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Enzymatic Oxidation of Cholesterol in Reverse Micelles

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We explore the use of the microheterogeneous system of reverse micelles as the reaction medium for the enzymatic oxidation of cholesterol to cholestenone catalyzed by cholesterol oxidase. Toward this goal, the activity of the enzyme has been measured in three reverse micellar systems based on the anionic AOT, the cationic CTAB, and the nonionic Triton X-100 surfactants as a function of the pH, the molar ratio R of water-to-surfactant in the reverse micelles, and the surfactant concentration. The Michaelis–Menten kinetic constants k_{cat} and K_m have been estimated in the three reverse micellar systems and are compared to those obtained in the aqueous medium. For the AOT system, the kinetic constants have been estimated also as a function of R and the surfactant concentration. Further, the kinetic constants in the reverse micelles are related to those in the aqueous medium using a model which assumes the partitioning of the substrate between the three microdomains of the reverse micelle system (the water pool, the interface, and the organic continuous phase) as well as the physical partitioning of the enzyme and/or the noncompetitive inhibition of the enzyme by the surfactant in the interfacial microdomain.

I. Introduction

Enzymatic biosynthesis constitutes an attractive approach for the commercial production of important pharmaceuticals, food products, and fine chemicals such as steroids, peptides, lipids, aromatics, and long-chain alcohols. However, to realize the full potential of enzymatic biosynthesis, we need to resolve the dilemma that many organic substrates are water-insoluble while an aqueous environment is necessary to maintain enzymatic activity. An intriguing solution to this problem is offered by the microheterogeneous system of reverse micelles (Luisi and Laane, 1986). Reverse micelles are aggregates of surfactant molecules that are spontaneously generated in an oil–water system. They consist of microdomains of water dispersed in a continuous domain of oil, stabilized by surfactant molecules present at the interface. The enzyme can be solubilized in the aqueous domain of the reverse micelles and allowed to react with the substrate present in the oil domain. A number of studies in recent years have focused on solubilizing enzymes in reverse micelles and determining the enzymatic activity (Shield et al., 1986; Levashov et al., 1986; Luisi and Steinmann-Hofmann, 1987; Martinek et al., 1989; Gupte et al., 1995). Methods for recovering the solubilized enzymes from the reverse micelles have also been developed (Carlson and Nagarajan, 1992; Gupta et al., 1994).

An important class of reactions involving enzymatic biosynthesis of water-insoluble compounds is that of steroid conversion to pharmaceutically important products. The oxidation of cholesterol to cholestenone, catalyzed by cholesterol oxidase, is an example of a steroid conversion where a 3β-sterol is converted to the corresponding ketone.

$$\text{cholesterol} + {}^1/_2O_2 \xrightleftharpoons{\text{cholesterol oxidase}} \text{cholestenone} + {}^1/_2H_2O_2$$

The coproduct hydrogen peroxide which can deactivate the enzyme can be removed as soon as it is produced by the action of the enzyme catalase via a decomposition reaction.

$$H_2O_2 \xrightleftharpoons{\text{catalase}} H_2O + {}^1/_2O_2$$

Cholestenone is a precursor of androst-1, 4-diene-3,17-dione which can be chemically modified to manufacture oral contraceptives. Cholesterol oxidase also oxidizes other 3β-steroids such as β-cholestanol, pregnelone and phytosterols, dehydroisoandrosterone, and ergosterol. This offers a relatively simple process for the preparative oxidation of 3β-alcohols. Also, the oxidation of the 3β-hydroxyl group is used as an analytical method for the detection of cholesterol in body fluids.

The oxidation of cholesterol has been investigated in various kinds of media where the organic phase constitutes a significant fraction of the total volume. Aleksandrovskii (1987) used an aqueous–organic media made up of a true molecular solution of the aqueous buffer and varying amounts of 2-propanol. Such miscible water–organic solvent systems are not capable of solubilizing highly lipophilic substrates like cholesterol to an appreciable extent and thus have limited potential as reaction media. Khmelnitsky et al. (1988) studied the oxidation of cholesterol in the so-called "detergentless microemulsions" formed in ternary systems composed of 2-propanol, hexane, and water in the absence of any surfactant. This reaction medium suffers from the drawback that the maximum amount of enzyme solubilized is limited to about 1 μM. In addition, the enzyme stability in this medium is severely affected at room temperature, and complete conversion can be achieved only if the reaction is carried out at very low temperatures. Cholesterol oxidation has been studied also in supercritical carbon dioxide (Randolph et al., 1988). The main limitation of this reaction medium stems from the fact that the solubility of cholesterol is increased only 5–10-fold in supercritical fluids compared to that in the aqueous medium. In contrast, a 10 000-fold increase in solubility is possible in reverse micelles.

Lee and Biellmann (1986) studied the enzymatic oxidation of cholesterol in reverse micelles. They com-

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pared the maximum velocity and the Michaelis–Menten constant for the reaction in aqueous medium to those in reverse micelles composed of the anionic surfactant SDS (sodium dodecyl sulfate), cationic surfactant CTAB (cetyltrimethylammonium bromide), and the nonionic surfactant Triton X-100 (isooctylphenoxypolyoxyethanol). Though the maximum velocity (or k_{cat}) in the reverse micelles was of the same order of magnitude as in the buffer, the Michaelis–Menten constant (K_m) was found to be higher by 3 orders of magnitude in the reverse micelles. The enzyme was rapidly inactivated in the anionic SDS reverse micelles but retained its activity for appreciable lengths of time in the cationic and the nonionic reverse micelles.

