0.5	5.5 ± 0.1	>10 000	>10 000	2.2 ± 0.1	24 ± 3
4	ePC/cholesterol 1:0.3	0.9 ± 0.1	>10 000	>10 000	3.8 ± 0.5	26 ± 2
5	ePC/cholesterol 1:0.1	80 ± 6	>10 000	>10 000	3.5 ± 0.11	26 ± 4
6	ePC/cholest-4-en-3-one 1:1	nm[e]	nm	<10[f]	na	na
7	ePC/cholest-4-en-3-one 1:0.5	nm	nm	<10[f]	na	na
8	ePC/cholest-4-en-3-one 1:0.1	nm	nm	<10[f]	na	na
9	ePC/cholesterol/cholest-4-en-3-one 1:0.9:0.1	4.5 ± 0.5	>1000	>1000	0.44 ± 0.11	17 ± 3
10	ePC/cholest-5-en-3-one 1:1	300	300	300	nm	na
11	POPC	>10 000	>10 000	>10 000	na	na
12	POPC/cholesterol 1:1	14 ± 1	>10 000	>10 000	0.4 ± 0.1	4 ± 1
13	POPC/POPE 1:1	>10 000	>10 000	>10 000	na	na
14	POPC/POPE/cholesterol 1:1:2	17 ± 1	>10 000	>10 000	1.5 ± 0.1	188 ± 2

[a] Times are for 10 μg/mL of the appropriate enzyme and are represented as the means ± standard errors, $n = 2-5$. [b] Measured as formation of cholest-4-en-3-one. [c] Measured as formation of cholest-5-en-3-one. [d] Not applicable. [e] Not measurable. [f] Only an approximation of the upper limit; vesicles lose > 70% of encapsulated CF during purification.

cholesterol/cholest-4-en-3-one 1:0.9:0.1 vesicles with encapsulated CF could be prepared. They leaked CF 1000 times more slowly than the ePC/cholest-4-en-3-one vesicles but about 10 times faster than ePC/cholesterol 1:1 vesicles (Table 2, entry 9). HPLC analysis of the ePC/cholest-5-en-3-one vesicles revealed that about 20% of the cholest-5-en-3-one had decompsed to conjugated 4-en-3-ones, i.e., cholest-4-en-3,6-dione, cholest-4-en-6-hydroperoxy-3-one, cholest-4-en-6-hydroxy-3-one, and cholest-4-en-3-one, during vesicle preparation and purification. Consequently, the rate of leakage of ePC/cholest-5-en-3-one vesicles is higher than that of ePC/cholesterol vesicles. This decomposition is due to the susceptibility of cholest-5-en-3-one to air oxidation at the 6-position (Dang et al., 1990).

Upon addition of wild-type cholesterol oxidase (10 μg/mL), cholesterol-containing vesicles became permeable to CF (Figure 3A−E and Table 2, entries 2−5, 9, 12, and 14). No CF leakage was observed when wild-type oxidase was added to pure phospholipid vesicles (Table 2, entries 1, 11, and 13). Upon addition of E361Q cholesterol oxidase to ePC, POPC, or POPE/POPC vesicles with and without cholesterol, no CF leakage was observed (Figure 3F,G and Table 2, entries 1−5 and 11−14). In the case of ePC/cholesterol/cholest-4-en-3-one 10:9:1, the leakage rate was the same before and after addition of E361Q (Table 2, entry 9).

DISCUSSION

Dye leakage experiments were undertaken to examine the membrane disruption properties of cholesterol oxidase. Inspection of the X-ray crystal structures of cholesterol oxidase suggested that an active-site lid opens in order to bind substrate (Li et al., 1993; Vrielink et al., 1991). This lid may insert into the membrane bilayer in order to form a hydrophobic channel for binding steroid. Alternatively, a mechanism whereby the oxidase binds cholesterol that is free in solution can be envisioned. The rates of substrate turnover catalyzed by cholesterol oxidase with vesicle substrates, however, are faster than the rate of sterol transfer out of membranes (Bar et al., 1989; Lund-Katz et al., 1988; Phillips et al., 1987). Furthermore, Slotte has demonstrated that tetramethylrhodamine-labeled cholesterol oxidase associates with monolayers composed of cholesterol and DMPC (Slotte, 1995). These experiments suggested that the oxidase penetrated the monolayer. Cholesterol oxidase was added to the aqueous subphase and fluorescence detected at the air/lipid interface.

