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Oxidation/isomerization of 5-cholesten-3β-ol and 5-cholesten-3-one to 4-cholesten-3-one in pure sterol and mixed phospholipid-containing monolayers by cholesterol oxidase

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In this study we have examined the cholesterol oxidase (*Streptomyces cinnamomeus*) catalyzed conversion of either 5-cholesten-3β-ol or 5-cholesten-3-one into 4-cholesten-3-one in pure sterol or mixed phospholipid-containing monolayers at the air/buffer interface. The mean molecular area requirement of 5-cholesten-3-one in a pure monolayer was slightly smaller than the comparable area required by 5-cholesten-3β-ol (although the collapse pressure was markedly lower for 5-cholesten-3-one), and both sterols were about equally capable of condensing the lateral packing density of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine at a lateral surface pressure of 20 mN/m. Both sterols were converted by cholesterol oxidase to 4-cholesten-3-one, the reaction being faster with 5-cholesten-3-one as compared to 5-cholesten-3β-ol. When the temperature-dependency of the cholesterol oxidase catalyzed conversion of the sterols to 4-cholesten-3-one was examined, the Arrhenius activation energy was calculated to +30 kJ/mol and +27 kJ/mol for 5-cholesten-3β-ol and 5-cholesten-3-one, respectively, when the sterols were presented to the enzyme as pure sterol monolayers at a lateral surface pressure of 20 mN/m. With a mixed monolayer containing 40 mol% sterol and 60 mol% EPC, the corresponding activation energies were +107 kJ/mol and +96 kJ/mol for 5-cholesten-3β-ol and 5-cholesten-3-one, respectively. With the monolayer system used, it appeared that the over all rate-limiting step in the enzyme-catalyzed conversion of 5-en-sterols to 4-en-3-one was the desorption of the sterol molecules from the monolayer into the active site of the enzyme at the interface. This appeared to be true both with pure sterol monolayers as well as with mixed monolayers containing phosphatidylcholine.

Introduction

Cholesterol oxidases (EC 1.1.3.6), or more generally β-hydroxy steroid oxidases, are a group of enzymes found in or secreted by various microorganisms [1–5]. These enzymes catalyze both the oxidation of 5-cholesten-3β-ol to 5-cholesten-3-one with production of H_2O_2, and the subsequent isomerization of the 5-cholesten-3-one to 4-cholesten-3-one [6,7]. Cholesterol oxidases have a fairly broad substrate specificity, and will act upon most 3β-hydroxy sterols which do not have obstructing functional groups in the A- or B-ring of the sterol molecule [5–9]. Mammalian steroidogenic cells also contain an enzyme (or group of enzymes) which convert 5-ene-3β-hydroxysteroids to their corresponding 4-ene-3-keto configuration (β-hydroxysteroid/5-ene-4-ene isomerase, EC 5.3.3.1) [10–15].

Previous studies from this laboratory have shown that cholesterol oxidases (from both *Brevibacterium* and *Streptomyces* species) can be useful tools with which one can examine sterol–phospholipid interactions in monolayer membranes [9,16–21]. The enzyme-catalyzed oxidation of cholesterol (or another β-hydroxy sterol) in a pure sterol or a mixed phospholipid-containing monolayer is influenced by the degree of substrate packing density (or lateral surface pressure), and by the type of nearest neighbor (a sterol or a phospholipid molecule). These parameters can be exploited to yield information about the relative affinity of a sterol for a particular phospholipid class [16,18],

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Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; EPC, egg phosphatidylcholine.

and was recently also used to estimate stoichiometries of lipid packing in binary mixed monolayers of cholesterol and different phospholipids [19].

Since 5-cholesten-3-one is an intermediate reactant in the oxidation/isomerization of cholesterol by cholesterol oxidase [7], we have in this study examined the effects of the β-hydroxy group (present in cholesterol but substituted for a 3-keto group in 5-cholesten-3-one) on the interactions between cholesterol oxidase (*Streptomyces cinnamomeous*) and the sterols, presented to the enzyme as pure or mixed monolayers. We report that both substrates are readily acted upon by the enzyme (i.e., that a β-hydroxy group is not an essential substrate property), and that the isomerization reaction is the one that leads to the measurable monolayer expansion (due to conformational changes in the molecule). We further report on the activation energies required for the oxidation/isomerization reactions in pure and mixed monolayers, and discuss about the plausible rate-limiting step in the overall process.

