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Kinetics of Cholesterol Oxidation by Cholesterol Oxidase

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ABSTRACT

Kinetic studies of cholesterol oxidation catalyzed by soluble cholesterol oxidase from *Brevibacterium* were conducted. The optimum temperature and pH were found to be 40–45°C and 7.0, respectively. A plot of initial reaction rate versus cholesterol concentration is sigmoidal in shape. Analysis of the data suggests that the reaction follows a concerted model and not a stepwise model.

Index Entries: Cholesterol; cholesterol oxidase.

Nomenclature: $[E_o]$, initial enzyme concentration; E_R, enzyme in strong binding (R) state; E_T, enzyme in weak binding (T) state; h, Hill coefficient; K_h, association constant of ES_h; K_m, Michaelis constant, mM; $K_{m,app}$, apparent Michaelis constant; K_{s1}, K_{s2}, dissociation constants in equation 2; K_{s1s2}, dissociation or composite constant in equation 2; K_R, dissociation constant of R state; K_T, dissociation constant of T state; $[S]$, $[S_1]$, $[S_2]$, substrate concentration; S_h, h number of substrate molecules bind to enzyme to form complex ES_h; R, strong substrate binding state; T, weak substrate binding state; v, initial reaction rate, mM/min; V_m, maximum reaction rate, mM/min; Y, fractional saturation of enzyme.

INTRODUCTION

Cholesterol oxidase from *Brevibacterium* is a flavin-dependent enzyme that catalyzes the oxidation and isomerization of 3β-hydroxy steroids.

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The enzyme contains one molecule of the cofactor flavin adenine dinucleotide (FAD) per mole of the protein. The highest enzyme activity is observed using cholesterol as a substrate. There are very limited kinetic data of the enzyme on steroid binding. Cheetham et al. (*1*) have studied the effect of pH on cholesterol oxidase from *Nocardia*. Other studies on cholesterol oxidase include the work of Smith and Brooks (*2,3*) and Hesslink et al. (*4*), again from *Nocardia*. In this article, the kinetics of cholesterol oxidase from *Brevibacterium* in the oxidation of cholesterol are examined.

MATERIALS AND METHODS

Materials

Ash-free cholesterol (C-3292) and cholesterol oxidase from *Brevibacterium* (C-8153) were obtained from Sigma (St. Louis, MO). The activity of cholesterol oxidase was measured in International units (U) and 1 U converts 1.0 mmol of cholesterol to 4-cholestene-3-one per min at pH 7.0 and at 37°C.

Preparation of Buffer Solution

A so-called biological buffer solution of pH 7.4 at 37°C, the same pH value as of human blood, was prepared by dissolving 1.360 g of KH_2PO_4 and 5.677 g of Na_2HPO_4 in 1 L of distilled and deionized water. After mixing this buffer with Triton X-100 at buffer to triton ratio of 9:1, we obtained a pH of 7.38–7.40 at 37°C. To investigate the pH effect on the enzyme activity at 37°C, buffers with different pH values but the same ionic concentration were prepared by adjusting the ratio of the two phosphate salts. The amount of Triton X-100 used was the same in all the experiments.

Preparation of Cholesterol Solution

Cholesterol solution was prepared according to the following procedure. Cholesterol (500 mg) was dissolved in 10 mL of Triton X-100 by slowly heating and stirring until the solution was clear; 90 mL of the prepared buffer solution was then added. This gave a cholesterol concentration of 500 mg/dL. Solutions of different concentrations were similarly prepared.

Preparation of Enzyme Solution

A known amount of enzyme was weighed and dissolved in the buffer solution to obtain a cholesterol oxidase concentration of 1 U/50 mL. The solution was made fresh just before each experiment.

Experimental Procedure

A Yellow Springs Instruments (YSI) Model 5300 Biological Oxygen Monitor (Yellow Springs, OH) was used in all the experiments. The system consisted of a 5300 Monitor, a 5301 Standard Bath Assembly and two 5331 Standard Oxygen Probes. A chart recorder was connected to the monitor to collect experimental data. An oxygen cylinder (99.6% oxygen) was used to supply oxygen to the experiments.