The average depth of a bilayer is approximately 39 Å (New, 1990), whereas a DMPC monolayer is about 22 Å thick (Bayerl et al., 1990). The maximum length of the putative lid is approximately 20 Å, based on inspection of the closed X-ray crystal structure (Li et al., 1993). Although the monolayer fluorescence experiments of Slotte suggest that the oxidase spans the lipid, in a bilayer this would simply be an insertion of protein. We sought other methods to characterize the interactions of cholesterol oxidase with the lipid bilayer, because the direct observation method of Slotte cannot be applied to vesicle systems. We chose, therefore, to determine the propensity of cholesterol oxidase to induce leakage of vesicle contents. This method has been used with other proteins, for example, melittin (Benachir & Lafleur, 1995), to demonstrate the perturbation of membrane permeability.

Moreover, in a monolayer system, affinities for phospholipid are not measured. We developed a method that would allow determination of apparent K_Ds, in order that we could optimize the concentration of vesicles in our leakage assays. The cholesterol oxidase used in our experiments, from *Streptomyces*, has 10 tryptophan residues, two of which are located in or near the putative active-site lid (W68 and W82). We reasoned that we might use change in intrinsic tryptophan fluorescence as a reporter for vesicle binding. This method has the added advantage that we have not perturbed the structure of the enzyme by attaching a reporter group. In fact, the tryptophan fluorescence of cholesterol oxidase is quenched upon binding to phospholipid vesicles. We do not, however, know which tryptophans are responsible for the quenched signal. As shown in Table 1, wild-type cholesterol oxidase binds with equal affinity to both phospholipid and phospholipid/cholesterol vesicles composed of either phosphatidylcholine or phosphatidylethanolamine. The apparent K_Ds range from 20 to 36 μM in monomeric phospholipid.

In order to confirm that we were measuring binding in our experiments, we tested the activity of cholesterol oxidase with ePC/cholesterol 1:1 vesicles as substrate using ePC vesicles as inhibitor. We expected that if cholesterol oxidase