Experimental procedures

Materials. Cholesterol (99 + %), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and egg phosphatidylcholine were obtained from Sigma (St. Louis, MO). The sterol was 99% pure by gas-liquid chromatography, and the phospholipid gave a single spot on thin-layer chromatography plates (Kieselgel 60 (Merck, Germany), eluted with chloroform/methanol/acetic acid/water (25:15:4:2, v/v); [22]. 5-Cholesten-3-one was purchased from Aldrich (Germany) and was reported to be better than 97% pure. A GC-M5-analysis revealed the contaminant to be 4-cholesten-3-one and the presence of a very minute trace of 4-cholesten-3,6-dione. Cholesterol oxidase (*Streptomyces cinnamomeous*) was purchased from Calbiochem, and was used as delivered. Buffer salts were of pro analysis grade, and the water used was double distilled and further purified with a Millex Q system (to better than 15 MΩ/cm).

Lateral surface pressure versus mean molecular area isotherms. Force-area isotherms of lipids on water at 22°C were determined for pure sterol monolayers, or for mixed monolayers containing either of the sterols and POPC or EPC, with a KSV 3000 Surface Barostat (KSV Instruments, Helsinki), as reported previously [16,19]. At least three different runs were performed at each lipid composition, and the variation between different runs was less than ±5%. The effect of temperature on the force-area isotherms of either pure s-cholesten-3β-ol and 5-cholesten-3-one, or a mixed monolayer containing 40 mol% 5-cholesten-3β-ol and 60 mol% EPC was negligible (less than 2% mean molecular area increase going from 22°C to 35°C).

Oxidation/isomerization of monolayer sterols. The oxidation and isomerization of sterols at 22°C in pure or mixed monolayers by cholesterol oxidase was determined at constant surface pressure in a thermostatted zero-order Teflon trough [16,23] with Tris buffer (50 mM, pH 7.4, with 140 mM NaCl), as previously described [19]. The specific experimental conditions are indicated in the legends to each relevant figure.

Calculation of enzyme activity. The area over the reaction chamber in the zero-order trough was 2550 mm^2. The mean molecular area determinations of pure and mixed monolayers made it possible to calculate the number of cholesterol molecules that would fit on the surface over the reaction chamber. Since only molecules over the reaction chamber were oxidized/isomerized, and since a reaction end-point was determined, this reaction time could be converted to an average reaction rate (molecules converted per s) [20]. Usually three experiments were performed with each monolayer or condition, and the measured reaction times deviated less than ±15% from the reported mean value.

Molecular modeling. Energy-minimized 3D-models of the sterol were made on an Evans-Sutherland computer running the Sybyl Molecular Modeling Software (version 5.41; Tripos Associates, St. Louis, MO). The Tripos internal parameter set was used for the mechanistic energy-minimization procedure.

Results

Molecular area isotherms of the sterols

Since one needs to know the mean molecular area requirement of a sterol in order to calculate the enzyme activity during oxidation/isomerization reactions in monolayers, we collected force–area curves for all three sterol derivatives, although similar isotherms have previously been published in the literature [20,24,26]. The isotherms in Fig. 1 show that 5-cholesten-3-one requires a slightly smaller mean molecular area as compared to 5-cholesten-β-ol, whereas 4-cholesten-3-one has a much larger mean molecular area requirement than both of the precursor sterols. In a previous report [25], the mean molecular area of 5-cholesten-3-one was reported to be slightly larger than the area reported here; however the presence of 4-cholesten-3-one as a contaminant would lead to a larger measured mean molecular area. In the present study, the 4-cholesten-3-one contamination was analyzed to be less than 10%. The monolayer collapse pressure was lowest for 4-cholesten-3-one, intermediate for 5-cholesten-3-one, and highest for 5-cholesten-3β-ol. A computer-performed mechanistic energy-minimization of the sterol structures (Fig. 2) indicate that both 5-cholesten-3β-ol and 5-cholesten-3-one have very similar ring conformations, and differ mainly in the projection angle of