The oxygen solubilities of sample solutions were determined using a YSI Model 57 Oxygen Meter. For free cholesterol, 6 samples with cholesterol concentrations ranging from 0 to 700 mg/dL were tested. The oxygen solubilities ranged between 6.65 and 6.75 mg/L under air saturation at a temperature of 37°C. An average value of 6.7 mg/L was used in the analysis of experimental data.

The following procedure was used in reactions catalyzed by soluble cholesterol oxidase. Five mL of the cholesterol sample solution were injected into the reaction chamber, and the solution was saturated with oxygen under agitation. Between 10–50 μL (0.2–1.0 U) of the cholesterol oxidase solution was then injected. The oxygen consumption in the first 30 s was used to determine the initial reaction rates.

Experimental runs were also conducted at different oxygen concentrations but at a fixed cholesterol level (50–700 mg/dL). For these runs, the sample solutions were either partially or completely saturated with pure oxygen to bring the relative dissolved oxygen to a certain saturation level (30–100%), and the runs were conducted following the procedure specified above.

RESULTS AND DISCUSSION

A plot of the initial reaction rate vs pH for samples at an initial cholesterol level of 500 mg/dL, fully saturated with oxygen and at a temperature of 37°C, is shown in Fig. 1. The results indicate that the cholesterol oxidase exhibits the highest activity around a pH of 7.0. These results are in agreement with that of Cheetham et al. (*1*) for the enzyme from *Nocardia*.

The effect of temperature on activity is shown in Fig. 2. It can be seen that the initial reaction rate increases very rapidly with reaction temperature up to 40°C, followed by a slow increase up to 45°C, and then a rapid decrease.

All the experiments on kinetics were conducted at 37°C and a pH of 7.4, with a cholesterol oxidase concentration of 0.12 U/mL. The reactions were conducted at oxygen saturations of 100%. The lowest cholesterol concentration in the reaction was 50 mg/dL (1.3 m*M*). Below this level, the monitor showed an unstable response and the oxygen depletion was very

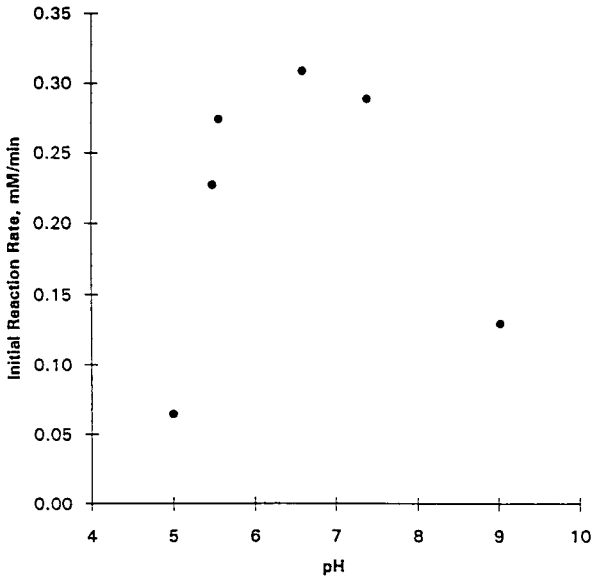

Fig. 1. Initial reaction rate vs pH of sample solutions. Experiment were performed at 37°C with cholesterol concentration of 500 mg/dL and cholesterol oxidase concentration of 0.12 U/mL.