Doddema et al. (1987) used a continuous stirred-tank reactor and a plug-flow reactor for carrying out the enzymatic oxidation of cholesterol using cholesterol oxidase. In this study, the enzyme was solubilized in reverse micelles formed by the CTAB–octane–hexanol–buffer system. They found that the operational stability of the enzyme was less than desirable and the half-life of the enzyme was only 20 h.

Recently, Bru and co-workers (1989a) investigated the kinetics of this reaction in reverse micelles composed of the anionic surfactant AOT (sodium bis(2-ethylhexyl) sulfosuccinate) in isooctane. They studied the dependence of the enzymatic reaction rate constant on the micelle size and found that the enzymatic activity in reverse micellar systems approaches that in the aqueous medium as the micelle size increases. In the absence of measurements of enzymatic activity over a range of pH and R (the molar ratio of water-to-surfactant in the reverse micelle), they concluded that cholesterol oxidase is active only in the free water present in the aqueous core of the micelles while the enzyme partitioned into the layers of bound water near the surfactant polar heads does not exhibit any catalytic activity.

The principal goal of this work is to examine comprehensively the kinetics of enzymatic oxidation of cholesterol in reverse micelles. Toward this goal, the following objectives have been achieved: (i) We compare the enzymatic activity in reverse micelles composed of the anionic surfactant AOT, the cationic surfactant CTAB, and the nonionic surfactant Triton X-100 with the activity in the aqueous medium. (ii) We investigate the combined effect of both pH and R on the Michaelis–Menten kinetic constants k_{cat} and K_m and thereby obtain an optimal micellar size. (iii) We determine the kinetic constants k_{cat} and K_m as a function of R as well as the surfactant concentration in the reverse micelles. (iv) We modify the "biphasic" model of enzyme kinetics in reverse micelles in order to relate the experimentally determined kinetic constants in the reverse micelles to the kinetic constants in the aqueous medium. The modification assumes the partitioning of the substrate between the water pool, the interface, and the organic continuous phase domains of the reverse micellar system. It also considers the partitioning of the enzyme and/or the noncompetitive inhibition of the enzyme by the surfactant in the interfacial domain. (v) We report preparative studies demonstrating a 100% conversion of cholesterol to cholestenone in AOT–isooctane reverse micelles and also show that the enzyme displays appreciable activity retention in the micelles.

II. Materials and Methods

Surfactants AOT and Triton X-100 were purchased from Aldrich, Milwaukee, WI; surfactant CTAB, octane, hexanol, cyclohexane, and 4-aminoantipyrine, were purchased from Fluka, Ronkonkomma, NJ; surfactant Thesit (poly(nonoxyethylene) lauryl ether), Trizma base, phenol, and cholestenone were purchased from Sigma Chemical Co., St. Louis, MO; enzymes cholesterol oxidase (EC 1.1.3.6) from *Nocardia erythropolis*, horse radish peroxidase (EC 1.11.1.7) Grade II, beef catalase (EC 1.11.1.6) and substrate cholesterol were purchased from Boehringer Mannheim, Indianapolis, IN; isooctane, HPLC-grade methanol, and silica quartz cuvettes were from Fisher Scientific, Pittsburgh, PA. All the solvents were obtained in UV spectroscopy grade. The aqueous component used throughout the experiments was a 0.05 M Tris-HCl buffer. The cholesterol oxidase suspension in ammonium sulfate was extensively dialyzed against buffers of different pH before its use in catalytic studies. The enzyme concentration was calculated using information specified by the manufacturer. All experiments were performed at 25 °C.

Kinetic Studies in Anionic AOT Reverse Micelles. (A) Solubilization of Enzymes in Reverse Micelles. The surfactant AOT was dissolved in isooctane, and this solution was used for the enzymatic reactions. The reverse micelles were prepared by adding varying volumes of aqueous buffer at the desired pH to the AOT–isooctane solution to obtain values of R ranging from 8 to 50. In addition to the enzyme cholesterol oxidase, the aqueous buffer also included the enzyme catalase in order to destroy the coproduct hydrogen peroxide generated from the cholesterol oxidation reaction.

(B) Activity Measurements. The substrate cholesterol was added to the AOT–isooctane–buffer system containing the solubilized enzymes cholesterol oxidase and catalase. The overall reaction mixture had a volume of 3.19 cm^3 and contained 2 μg of cholesterol oxidase and 4 μg of catalase. The cholesterol concentration in the reaction mixture was 2.91 mM. This concentration corresponds to the solubility limit of cholesterol in the solvent isooctane. Following the addition of cholesterol, the mixture was slightly shaken. The reaction was thus initiated. Initial velocities of enzymatic cholesterol oxidation were measured spectrophotometrically at 25 °C using a Gilford Response spectrophotometer (Corning Glassworks, Corning MI) by continuously recording the increase in absorbance at 240 nm, the absorption maximum of cholestenone. The activity was calculated on the basis of the specified molar absorption coefficient of cholestenone of 15.5 mM^{-1} cm. The enzymatic activity a is expressed in the figures as units/mg of enzyme, where one unit represents 1 μmol of cholestenone formed/min at 25 °C.