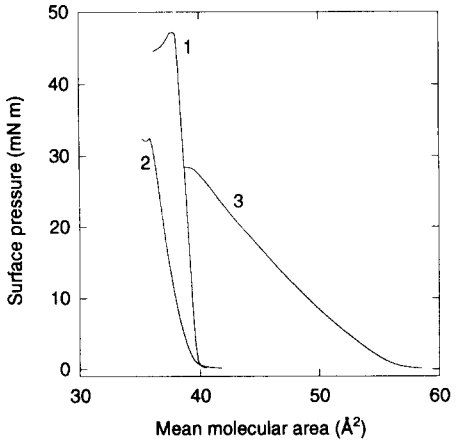

Fig. 1. Force-area isotherms of pure sterol monolayers at the air/water interface. Data were collected at 22°C and line (1) represents 5-cholesten-3β-ol, line (2) 5-cholesten-3-one, and line (3) 4-cholesten-3-one. The compression rate was 9 Å² per molecule per min, and each curve is from one representative experiment.

the functional group at position 3. 4-Cholesten-3-one, on the other hand, has a very different conformation of the A-ring compared to either 5-cholesten-3β-ol or 5-cholesten-3-one. These models agree with the isotherm data presented in Fig. 1, and suggest that it is mainly the changed conformation of ring A in 4-cholesten-3-one that leads to the significantly larger mean molecular area requirement of this sterol compared with the other two. Since the only apparent difference between 5-cholesten-3β-ol and 5-cholesten-3-one is the functional group at position 3, this difference obviously is responsible for the lowered collapse pressure observed in monolayers of 5-cholesten-3-one,

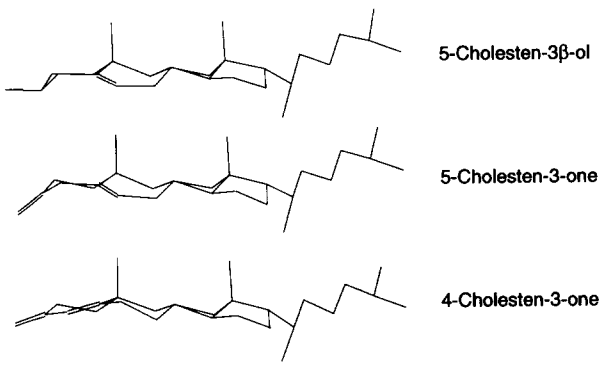

Fig. 2. Computer-modeled 3D structures of the used sterols. The structure of 5-cholesten-3β-ol was drawn and subjected to a mechanistic energy-minimization (with Tripos internal parameter set). After the minimization, the hydrogen atoms were removed, the molecule tilted so that carbons 8 and 11 were superimposed, and the wire-frame structure plotted as shown. To obtain the structures of 5-cholesten-3-one and 4-cholesten-3-one, the minimized structure of 5-cholesten-3β-ol was changed to reflect the altered position of the double bond or the functional group at position 3, and then the hydrogen atoms were added and the molecule subjected to the energy-minimization procedure. The three molecules are tilted similarly with regard to the carbons 8 and 11.