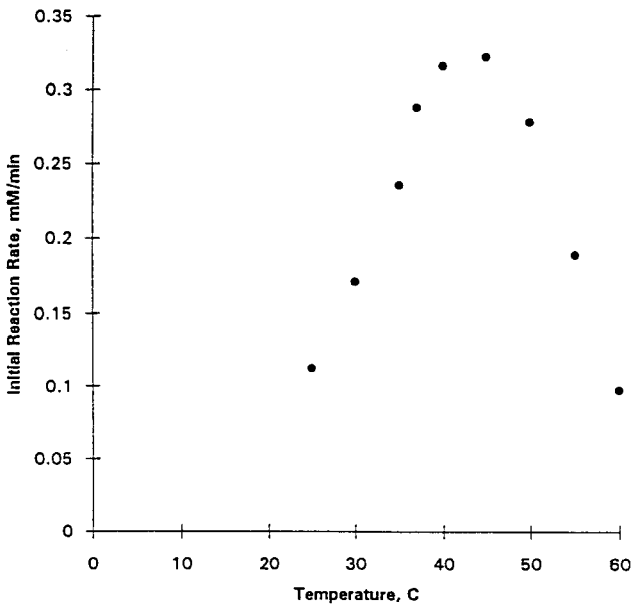

Fig. 2. Initial reaction rate vs temperature. Experiment were performed at pH of 7.4 with cholesterol concentration of 500 mg/dL and cholesterol oxidase concentration of 0.12 U/mL.

slow. The highest cholesterol concentration used in our runs was 750 mg/dL (19.4 m*M*), which was the highest cholesterol concentration we could obtain by this preparation procedure. Figure 3 shows that a plot of the initial reaction rate v vs cholesterol concentration is sigmoidal. The experiments were repeated a number of times to ensure reproducibility of

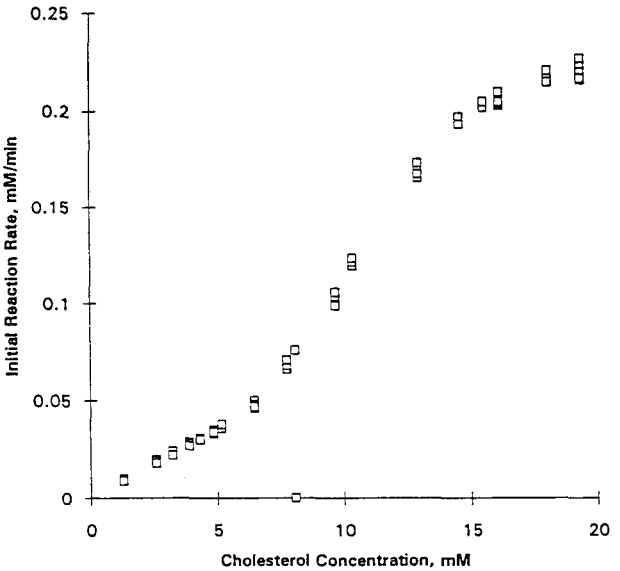

Fig. 3. Initial reaction rate vs cholesterol concentration. Samples were saturated with oxygen and experimental runs were conducted at 37°C and pH of 7.4. Cholesterol oxidase concentration was 0.12 U/mL. Reproducibility of data indicated by spread in square symbols.

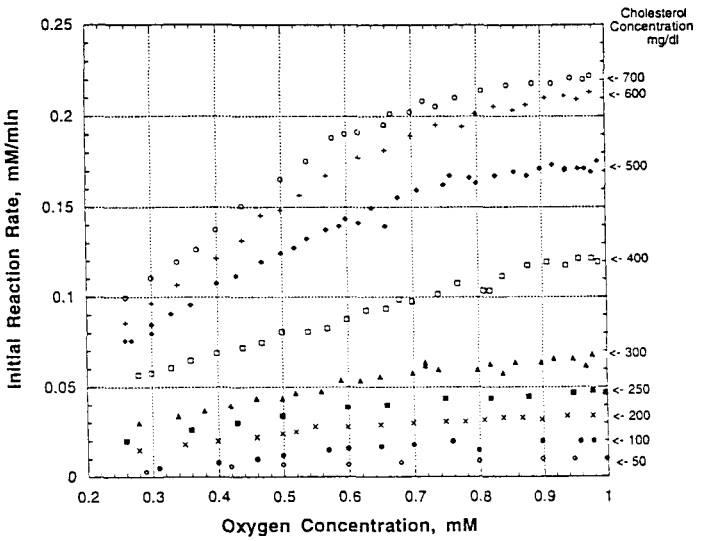

Fig. 4. Initial reaction rate vs oxygen concentration for different cholesterol concentrations. Experiments were conducted at 37°C and pH of 7.4. Cholesterol oxidase concentration was 0.12 U/mL.

data. The spread of data (indicated by □ on the plot) clearly suggests that the data are highly reproducible.