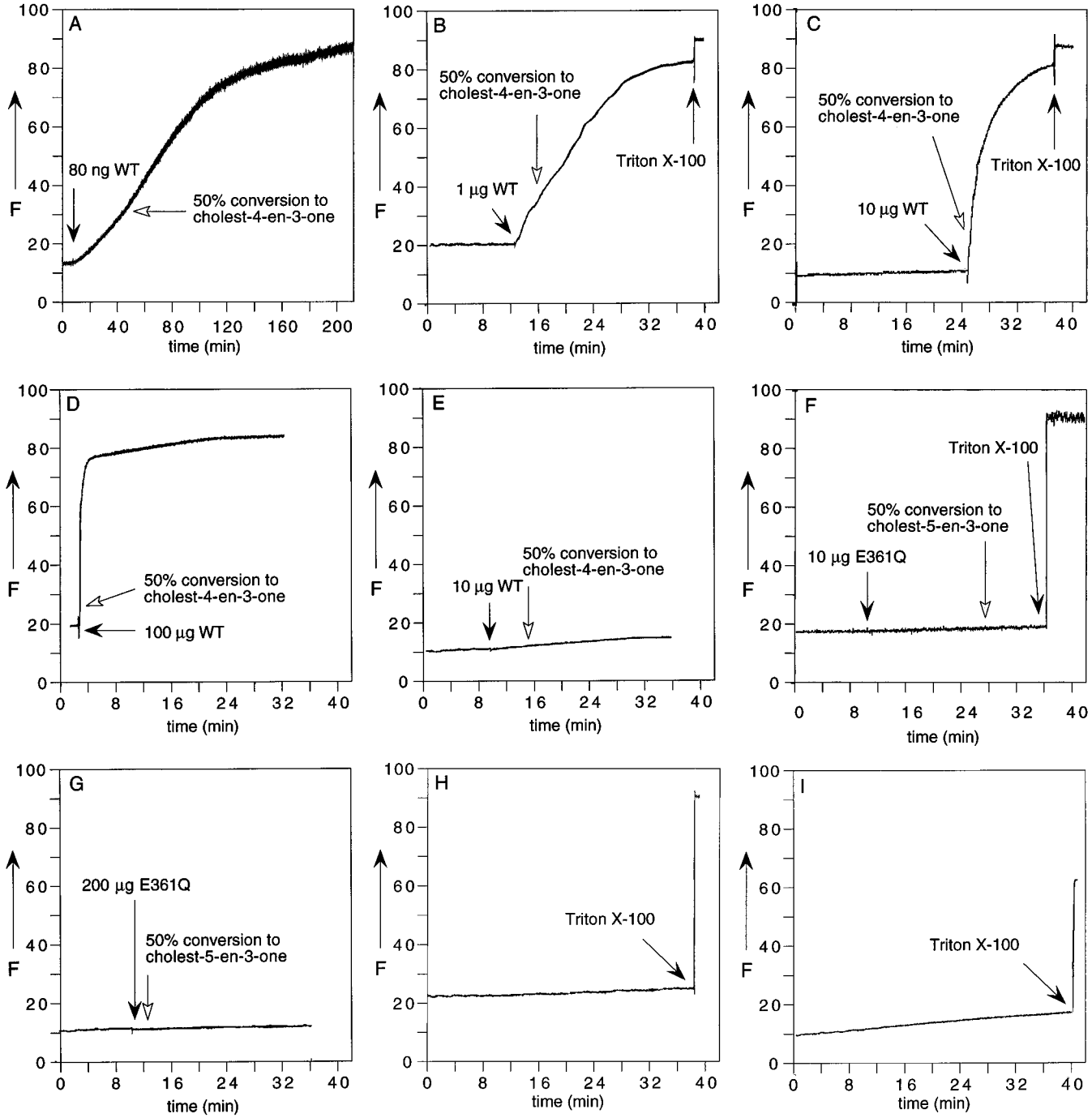

FIGURE 3: CF leakage curves of ePC/cholesterol 1:1 vesicles with (A) 80 ng/mL wild type, (B) 1 μg/mL wild type, (C) 10 μg/mL wild type, and (D) 100 μg/mL wild type; CF leakage curves of ePC/cholesterol 10:1 vesicles with (E) 10 μg/mL wild type. CF leakage curves of ePC/cholesterol 1:1 vesicles with (F) 10 μg/mL E361Q cholesterol oxidase, (G) 200 μg/mL E361Q cholesterol oxidase, and (H) no enzyme; and CF leakage curve of ePC/cholest-5-en-3-one 1:1 vesicles with (I) no enzyme. Leakage assays were conducted at 37 °C in 100 mM TEA·HCl buffer, pH 7.0, and 100 mM NaCl with 100 μM lipid. The samples were excited at 430 nm and the emission at 535 nm was measured as a function of time. Fluorescence intensity is in arbitrary units. Solid arrows indicate the time of addition of enzyme or Triton X-100, the latter being added to achieve complete release of encapsulated CF. The open arrow indicates 50% conversion of cholesterol to cholest-4-en-3-one or cholest-5-en-3-one (see Table 2).

were binding to ePC vesicles, a nonproductive complex would be formed and the effective concentration of active cholesterol oxidase would be decreased. Indeed, upon addition of increasing amounts of ePC vesicles, the catalytic reaction was inhibited (Figure 2A). In a fit of approximate initial velocity values from Figure 2A versus [PC], a K_i (e.g., apparent dissociation constant) of 43 ± 9 μM is obtained, in agreement with the spectroscopically determined value. (This method is only approximate because of the inaccuracy in measuring initial velocities on sigmoidal reaction progress curves, *vide infra*.) Thus, we have a simple direct fluorescence assay for measuring binding affinity. With the phospholipids surveyed thus far, the binding affinity is insensitive to the amount of cholesterol present in the membrane or the steric bulk of the head group.

In addition, note the sigmoidicity of the reaction progress curves. If ePC/cholesterol vesicles with different mole fractions of product cholest-4-en-3-one are incubated with wild-type cholesterol oxidase, the progress curves are superimposable on the progress curve of ePC/cholesterol vesicles at the appropriate percentage reaction (Figure 2B). The sigmoidicity is due, therefore, to the increasing mole

fraction of product, cholest-4-en-3-one in the membrane, not to mixing or enzyme activation phenomena. Originally, we hypothesized that the apparent increase in initial velocity as a function of time was due to increasing affinity of the enzyme for the vesicle surface as the mole fraction of product increased. This sort of phenomenon has been well-documented in the case of phospholipases (Jain & Berg, 1989). The apparent K_Ds for both substrate and product vesicles, however, are essentially the same. We now hypothesize that the sigmoidicity is due to expansion of the lipid bilayer upon the conversion of cholesterol to cholest-4-en-3-one. Upon formation of cholest-4-en-3-one, the remaining cholesterol is extracted more easily from the bilayer by the oxidase. The facilitated extraction correlates with the increased permeability observed by us (*vide infra*) and by others (Brasaemle & Attie, 1990; Demel et al., 1972), as well as with the monolayer experiments of Slotte (1995).