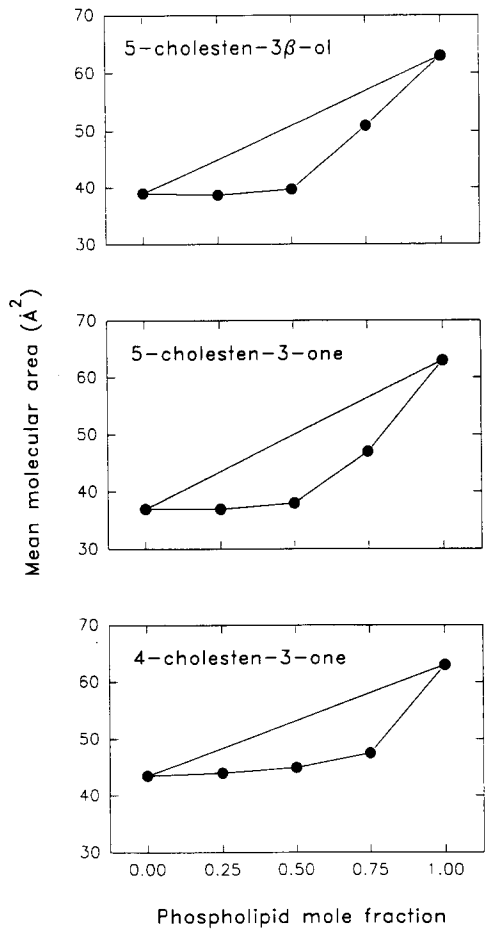

Fig. 3. Mean molecular area–mol fraction plot for the mixed monolayers of sterols and POPC. The mean molecular areas were determined at a lateral surface pressure of 20 mN/m for mixed monolayers of POPC and 5-cholesten-3β-ol, 5-cholesten-3-one, or 4-cholesten 3 one. The straight line connecting the two pure monolayer systems represents ideal mixing (additive molecular areas of each component). Values are averages from three separate experiments at each indicated mol fraction (individual values deviated less than ±5% from the calculated average).

as well as for the slightly smaller mean molecular area requirement or 5-cholesten-3-one.

Interaction of the sterols with POPC

3β-Hydroxy sterols with an isooctyl side chain at position C(17) are known to condense the lateral packing density of phospholipids in mixed monolayers [24–27]. To compare the effects of the present sterols (5-cholesten-3β-ol, 5-cholesten-3-one, 4-cholesten-3-one) on the lateral packing of POPC in mixed monolayers, force–area isotherms of each of the sterol/POPC mixed monolayers were obtained, and the mean molecular area determined at a lateral surface pressure of 20 mN/m. These plots are shown in Fig. 3 for 5-cholesten-3β-ol, 5-cholesten-3-one, and 4-cholesten-3-one, respectively. It is evident that with each sterol, the condensing effect on the lateral packing of POPC is remarkably similar. Qualitatively similar findings have

previously been reported by Demel at al. [25] for a system with 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine. This finding suggests that both the substitution of the β-hydroxy group with a 3-keto group, and the A-ring deformation (in 4-cholesten-3-one) has only minor effects on the capacity of the 3-keto sterols to interact with (i.e., condense) POPC packing as compared to the ideal sterol, 5-cholesten-3β-ol.

Isomerization of 5-cholesten-3-one in pure and mixed monolayers by cholesterol oxidase

Data for oxidation of cholesterol in pure monolayers by cholesterol oxidase has recently been reported by this laboratory [20]. In the present study we have examined the isomerization of 5-cholesten-3-one to 4-cholesten-3-one in pure sterol monolayers by cholesterol oxidase. A plot of the apparent enzyme activity versus the lateral surface pressure of the pure 5-cholesten-3-one monolayer is shown in Fig. 4. The velocity of the reaction increased linearly with the surface pressure, a finding which is qualitatively similar to that reported for the oxidation of a pure cholesterol monolayer [20]. It is noteworthy that the average reaction rate was significantly faster when 5-cholesten-3-one was the substrate compared to the average rate previously published for cholesterol (at $\pi = 20$ mN/m, the average rates were about $4.5 \cdot 10^{13}$ and $2.8 \cdot 10^{13}$ molecules converted per s for 5-cholesten-3-one and 5-cholesten-3β-ol, respectively).

In a mixed phospholipid-containing monolayer (50 mol% each of 5-cholesten-3-one and POPC, respectively), the isomerization reaction catalyzed by cholesterol oxidase had a lateral surface pressure optimum around 15 mN/m (Fig. 5). This surface pressure dependency in mixed monolayers is clearly different from that observed in pure sterol monolayers (Fig. 4), but is similar to that reported for a cholesterol/POPC system [16], although the amplitude of the surface pressure-dependent changes was smaller with 5-cholesten-3-one compared to that found with cholesterol [16].