Figure 4 shows results of initial reaction rate vs oxygen concentration at fixed cholesterol concentrations. These runs were performed with the same cholesterol oxidase concentration under the conditions specified above. All the runs were conducted at oxygen levels greater than that corresponding to air saturation (21% oxygen).

Table 1
Values of V_m Calculated from Michaelis-Menten Kinetics

Cholesterol, mg/dL	50	100	200	250	300	400	500	600	700
V_m, m*M*/min	−0.296	−0.035	0.078	0.099	0.142	0.22	0.376	0.534	0.47
Corr. Coeff.	0.953	0.948	0.991	0.996	0.989	0.992	0.994	0.996	0.993

Initial examination of Fig. 4 suggests that the relationship between v and oxygen concentration at certain cholesterol levels may follow Michaelis-Menten kinetics. Table 1 shows the calculated V_m for certain cholesterol concentrations. Here V_m is the apparent maximum reaction rate described by Michaelis-Menten equation. The calculated maximum reaction velocities V_m appear to be unreasonable, especially at lower cholesterol concentrations. This suggests that the kinetic model may be more complex. The sigmoidal shape observed in Fig. 3 is not immediately obvious when the initial reaction rates are plotted as a function of oxygen concentration (Fig. 4). This is probably because the data sets are close to one another and the increase in reaction rate as a function of oxygen concentration is not so prominent as is the case in Fig. 3.

Cholesterol oxidase catalyzes the reaction

$$\text{Cholesterol} + O_2 \rightarrow \Delta^4 - \text{cholestenone} + H_2O_2 \quad (1)$$

This may be considered as a two-substrate reaction in which the binding of the substrates can be random, ordered, or follow a ping-pong mechanism. If the reaction of the two substrates is of the first degree for both random and ordered binding mechanisms, all the steps leading to the formation of a ternary complex are rapid and approach equilibrium, except in the breakdown of this complex, which is the rate-limiting step. A general rate equation can be written as

$$v = V_m/\{1 + (K_{s1}/[S_1]) + (K_{s2}/[S_2]) + (K_{s1s2}/[S_1][S_2])\} \quad (2)$$

If one of the substrates (say S_2) is set constant, the equation becomes

$$v = V_{m,app}[S_1]/(K_{m,app} + [S_1]) \quad (3)$$

where

$$V_{m,app} = V_m/(1 + K_{s_2}/[S_2]) \quad (4)$$

and

$$K_{m,app} = (K_{s1} + K_{s1s2}/[S_2])/(1 + K_{s2}/[S_2]) \quad (5)$$

If, instead of equilibrium, a quasi steady-state approach or ping-pong model is used, then the following initial rate expression is obtained:

$$[E_o]/v = \phi_1 + \phi_2/[S_1] \quad (6)$$

where ϕ_1 and ϕ_2 are related to the individual rate constants and are functions of S_2 as well. However, it is clear that neither of these general rate equations (Eq. 3 or 6) can explain the data of Fig. 3 (due to the sigmoidal

shape observed). Lineweaver-Burk plots of both Eqs. 3 and 4 should result in a straight line, but the data of Fig. 3 indicate that the reaction is a complicated one. Cholesterol oxidase from *Brevibacterium* is a flavin-dependent oxidase containing 1 mol of tightly bound flavin adenine dinucleotide (FAD) per mol of protein (*5*). The structure of the enzyme is made up of two domains: an FAD-binding domain and steroid-binding domain. The active site lies at the interface of the two domains, in a large cavity filed with a well-ordered lattice of 13 solvent molecules (*6*). The cavity is buried inside the protein molecule, but three hydrophobic loops at the surface of the molecule show relatively high temperature factors, suggesting a flexible region that may be a possible path by which the substrate could enter the cavity. This might be the reason that substrate does not bind in a depression, cleft, or gorge, but in an internal cavity which is sealed from the solvent (*5*).