Incorporation of cholest-4-en-3-one into membranes increases the permeability of vesicles to small molecules such as glucose (Demel et al., 1972). Similar effects are seen with mammalian cells. Furthermore, addition of cholest-4-en-3-one to bilayers increases the disorder of the liquid crystal phase (Ben-Yashar & Barenholz, 1989). Addition of cholest-4-en-3-one to monolayers results in reduced condensation of the membrane compared to the condensation observed upon addition of cholesterol (Grönberg & Slotte, 1990). Increased disorder and reduced condensation are consistent with increased permeability. Cholest-5-en-3-one, however, behaves differently than cholest-4-en-3-one. Incorporation of cholest-5-en-3-one into phospholipid vesicles does not increase their permeability to glucose or glycerol (Demel et al., 1972). In our experiments, we observed the same effects. Vesicles with encapsulated CF containing cholesterol or cholest-5-en-3-one or no steroid, leaked very slowly (Figure 3H,I and Table 2, entries 1−5 and 10−14). The ePC/cholest-5-en-3-one vesicles partially decomposed during their preparation to conjuated 4-enones, and consequently, they have a measurable, although slow, rate of leakage. The ePC/cholest-4-en-3-one vesicles with encapsulated CF rapidly leaked their contents during their preparation (Table 2, entries 6−8). More than 70% of the encapsulated CF was lost during the 20 min gel-filtration process required to purify the encapsulated vesicles. However, ePC/cholesterol/cholest-4-en-3-one vesicles with encapsulated CF could be prepared. Their leakage rate in the absence of enzyme was slower than ePC/cholest-4-en-3-one vesicles, although 10 times greater than ePC/cholesterol (Table 2, entry 9). The presence of cholesterol in the vesicle must condense the membrane sufficiently to impede leakage. We desired to determine if cholesterol oxidase *binding* to the lipid caused leakage. We needed a method, therefore, to separate the effects of cholesterol oxidase binding to the membrane from the effects of catalytic conversion of cholesterol to cholest-4-en-3-one and consequently a permeable vesicle. Moreover, we wanted to study binding to *sterol*-containing vesicles.

Our solution was to use an active-site mutant, E361Q, that still binds to the membrane and to substrate but is slow to convert cholesterol, and when it does, the predominant product is cholest-5-en-3-one, not cholest-4-en-3-one; i.e., oxidation is separated from isomerization (Figure 1) (Sampson & Kass, 1997). However, this active-site mutant has the same affinity for vesicles as wild-type oxidase (Table 1), with the exception of POPC:POPE:cholesterol vesicles. It is not clear why E361Q has a different affinity (and activity) than wild type for this substrate. This observation warrants further study but does not affect the interpretation of the data presented here. We could study binding to phospholipid/cholesterol vesicles and phospholipid/cholest-5-en-3-one vesicles because of the catalytic activity of the E361Q mutant.

With binding assay and active-site mutant E361Q in hand, we initiated vesicle leakage experiments. We conducted our assays with 100 μM phospholipid. This concentration was chosen to maximize binding of the enzyme to the vesicle surface without overwhelming interference from light-scattering of the vesicles. Varying amounts of cholesterol oxidase were added up to 200 μg/mL. In the experiments summarized in Table 2, 10 μg/mL enzyme was used.

At concentrations of 10 μg/mL, the ratio of enzyme molecules per vesicle is approximately 1.2×10^4. Thus, any perturbation of the vesicle membrane is magnified by 10^4-fold. However, at this concentration, 50% conversion of cholesterol (in 1:1 ePC/cholesterol vesicles) to cholest-4-en-3-one by wild-type enzyme requires less than 1 min. With the wild-type enzyme, we really observed the combined effects of increased permeability due to cholest-4-en-3-one formation and cholesterol oxidase binding to cholest-4-en-3-one vesicles. The rate of leakage was dependent on the rate of cholest-4-en-3-one formation (Figure 3A−D) and dependent on cholest-4-en-3-one mole fraction (Figure 3E and Table 2, entries 2−5, 9, 12, and 14). These leakage results are consistent with the known properties of phospholipid/cholest-4-en-3-one membranes (Ben-Yashar & Barenholz, 1989; Brasaemle & Attie, 1990; Demel et al., 1972; Grönberg & Slotte, 1990).