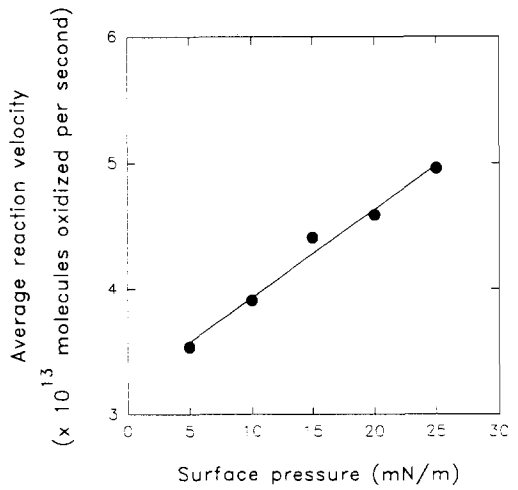

Fig. 4. Isomerization of pure 5-cholesten-3-one monolayers by cholesterol oxidase at different lateral surface pressures. The pure sterol monolayer was spread on a Tris buffer (50 mM, pH 7.4, with 140 mM NaCl) at 22°C in a zero-order Teflon trough. Cholesterol oxidase (16 mU/ml) was added to the thermostatted reaction compartment, and the monolayer expansion was determined at constant surface pressure. Values are calculated averages from three separate experiments.

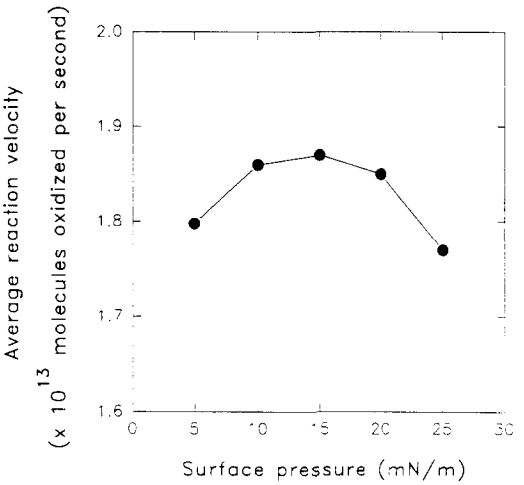

Fig. 5. Isomerization of mixed 5-cholesten-3-one/POPC monolayers by cholesterol oxidase at different lateral surface pressures. The monolayer (50 mol% each of 5-cholesten-3-one and POPC) was exposed to cholesterol oxidase (16 mU/ml, 22°C) at different lateral surface pressures, and the conversion of 5-cholesten-3-one to 4-cholesten-3-one was registered as a monolayer area expansion at constant surface pressure. Values are calculated averages from three different experiments.

Activation energy for the action of cholesterol oxidase

To gain information about potential rate-limiting steps in the overall process of converting monolayer sterols (either in pure of mixed monolayers) to their 4-en-3-one derivative, we measured the activity of cholesterol oxidase over a temperature range with the substrate (5-cholesten-3β-ol or 5-cholesten-3-one) presented to the enzyme either as a pure monolayer or as a mixed monolayer containing EPC. At a lateral surface pressure of 20 mN/m, an Arrhenius plot of the temperature-dependency of the oxidation of cholesterol (in a pure sterol monolayer) to 4-cholesten-3-one (Fig. 6A) indicate that the activation energy was in the order of +30 kJ/mol, whereas the corresponding activation energy for the isomerization of 5-cholesten-3-one to 4-cholesten-3-one was about +26 kJ/mol. With a mixed monolayer containing 40 mol% sterol and 60 mol% EPC, the corresponding activation energies were +107 and +97 kJ/mol for the oxidation/isomerization of cholesterol and the isomerization of 5-cholesten-3-one, respectively (Fig. 6B). The mixed