(C) Preparative Conversion of Cholesterol to Cholestenone. Cholesterol was preparatively converted to cholestenone in AOT–isooctane–buffer reverse micelles at optimum conditions of pH = 5.0 and $R = 8$. The overall reaction mixture volume was 20 cm^3 and contained 1.16 mg of cholesterol oxidase and 2 mg of catalase. The initial concentration of cholesterol in the system was 3 mM. The concentration of cholestenone was assayed at various times using HPLC (Perkin-Elmer) with a C_{18} reverse phase column employing methanol flowing at 1.5 mL/min as the eluant. The retention time for cholestenone was 9.47 min. A detection wavelength of 240 nm and a sample size of 5 μL were employed.

Kinetic Studies in Cationic CTAB Reverse Micelles. (A) Solubilization of Enzymes in Reverse

Micelles. The cationic surfactant CTAB was dissolved in octane, and this solution was used for the preparation of the reverse micelles. The reverse micelles were prepared by adding hexanol and varying amounts of buffer to a 0.186 M solution of CTAB in octane. The volume of hexanol added was one-tenth the volume of octane in the reaction mixture. The aqueous buffer at the desired pH containing cholesterol oxidase was added to the CTAB–octane–hexanol–buffer reverse micelles. Catalase was also added to destroy the hydrogen peroxide formed as a byproduct of the cholesterol oxidation reaction.

(B) Activity Measurements. The substrate cholesterol was added to the reverse micelles containing the solubilized cholesterol oxidase and catalase. The total volume of the reaction mixture was 3.19 cm^3. It contained 2 μg of cholesterol oxidase and 4 μg of catalase. The total concentration of cholesterol in the reaction mixture was 21.53 mM. The enzymatic activity was determined using absorbance measurements at 240 nm as described earlier.

Kinetic Studies in Nonionic Triton X-100 Reverse Micelles. (A) Solubilization of Enzymes in Reverse Micelles. Nonionic reverse micelles were prepared by the addition of hexanol and varying amounts of buffer at the desired pH to a solution of 0.177 M Triton X-100 in cyclohexane. Cholesterol oxidase in buffer at the desired pH was added to the reverse micelles. The total volume of the reaction mixture was 3.19 cm^3, and it contained 2 μg of cholesterol oxidase. The volume of hexanol used was 0.1 cm^3.

(B) Activity Measurements. Triton X-100 absorbs very strongly in the UV region, and this prevents the determination of the enzyme activity by monitoring the formation of cholestenone through absorption at 240 nm. Consequently, the activity measurements were done using a coupled enzyme assay (Lee and Biellman, 1986) in the presence of horse radish peroxidase. The method involves monitoring the reaction between the coproduct hydrogen peroxide and the reagent phenol which is catalyzed by horse radish peroxidase, using 4-aminoantipyrine as the redox indicator. The aromatic chromophore formed in this reaction absorbs at 500 nm, and by following the increase in this absorbance one can determine the activity of cholesterol oxidase with respect to the formation of cholestenone. The molar absorptivity of cholestenone at 240 nm is 15.5 mM^{-1} cm^{-1} while that of the aromatic chromophore formed in the coupled enzyme assay has a molar absorptivity of 6 mM^{-1} cm^{-1} (Allain et al., 1974). Therefore, to standardize the activity measurements, the concentrations of the reagents involved in the coupled assay (phenol, 4-aminoantipyrine, and peroxidase) were adjusted such that in the cationic reverse micelles, where there is no interference from the surfactant to the absorbance, the absorbance at 500 nm was 0.357 (the ratio between the two molar absorptivities) that at 240 nm. The overall concentrations of 0.1 M phenol, 0.3 mM 4-aminoantipyrine, and 20.96 units of peroxidase were used throughout the activity measurements, when the coupled enzyme assay was employed. The total volume of the reaction mixture was 3.19 cm^3. The concentration of cholesterol in the reaction mixture was 20.64 mM. Since peroxidase effectively destroys the hydrogen peroxide formed, the enzyme catalase was not necessary in the reaction mixture. As before, the activity of the enzyme is expressed as units/mg of enzyme, where one unit corresponds to the formation of 1 μmol of cholestenone/min at 25 °C.

Kinetic Studies in the Aqueous Medium. Since cholesterol has extremely low solubility in the aqueous phase, the activity of cholesterol oxidase was determined in the presence of either of two nonionic detergents Thesit or Triton X-100, which were used to increase the effective solubility of cholesterol in the aqueous medium.

(A) Activity Measurements in the Presence of Thesit. The aqueous phase was a 0.05 M Tris-HCl buffer containing 2 μg of cholesterol oxidase and 4 μg of catalase. The substrate cholesterol was added to this aqueous phase. In order to promote the solubility of cholesterol in water, approximately 3% by volume of 1-propanol and 4 mg/cm^3 of the nonionic detergent Thesit were added as suggested by the enzyme supplier. The total volume of the reaction mixture was 3.19 cm^3 as before. The concentration of cholesterol that could be reached in this reaction mixture was much larger than the aqueous phase solubility of 4.7 μM. On the addition of the substrate cholesterol, the reaction was initiated. The enzyme activity was determined by measuring absorbance at 240 nm as described previously.