With the mutant enzyme E361Q, we observed initially the effects of binding to cholesterol-containing vesicles and, as the catalytic reaction progressed, to cholest-5-en-3-one-containing vesicles. We analyzed the steroid content of the vesicles as a function of time by UV assay of H_2O_2 formation (and thus, indirectly, cholest-5-en-3-one) to confirm that the phospholipid/cholesterol vesicles were substrates for E361Q cholesterol oxidase and by HPLC to confirm that the expected products were formed. The time for 50% conversion of cholesterol to cholest-5-en-3-one is 17 min with 10 μg/mL E361Q cholesterol oxidase and 1:1 ePC/cholesterol vesicles. This slow turnover allowed us to test for leakage with phospholipid/cholesterol vesicles immediately after addition of E361Q cholesterol oxidase. In the case of ePC/cholesterol, POPC/cholesterol, and POPE/POPC/cholesterol vesicles, no leakage of CF above the background rate was observed (Figure 3F,G and Table 2, entries 2−5, 9, 12, and 14). After conversion to phospholipid/cholest-5-en-3-one vesicles, still no leakage of CF was observed (Figure 3F and Table 2, entries 2−5, 9, 12, and 14). Even with 200 μg/mL mutant enzyme, i.e., at 20 times the previous concentration, no leakage was observed (Figure 3G).

Furthermore, CF leakage did not occur upon incubation of wild-type or E361Q cholesterol oxidase with ePC, POPC, or POPC/POPE vesicles (Table 2, entries 1, 11, and 13). Thus, regardless of the cholesterol mole fraction, binding of cholesterol oxidase to the membrane does not sufficiently perturb the bilayer to cause leakage of fluorescent dye. Conversion of cholesterol to cholest-4-en-3-one is necessary for the membrane to become permeable.

We conclude from these experiments that the binding of cholesterol oxidase to the vesicle membrane and binding of

sterol in the active site of cholesterol oxidase do not cause membrane leakage. Both wild type and E361Q bind to the vesicle membrane with the same affinity and transform membrane cholesterol to product; thus we may infer that their interactions with the membrane are nearly identical. These measurements and the fact that fluorescence quenching of cholesterol oxidase occurs upon binding to the lipid bilayer suggest that interactions with the phospholipid head groups predominate over interactions with the lipid moiety of the fatty acids. The absence of significant membrane perturbation and the relatively low affinity of cholesterol oxidase for the membrane suggest that the enzyme sits on the membrane surface.

These results have implications for the use of cholesterol oxidase as an insecticide and for its use as a tool in cell biology. The most likely mechanism of cholesterol oxidase larvicidal activity requires conversion of cholesterol to cholest-4-en-3-one. This conversion increases the permeability of the endothelium and, with a large osmotic pressure differential, causes lysis, as is observed (Purcell et al., 1993). This lytic behavior will only occur with membranes that contain a sufficient mole fraction of 3β-hydroxy steroids to cause leakage upon their conversion to conjugated 4-en-3-ones. This mechanism suggests that insects, or other organisms, with a high content of free 3β-hydroxy steroids in their mid-gut endothelial cells will be the most susceptible to cholesterol oxidase in their diet. Membranes with 10 mol % free 3β-sterol content have sufficient sterol to undergo leakage in the presence of wild-type cholesterol oxidase. In fact, mammalian cells are also susceptible to leakage upon cholesterol oxidase treatment. For example, cholesterol oxidase treatment of Chinese hamster ovary cells increases their permeability and 80% of the cellular K^+ is released (Brasaemle & Attie, 1990), and erythrocytes may lyse upon oxidase treatment depending on the experimental conditions (Brasaemle et al.; 1988, Lange et al., 1984; Linder et al., 1989).

The use of cholesterol oxidase as a tool in cell biology has been recently reviewed by Lange (1992). Although some of the conditions under which cholesterol oxidase is most active have been determined, the basis for the conditionality of the activity has not. The binding affinity constants reported here are certainly one variable that has been overlooked and should be considered in the design of experiments utilizing cholesterol oxidase as a probe. Our experiments demonstrate that cell lysis or increased permeability is a result of cholest-4-en-3-one formation and not binding of cholesterol oxidase.

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