The enzyme is believed to carry out the stoichiometric oxidation and isomerization steps as follows: cholesterol (5-cholestan-3-ol) binds to the enzyme (E-FAD); an oxidation step takes place by utilizing tightly bound FAD; this is followed by an isomerization step, which brings the transit state 5-cholesten-3-one to 4-cholesten-3-one. The reduced enzyme (E-$FADH_2$) is regenerated by molecular oxygen, yielding H_2O_2, hence, cholesterol oxidase could be classified as a dioxygenase. It has been suggested that the reaction follows a concerted mechanism or a stepwise procedure (*7*). As discussed above, the stepwise mechanism cannot describe our experimental data. The symmetry mechanism will be considered in the following paragraphs.

We decided to fit the data to the Hill equation in view of the sigmoidal shape of reaction rate vs cholesterol concentration in Fig. 3. If an enzyme binds h molecules of the substrate in a single step

$$E + hS \overset{K_h}{\rightleftharpoons} ES_h \tag{7}$$

then the concentration of the complex ES is given by

$$[ES_h] = K_h [E][S]^h \tag{8}$$

where K_h is the appropriate association constant.

The fractional saturation can be expressed as

$$Y = [ES_h]/([E] + [ES_h]) \tag{9}$$

Assuming that the protein exists solely in the two forms E and ES_h, and combining Eqs. 8 and 9, we get

$$Y = K_h[S]^h/(1 + K_h[S]^h) \tag{10}$$

This equation is known as Hill equation, which fits available data accurately, if the h values falls in the range 1.0–3.2 (*8*).

If Eq. 10 is rearranged as follows

$$\log(Y/(1-Y)) = \log K_h + h \log[S] \tag{11}$$

Table 2
Results of Hill Fitting for Data of Initial Reaction Rate
Versus Oxygen Concentration at Various Cholesterol Concentrations

Cholesterol concentration, mg/dL	200	250	300	400	500	600	700
Hill coefficient, h	*1.84*	*1.54*	*1.77*	*2.30*	*2.83*	*2.97*	*2.86*

then a plot of log $(Y/(1-Y))$ vs log[S] should be a straight line with slope h. This plot is known as a Hill plot and provides a simple means for evaluating h and K_h. It has been found to fit a wide variety of data remarkably well for values of Y in the range 0.1–0.9, but deviations always occur at the extremes because the equation is at best only an approximation of a more complex relationship.

The sigmoidal shape of Fig. 3 suggests a cooperative binding of substrate molecules to the enzyme; that is, at low cholesterol concentrations, the protein acts as if it is binding the substrate very weakly, and as more cholesterol is bound, the affinity becomes greater. There is no evidence in the literature that the regulation of cholesterol oxidase from *Brevibacterium* follows homoallostery. Hill was careful to disclaim any physical meaning for K_h and h, but many recent workers have hypothesized that h ought to be equal to n, the number of subunits in the fully-associated protein. They have accordingly been puzzled that h is generally a non-integer, and is rarely equal to n. The assumption that $Y = v/V_m$ is unfortunate, because it is difficult or impossible to prove. It has been suggested that this assumption is at least reasonably plausible (*8*).

Since $Y = v/V_m$, and replacing $1/K_h$ with K_m, Eq. 11 can be rewritten as:

$$\log(v/(V_m - v)) = h\log[S] - \log K_m \quad (12)$$

Since the Hill fitting will give reliable values of h only in the range of $Y = 0.1$ to 0.9, data that fall in this range (initial reaction rates corresponding to cholesterol concentrations from 150 to 625 mg/dL) from Fig. 3 were regressed. The value of h was found to be 2.8.

For rate vs oxygen concentration data at different cholesterol levels (*see* Fig. 4), regression based on a Hill plot was carried out for data sets with cholesterol concentration greater than 200 mg/dL (5.17 m*M*), to ensure that the cholesterol is in excess compared to oxygen. The h values from the regression are shown in Table 2. It is clear that h values from Hill fitting of rate vs cholesterol concentration is very close to that obtained by fitting rate vs oxygen concentration at high cholesterol concentration (greater than 500 mg/dL). It has been shown (*8*) that for any value of the association constants, h must approach unity at the extremes of [S].