(B) Activity Measurements in the Presence of Triton X-100. The activity of cholesterol oxidase in the aqueous phase was determined in the presence of the nonionic detergent Triton X-100 as suggested by Smith and Brooks (1974). The aqueous phase was a 0.05 M Tris-HCl buffer containing 2 μg of cholesterol oxidase and 4 μg of catalase. In order to promote the solubility of cholesterol in water, approximately 3% by volume of 2-propanol and 1 mg/cm^3 of the nonionic detergent Triton X-100 were added. The total volume of the reaction mixture was 3.19 cm^3 as before. The concentration of cholesterol that could be reached in this reaction mixture was much larger than the aqueous phase solubility of 4.7 μM. On the addition of substrate cholesterol, the reaction was initiated. The enzyme activity was determined via absorbance measurements at 240 nm as described above. One may note that the low concentrations of the detergent Triton X-100 added in these experiments allows the detection of the product cholestenone at 240 nm without requiring the coupled assay employed in the case of Triton X-100 reverse micelles.

Method of Conducting Kinetic Measurements. Before initiating a systematic kinetic study, we tested whether different procedures of contacting the substrate and the enzyme will affect the initial rate of the enzymatic reaction. For this purpose, experiments were carried out in AOT–isooctane–Tris-HCl buffer reverse micelles at a pH of 7.5 and water content specified by $R = 20$. Four contacting procedures were tested, each requiring a different sequence of molecular events to take place before the enzyme and the substrate come into contact and the reaction can occur.

In the first contacting technique, the substrate cholesterol was added to the enzyme already present in the reverse micelles. This necessitated the substrate to partition between the water core of the micelles, the surfactant region constituting the interface, and the organic continuous phase before the reaction could take place.

In the second contacting procedure, the aqueous buffer containing the enzyme was added to cholesterol already present in the reverse micelles. This requires

the incorporation of the enzyme in the water pool of the preformed reverse micelles before the reaction could take place.

The third contacting method involved the addition of a solution of cholesterol and AOT in isooctane to the reverse micelles containing the enzyme. In this case, the further addition of AOT to the preexisting reverse micelles results in an increase in the number and a decrease in the size of the water droplets containing the enzyme. Thus, the exchange of water molecules between the micelles has to occur. Simultaneously, the substrate cholesterol has to partition between the various microdomains of the reverse micelles.

In the last contacting procedure used, reverse micelles containing cholesterol were contacted with reverse micelles containing the solubilized enzyme. In this situation, a rearrangement of the water pools and a redistribution of the enzyme in the water pool of the resultant reverse micelles should occur prior to the reaction. Also, the substrate has to simultaneously repartition into the re-formed reverse micelles.

For the four contacting procedures tested, there was no difference between the observed rates of the enzymatic reaction. The second contacting procedure described above was employed in all the kinetic studies in the present work. However, the experiments investigating the storage stability of the enzyme employed the first contacting method where substrate was added to the reverse micelles containing the enzyme.

Representation of Kinetic Data. Initial rate kinetics of enzyme-catalyzed reactions follow a substrate-saturation mechanism, well-known as the Michaelis–Menten kinetics.

$$E + S \underset{k_-}{\overset{k_+}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Here, E, S, ES, and P represent the concentration of enzyme, the substrate, the enzyme–substrate complex and the product, respectively, while the various phenomenological rate constants are denoted by k_+, k_-, and k_{cat}. Using the quasi steady-state approximation, one can represent the initial rate kinetics by the relation

$$v = -\frac{dS}{dt} = \frac{v_{max}S}{K_m + S} = \frac{k_{cat}E_oS}{K_m + S} \quad \text{where}$$

$$K_m = \frac{k_- + k_{cat}}{k_+} \quad (1)$$

K_m is the steady-state dissociation constant of the enzyme–substrate complex, also known as the Michaelis constant, v is the reaction velocity, and $v_{max} = k_{cat}E_o$ is the maximum velocity attained at saturating substrate concentrations. E_o refers to the total concentration of the enzyme within the system, i.e., the sum of the free enzyme, E, and that bound to the substrate, ES. The experimentally measured enzyme activity a (expressed as units/mg of enzyme) is related to the reaction velocity v (expressed as mM/s) through the conversion relation, $v = (10^{-3}\ a)E_oM_E/60$, where E_o is the concentration of the enzyme (expressed as mM) and M_E is the molecular weight of the enzyme (59 000 for cholesterol oxidase, as specified by the manufacturer). By fitting the observed reaction velocity v as a function of the substrate concentration S to eq 1 at a given enzyme concentration E_o, we can obtain the kinetic constants K_m and k_{cat}. Since the enzymatic reactions in reverse micelle media involve the presence of various microdomains, the kinetic constants obtained by fitting experimental activity data and the overall substrate and enzyme concentrations ($S_{o,t}$ and $E_{o,t}$) to eq 1 will be referred to as the apparent kinetic constants, denoted by $k_{cat,app}$ and $K_{m,app}$, respectively.

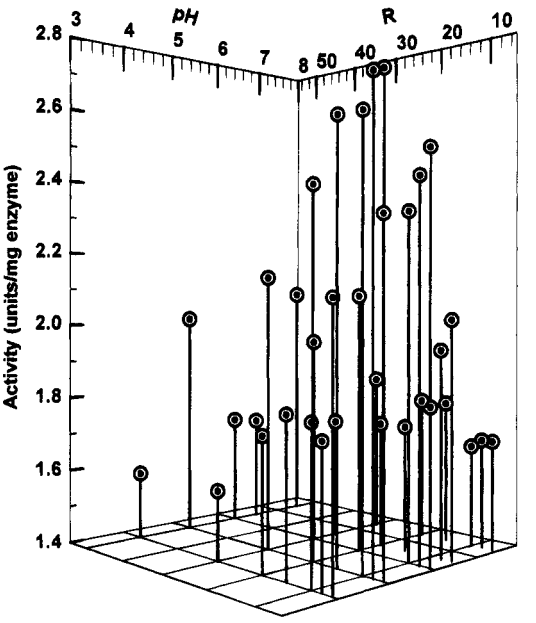

Figure 1. Specific activity of cholesterol oxidase in the 0.068 M AOT–isooctane reverse micelle system at various values of pH and the molar ratio R of water-to-surfactant in the reverse micelle. The specific activity can be converted to the reaction velocity v through the relation described in the text. The overall initial concentration of the substrate cholesterol is 2.91 mM.