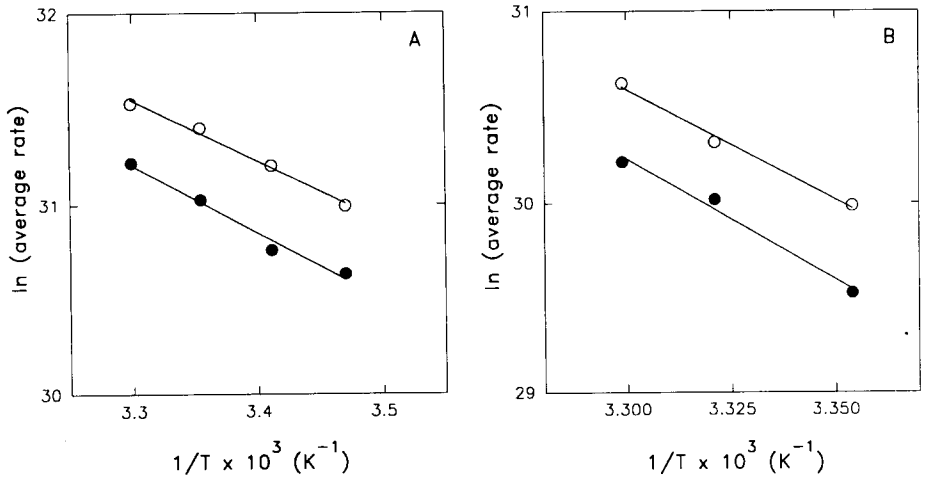

Fig. 6. Effect of temperature on the cholesterol oxidase-catalyzed conversion of 5-cholesten-3β-ol and 5-cholesten-3-one to 4-cholesten-3-one in pure or mixed monolayers. Monolayers of pure 5-cholesten-3β-ol (filled circle) or 5-cholesten-3-one (open circle; panel A) or a mixture of 5-cholesten-3β-ol (filled circle) or 5-cholesten-3-one (open circle) and phosphatidyl choline (40 mol% sterol and 60 mol% EPC; panel B) were spread on Tris buffer kept at the indicated temperatures ($\pm$0.5°C). Cholesterol oxidase (16 mU/ml) was added to the subphase, and the monolayer expansion was registered as a time function. Each value is a calculated average from three to four different experiments.

monolayer composition of 40 mol% sterol and 60 mol% phospholipid was chosen in order to avoid the possibility of free cholesterol clusters (which appear above a 1:1 stoichiometry) [19].

Discussion

This study has measured the average rate of the cholesterol oxidase (*Streptomyces cinnamomeus*) catalyzed oxidation/isomerization of 5-cholesten-3β-ol, and the isomerization of 5-cholesten-3-one, to the common product 4-cholesten-3-one. We have presented the substrate to the enzyme in either pure sterol monolayers or in mixed phosphatidylcholine-containing monolayers. The monolayer technique is favored over other assay systems because it allows for the appropriate control of the substrate orientation and also allows for the variation in substrate packing densities [9].

It is well recognized that cholesterol oxidases (from different microorganisms) have a broad substrate specificity, converting many 3β-hydroxy sterols to their corresponding 3-keto derivative [5–9]. In the conversion of 5-cholesten-3β-ol to 4-cholesten-3-one, it is known that the intermediate metabolite is 5-cholesten-3-one [7]. The first reaction step, i.e., the conversion of the β-hydroxy to a corresponding keto group, also produces H_2O_2, whereas the second step (isomerization of the Δ^5 double bond to the Δ^4 position) does not [6]. The liberation of H_2O_2 is the basis for numerous assay systems used to measure cholesterol oxidase activity [28].

The assay for cholesterol oxidase activity in monolayer membranes is, however, based upon the fact that a 4-cholesten-3-one monolayer has a larger mean molecular area requirement compared to a pure 5-cholesten-3β-ol monolayer [20]. Therefore, at constant lateral surface pressure, the oxidation/isomerization of 5-cholesten-3β-ol to 4-cholesten-3-one leads to a monolayer area increase which can easily be recorded and converted to an average reaction rate [20]. Further, in the present study it was shown that the observable mean molecular area increase, as induced by the cholesterol oxidase catalyzed attack on 5-cholesten-3β-ol, must arise from the second reaction step, i.e. the isomerization reaction. This conclusion is based on the following findings: (i) the product of the oxidation step, 5-cholesten-3-one, does not have a larger mean molecular area compared to the precursor sterol, 5-cholester-3β-ol (actually its mean molecular area is slightly smaller at 20 mN/m; Fig. 1); on the other hand, the product of the isomerization reaction, 4-cholesten-3-one, has the largest mean molecular area requirement of the sterols; (ii) the computer-constructed 3D models of the sterol backbones suggest that the ring conformations of both 5-cholesten-3β-ol and 5-cholesten-3-one are very similar, whereas that of 4-cholesten-3-one differs (the A- and B-ring being markedly distorted compared to the other sterols).