As mentioned earlier, it has been suggested that the reaction follows a stepwise or concerted mechanism (*7*). Monod, Changeux, and Jacob (*9*) studied many examples of cooperative phenomena and concluded that the results can be attributed to conformational flexibility of the protein molecule. Subsequently, Monod, Wyman, and Changeux (*10*) proposed

Table 3
Parameters Used in the Mathematical Model Fitting in Eq. 13

n	K_R, mM	L	c
3	0.03	4E7	0.0005
4	0.03	1E10	0.0007
5	0.03	2.7E12	0.00085
6	0.03	5.2E14	0.00095

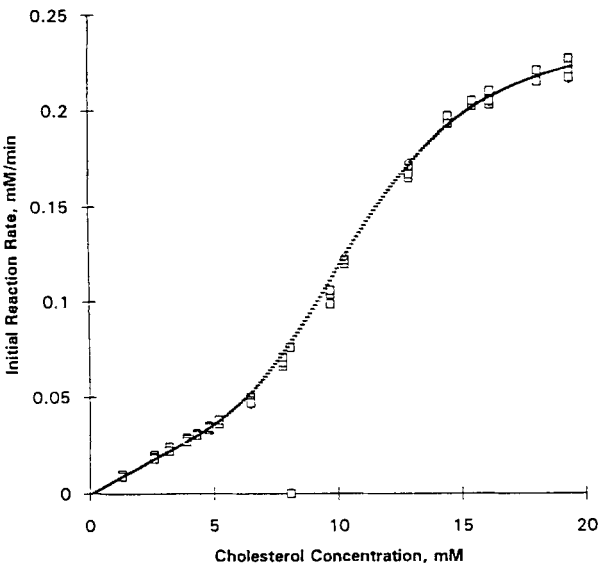

Fig. 5. Symmetry model fitting for initial reaction rate vs cholesterol concentration. Samples were saturated with oxygen and experiments were conducted at 37°C and pH of 7.4. Dashed line represents theoretical fit.

a general model (MWC model) and this has generally been called the symmetry model, to avoid any association between allosteric and cooperative phenomena. Remembering that $Y = v/V_m$, the fractional saturation based on the MWC model can be written as

$$v = \{L_c[S]/K_R(1+c[S]/K_R)^{n-1} + [S]/K_R(1+[S]/K_R)^{n-1}\}V_m/ \{L(1+c[S]/K_R)^n + (1+[S]/K_R)^n\} \quad (13)$$

In the above expression, $c = K_R/K_T$ and $L = [T_o]/[R_o]$. R (relaxed) and T (tense) are the two different conformations of the molecule, and K_R and K_T are the dissociation constants.

In our situation, setting $n = 3, 4, 5, 6$, a good fit was obtained when $n = 6$, compared to other n values. The K_R, L and c values for different n values are listed in Table 3. For $n = 6$, the calculated results and data from experiments are shown in Fig. 5, and there appears to be fairly good agreement between the two. The dashed line in Fig. 5 represents the theoretical

fit. The results simply suggest that kinetics probably follows a concerted (symmetry) model and not a stepwise mechanism, suggesting a cooperative binding of the substrate to the enzyme. However, it must be emphasized that there is no evidence from the literature that suggests that more than one binding site is present. Both the Hill equation and MWC model provide a good fit to the experimental data and provide an indication of the complex nature of the reaction. No physical meaning can be given to either the value of h or n obtained from the models.

CONCLUSIONS

The kinetics of cholesterol oxidation catalyzed cholesterol oxidase (from *Brevibacterium*) was investigated. The effects of pH and temperature on the enzyme were also determined. It was found that cholesterol oxidase has an optimum activity at a temperature of 40–45°C and pH of 7.0. The relationship between initial reaction rate and cholesterol or oxygen concentration is fairly complex and does not appear to follow Michaelis-Menten kinetics. A plot of initial reaction rate vs cholesterol concentration is sigmoidal in shape. Fitting of the data to the Hill equation gave a value of 2.8 for the Hill coefficient. Fitting of the data to the MWC equation suggests that the reaction follows a concerted (symmetry) model and not a stepwise model.

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