III. Results and Discussion

Effect of pH and R on the Enzyme Activity in AOT Reverse Micelles. The activity of cholesterol oxidase was studied in 0.068 M AOT–isooctane reverse micelles as a function of the pH and the molar ratio R of water-to-surfactant in the reverse micelles. The concentration of the substrate cholesterol in the overall reaction mixture was 2.91 mM, which is the saturation solubility of cholesterol in isooctane. The local concentration of cholesterol in the vicinity of the enzyme will be much lower than this overall concentration because of the partitioning of cholesterol between the water pool, the interface, and the organic continuous phase. One may expect the local concentration of cholesterol in the aqueous core to be comparable in magnitude to (but smaller than) the aqueous phase solubility of 4.7 μM. The measured activities of cholesterol oxidase at various values of pH and R are presented in Figure 1. The enzyme activities in reverse micelles are compared to that in the aqueous medium in Figure 2 as a function of the pH. For determining the enzyme activity in the aqueous medium, the nonionic detergent Thesit was added to the aqueous phase to enhance the solubility of cholesterol, and a cholesterol concentration of 0.323 mM was employed (approximately 70 times larger than the solubility in water). The amount of enzyme and the total volume of the reaction mixture were the same as those in the experiments with the reverse micelles. If the low local concentration of cholesterol in the micellar core is taken into account, the results show that the measured enzymatic activity in AOT reverse micelles is appreciable compared to that in the aqueous medium.

For values of R less than 30, an optimum in the pH of about 5 can be discerned from Figure 1, which is lower

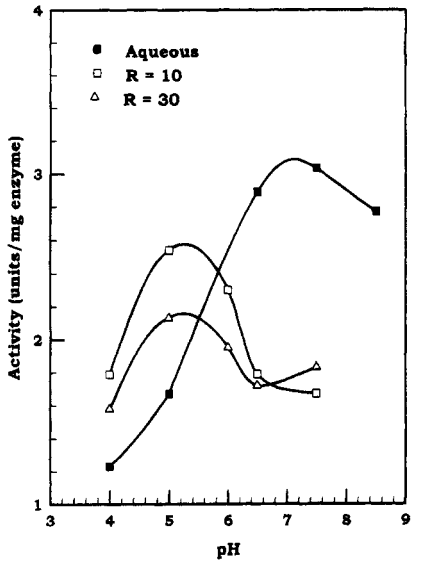

Figure 2. Effect of pH on the specific activity of cholesterol oxidase in the anionic 0.068 M AOT–isooctane reverse micelles for an overall initial cholesterol concentration of 2.91 mM. Also shown is the activity in the aqueous phase where a small amount of the nonionic surfactant Thesit is added to enhance the concentration of cholesterol to 0.323 mM compared to the aqueous phase solubility of 4.7 μM.

than the optimum pH of 7.5 for the reaction in the aqueous solution (Figure 2). This acidic shift in the pH profile is in accordance with the theory developed by Ruckenstein and Karpe (1990, 1991) for the variation of enzymatic activity with pH and R in ionic surfactants. According to this theory, the electrical double layer set up by the ionic groups of the surfactants in the reverse micelle leads to a pH distribution inside the water pool. Solving the Poisson–Boltzmann equation for this case, Ruckenstein and Karpe showed that the pH distribution in the water pool of reverse micelles composed of AOT was dependent on R and the method of preparation of the reverse micelles. In the case of the phase-transfer method of reverse micellar preparation, the local pH in the water pool is more acidic than the stock solution pH. However, in the case of the more widely used injection technique, the water pool of the reverse micelles is found to be more alkaline at the center and more acidic at the micellar surface than the stock buffer solution used for reverse micellar preparation. Since the injection method has been used throughout the present study, and the enzymatic oxidation is confined to the water pool, the increased alkalinity in the micellar core causes an acidic shift in the pH profile. The apparently contradictory alkaline shift in pH profile observed in earlier studies of enzymatic reactions in reverse micelles is now known to be due to slight amounts of acidic impurities present in the AOT samples (Luisi et al., 1988).

However, it is apparent from Figure 1 that for larger values of R ranging from 30 to 50, there is a tendency for the optimum pH in the reverse micelles to shift toward the optimal pH of 7.5 in the aqueous medium. The microenvironment of the enzyme in the reverse micelle thus differs from that in the aqueous medium only at low water content of the reverse micelles. This observation can be rationalized on the basis that a minimum of eight water molecules are required to hydrate one molecule of AOT. This water layer hydrating the polar head groups of the surfactant constitutes the bound water layer. As R increases beyond 8, the excess water constitutes the free water in the aqueous micellar core. The thickness of the bound water shell for AOT reverse micelles is estimated to be about 5 Å at low values of R and less than 3 Å at higher values of R (Luisi and Magid, 1986). The bound water exhibits a much higher microviscosity and lower polarity or dielectric constant compared to the free water (Wong et al., 1977). This marked difference in the properties of the bound water near the surfactant head groups and the free water leads to a difference in the catalytic behavior of the enzyme as a function of R. As R increases, the dielectric constant and the properties of water in the micelle core approach that of bulk water. Therefore, at large values of R, the increase in the proportion of free water in the micellar core causes the enzyme to experience an environment that increasingly resembles that in the aqueous medium.