The oxidation of a pure cholesterol monolayer by cholesterol oxidase was recently shown to be a fairly rapid process, and the apparent reaction velocity was observed to increase with increasing lateral packing density [20]. A similar finding was now also seen in the isomerization reaction (of 5-cholesten-3-one to 4-cholesten-3-one; Fig. 4). A direct comparison of the apparent reaction velocities for the two 5-en-3β-ol/3-one sterols indicated that the isomerization reaction was consistently faster than the complete oxidation/

isomerization reaction. Since the isomerization reaction of 5-cholesten-3-one theoretically requires less steps at the active site of the enzyme than the oxidation/isomerization reaction of 5-cholesten-3β-ol, this fact may to some extent explain why 5-cholesten-3-one is isomerized faster than 5-cholesten-3β-ol is oxidized/isomerized. However, both reactions should proceed with extreme rapidness once the substrate molecule is bound to the active site. This has been demonstrated with the analogous system of the Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* [29]. It is therefore possible that factors which affect the rate of sterol diffusion into the active site of cholesterol oxidase also will affect the overall reaction rate. The interfacial properties of the substrate monolayers are therefore likely to be of major importance for the cholesterol oxidase catalyzed reactions.

The cholesterol oxidase from *Brevibacterium cinnamomeus* does not readily penetrate into an inert sterol monolayer above a lateral surface pressure of about 10 mN/m [20]. The same lack of surface activity for the *Streptomyces* enzyme has also been observed (Slotte, unpublished observations). The low surface activity of cholesterol oxidase would suggest that the rate-limiting step in substrate supply is the desorption of the sterol molecule from the monolayer to the enzyme at the interface. It has been shown that the *Brevibacterium sterolicum* enzyme has a sterol-binding hydrophobic cavity [30]. It is likely that the *Streptomyces* enzyme has a similar sterol binding cavity, and that sterols probably have to desorb from the interface into the cavity before the catalytic process can be accomplished. The desorbing sterols may not necessarily have to enter the aqueous phase, if (which is plausible) the enzyme acts tightly at the water/lipid interface. 5-Cholesten-3-one would be expected to desorb more easily from the interface than 5-cholesten-3β-ol, since the desorption rate of oxysterols (including 4-cholesten-3-one and 4,6-cholestadien-3-one) from model membranes is significantly faster than the rate observed with cholesterol [31,32]. A faster desorption rate would be consistent with a faster reaction rate.

The apparent activation energy for the conversion of 5-cholesten-3-one or 5-cholesten-3β-ol to 4-cholesten-3-one was similar (both in pure and mixed monolayers), whereas the activation energies were much higher for oxidation/isomerization in mixed phospholipid-containing monolayers as compared to pure sterol monolayers (Fig. 6). The observed lower activation energy in pure sterol monolayers indicates that sterol molecules have a lower energy barrier to overcome, when they desorb from a pure sterol environment (to the enzyme) as compared to a phospholipid environment. This interpretation includes the assumption that the surface concentration of cholesterol oxidase (which could not be determined in this study) is similar in pure and mixed monolayers at different temperatures. The activation energy for the conversion of s-cholesten-3β-ol or 5-cholesten-3-one to 4-cholesten-3-one in a mixed monolayers (107 and 97 kJ/mol, respectively), was somewhat higher than the activation energy measured for the desorption of cholesterol from phosphatidylcholine vesicles (67.9 $\pm$ 5.7 kJ/mol; 40 and 60 mol% of cholesterol and phosphatidylcholine, respectively; [33]), but still close enough to suggest that a desorption process may be involved as a rate-limiting step.

Taken together, this work has demonstrated that cholesterol oxidase can accept a monolayer-associated 3-keto sterol as its substrate, consistent with previous reports on the substrate specificity of cholesterol oxidases (Ref. 6 and references therein). With the monolayer system used, it appears that the over all rate-limiting step in the enzyme-catalyzed conversion of sterols to 4-en-3-one is the desorption of the sterol molecules from the interface into the active site of the enzyme. This appears to be true both with pure sterol monolayers as well as with mixed monolayers containing phospholipids.

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