The effect of R on the activity of enzymes in reverse micelles has been an active subject of theoretical studies (Kabanov et al., 1988; Bianucci et al., 1990; Bru et al., 1989b, 1990; Ruckenstein and Karpe, 1989, 1990; Maestro and Walde, 1992). Several investigations of enzymatic activity in reverse micelles have shown that enzymes in reverse micelles exhibit an optimum in the activity at a certain value of the ratio R. This could be in the form of a characteristic "bell-shaped" curve or a monotonic increasing or decreasing dependence on R. From Figure 1 it is apparent that for pH values of 6.5 and below, as R decreases, the activity of cholesterol oxidase increases and the highest activity is observed at the lowest value of $R = 6$. It is believed that at the optimum value of R, the enzyme attains a biologically active conformation causing it to exhibit increased activity. The high catalytic activity exhibited by cholesterol oxidase at a value of R where the AOT polar head groups are not completely hydrated shows that the enzyme is most active when the water domain reflects the properties of the bound water with the microscopic dielectric constant (or equivalently the micropolarity) being lower than that of bulk water phase. For pH = 7.5, the enzyme activity increases with increasing R in contrast to the behavior observed for pH $\leq$ 6.5.

The measured activities show that the optimum enzyme activity occurs at pH = 5 and $R = 8$, in contrast to the conclusions reached in the earlier study by Bru and co-workers (1989a). They observed a pH optimum of 7.5 which is in accord with our studies at large values of R. Further, the investigation of the effect of R on the enzymatic activity at pH = 7.5 showed an increase in enzymatic activity with increasing R as is observed in this study. This led them to the conclusion that the enzymatic activity is the highest in free water. One may note from Figure 1 that at pH = 5, the enzymatic activity increases with decreasing R in direct contrast to the behavior at pH = 7.5. Thus, the combined effect of pH and R over a wider range investigated in the present study allows the identification of the micellar size and pH corresponding to optimal enzyme activity.

Effect of pH and R on the Enzyme Activity in CTAB Reverse Micelles. The specific activity of cholesterol oxidase was studied as a function of pH and R in cationic reverse micelles formed with CTAB–octane–hexanol–buffer system. The cholesterol concentration in the reaction mixture was 21.53 mM which corresponds to the saturation solubility of cholesterol in this system. The pH profile of the enzyme is shifted toward alkaline values in the cationic reverse micelles and the optimum occurs at a pH of 8.5 (Figure 3). However, since the enzyme catalase is rapidly inacti-

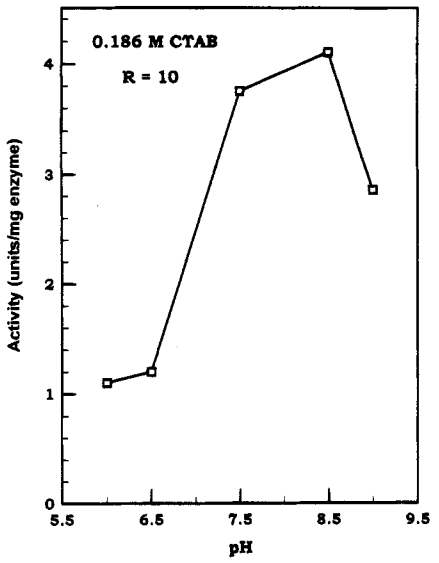

Figure 3. Effect of pH on the specific activity of cholesterol oxidase in the cationic 0.186 M CTAB–octane–hexanol reverse micelles at R = 10. The overall initial concentration of the substrate cholesterol is 21.53 mM.

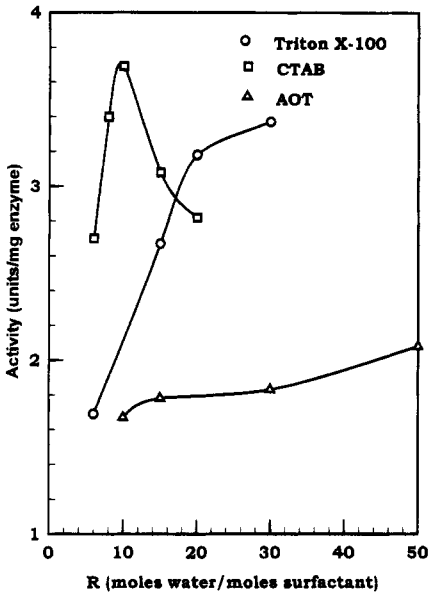

Figure 4. Effect of R on the specific activity of cholesterol oxidase at pH = 7.5 in the cationic 0.186 M CTAB–octane reverse micelles (overall initial concentration of cholesterol is 21.53 mM), the nonionic 0.177 M Triton X-100–cyclohexane reverse micelles (overall initial concentration of cholesterol is 20.64 mM), and the anionic 0.068 M AOT–isooctane reverse micelles (overall initial concentration of cholesterol is 2.91 mM).

vated at alkaline pH, the kinetic studies described in Figure 4 were conducted at a pH of 7.5. Figure 4 shows the characteristic bell-shaped curve for the enzymatic activity as a function of R and the maximum specific activity of cholesterol oxidase occurs at R = 10.

Effect of R on the Enzyme Activity in Triton X-100 Reverse Micelles. The specific activity of cholesterol oxidase was studied as a function of R in nonionic reverse micelles formed with Triton X-100–cyclohexane–hexanol–buffer system, at a pH of 7.5 and cholesterol concentration of 20.53 mM. These results are also included in Figure 4. Since nonionic reverse micelles are not expected to cause a change in the pH profile of the enzyme, the pH optimum of the enzyme in Triton X-100 reverse micelles can be intuitively expected to be the same (i.e., 7.5) as in the aqueous

Table 1. Michaelis–Menten Parameters $k_{cat,app}$ and $K_{m,app}$ for the Oxidation of Cholesterol by Cholesterol Oxidase

system	pH	R	$k_{cat,app}$ (s^{-1})	$K_{m,app}$ (mM)
Aqueous Medium				
(1 mg/mL Triton X-100)	7.5	na[a]	9.381	0.008
(4 mg/mL Thesit)	7.5	na	9.962	1.3215
Reverse Micelles				
0.186 M CTAB in octane–hexanol	7.5	6	4.55	2.408
0.177 M Triton X-100 in cyclohexane–hexanol	7.5	20	3.792	4.885
0.068 M AOT in isooctane	5	8	3.071	0.515
Effect of R in AOT Reverse Micelles				
0.068 M AOT in isooctane	5	30	2.566	0.182
0.068 M AOT in isooctane	5	15	2.76	0.29
0.068 M AOT in isooctane	5	8	3.071	0.515
Effect of AOT Concentration				
0.068 M AOT in isooctane	5	8	3.071	0.515
0.099 M AOT in isooctane	5	8	2.505	1.649
0.131 M AOT in isooctane	5	8	1.87	2.087
0.164 M AOT in isooctane	5	8	1.391	2.206

[a] na refers to not applicable.

medium (Balasubramaniam, 1981). The specific activity of cholesterol oxidase was found to increase with increasing R (Figure 4). The maximum water uptake in these reverse micelles corresponded to R = 30, beyond which any further addition of water caused a phase separation. The increase in enzyme activity with increasing R in Triton X-100 reverse micelles is in agreement with the results obtained earlier by Lee and Biellmann (1986) for the same system.

Estimation of $k_{cat,app}$ and $K_{m,app}$ in Reverse Micelles from Activity Data. The kinetics of cholesterol oxidation to cholestenone was studied as a function of the substrate concentration in two aqueous buffer systems (one containing Triton X-100 and the other containing Thesit, which are added to increase the solubility of cholesterol in water) and in reverse micelles composed of anionic, cationic, and nonionic surfactants. In all the systems studied, the cholesterol oxidation followed the classical Michaelis–Menten kinetics. The apparent or observed values of the first-order rate constant $k_{cat,app}$ and the Michaelis constant $K_{m,app}$ were determined by fitting the measured activity at various substrate concentrations to eq 1 and the results are summarized in Table 1.

The results show that $K_{m,app}$ in AOT reverse micelles is 100-fold higher than that in the aqueous medium containing Triton X-100, while in the CTAB and Triton X-100 reverse micelles, it is 1000-fold higher than that in the aqueous medium. It is important to note that the values of the Michaelis–Menten kinetic constants in the buffer system are not true aqueous phase kinetic constants since the presence of the added 2-propanol and the detergents Triton X-100 or Thesit permit a 100-fold increase in the solubility of the substrate cholesterol compared to its aqueous phase solubility of 4.7 μM. The larger value of K_m for CTAB and Triton X-100 reverse micelle systems compared to the AOT system is due to the increased partitioning of the substrate cholesterol in the interface and the organic continuous phase in the CTAB and Triton X-100 reverse micelles due to the presence of hexanol.

The large value of the Michaelis constant K_m for reverse micelles does not affect the velocity of the reaction at high substrate loadings which are used in industrial situations. The enormous increase in overall substrate solubility in reverse micelles more than

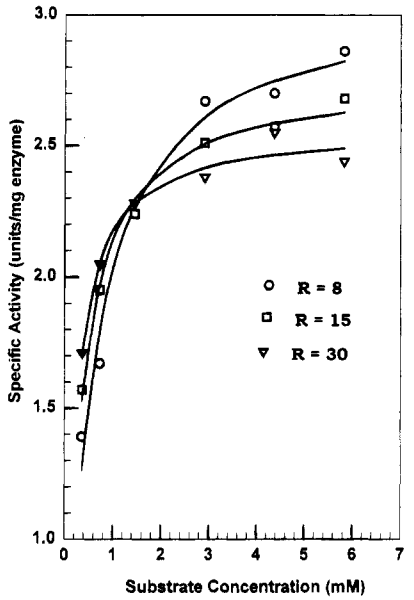

Figure 5. Correlation of experimental activity data by the Michaelis–Menten kinetic scheme (eq 1) for the oxidation of cholesterol to cholestenone in 0.068 M AOT–isooctane reverse micelles at pH = 5 and at various values of the molar ratio R of water-to-surfactant.

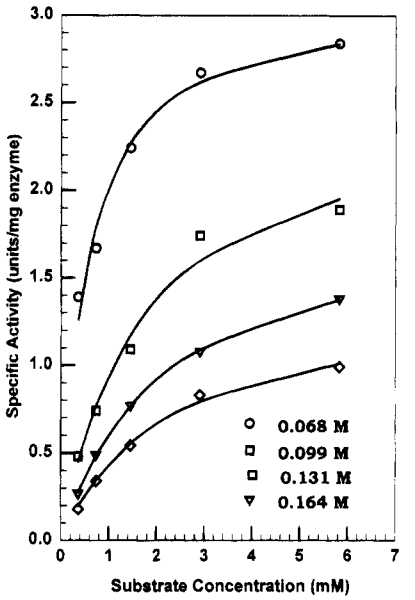

Figure 6. Correlation of experimental activity data by the Michaelis–Menten kinetic scheme (eq 1) for the oxidation of cholesterol to cholestenone in AOT–isooctane reverse micelles at R = 8 and pH = 5, at various values of the AOT concentration.

compensates for the slight lowering of the maximum velocity in the reverse micelles. Consequently, it is possible to obtain much greater yields in the same reaction volume by using reverse micelles as a medium for the enzyme-catalyzed synthesis of water-insoluble organic compounds.

The results summarized in Table 1 show that $k_{cat,app}$ in the reverse micelles is lowered 2–4-fold compared to that in the aqueous media, depending on the surfactant–solvent system used for the formation of the reverse micelles. A difference in k_{cat} indicates that there is an intrinsic change in the activity of the enzyme upon incorporation into the reverse micelles. This is in contradiction with the original "biphasic" model of enzyme kinetics in reverse micelles developed by Martinek et al. (1981) according to which the apparent rate constant $k_{cat,app}$ must equal the true rate constant $k_{cat,wp}$ in the aqueous phase.

Dependence of $k_{cat,app}$ and $K_{m,app}$ on R in AOT Reverse Micelles. We have examined the influence of R on the apparent kinetic constants, $k_{cat,app}$ and $K_{m,app}$. Figure 5 shows the fitting of experimental activity data to eq 1 for 0.068 M AOT reverse micelles at a pH of 5 and at three different values of R. The estimated kinetic constants are listed in Table 1. As R increases from 8 to 30, $k_{cat,app}$ decreases from 3.071 to 2.566 while $K_{m,app}$ decreases from 0.515 to 0.182. This behavior possibly originates from the fact that the proportion of free water molecules in the aqueous domain of the reverse micelle increases with increasing R. As mentioned before, R = 8 corresponds to the number of water molecules necessary to fully hydrate the surfactant polar group. At R = 8, the effective polarity or the dielectric constant of the water domain is smaller compared to that of bulk water and allows for larger solubility of the substrate in the domain. This could account for the larger value estimated for $K_{m,app}$ at R = 8 and for the decrease in $K_{m,app}$ toward the value in the aqueous phase as R increases.

The variation in $k_{cat,app}$ with R is probably due to the difference in the activity of the enzyme depending on whether the water core reflects the properties of bound water or free water. As R increases above a value of 8, the amount of free water present in the micellar core increases. Evidently, cholesterol oxidase exhibits the highest activity when the water core reflects the characteristics of bound water adhering to the surfactant polar head groups. An increase in the proportion of free water caused by an increase in R results in a decrease in enzymatic activity. As R decreases, the enzyme is experiencing an effective decrease in the size of the micelle it is solubilized in. This could potentially alter the exposed aromatic chromophores on the surface of the enzyme, or more importantly could change the backbone conformation of the enzyme, altering its intrinsic catalytic activity and thus $k_{cat,app}$ with R. Spectroscopic studies have revealed significant conformational differences in enzymes at low water contents of the reverse micelles (Luisi and Steinmann-Hofmann, 1987). Indeed, UV spectroscopy has shown that at low values of R, cholesterol oxidase exhibits significant conformational differences in block copolymer microdomains which are analogs of reverse micelle structures (Gupte et al., 1991). Interestingly, in the block copolymer microdomains, cholesterol oxidase was found to exhibit the highest activity at the lowest value of R. It is thus plausible that conformational differences of the enzyme are responsible for the dependence of the enzyme activity on R.

Dependence of $k_{cat,app}$ and $K_{m,app}$ on AOT Concentration. To determine the effect of surfactant concentration on the kinetic constants $k_{cat,app}$ and $K_{m,app}$ in AOT reverse micelles, experiments were conducted at pH = 5.0 and R = 8 where optimal enzymatic activity was observed. Figure 6 is an activity plot according to eq 1 at various AOT concentrations. The estimated kinetic constants are included in Table 1. As the AOT concentration is increased from 60 to 160 mM (which is the typical range used to formulate AOT reverse micelles in the literature), $K_{m,app}$ increases from 0.515 to 2.21, whereas $k_{cat,app}$ decreases from 3.1 to 1.4 s^{-1}.

The above dependences of $k_{cat,app}$ and $K_{m,app}$ on the surfactant concentration are not described by the original "biphasic" model of Martinek et al. (1981). This model assumes the micellar system to be composed of

two pseudophases, the aqueous core and the organic continuous phase, and does not account explicitly for the presence of the interface composed of the surfactant. In a latter paper, Martinek and co-workers have extended the "biphasic" model by considering the interfacial domain as another pseudophase (Khmelnitsky et al., 1990). They postulate that substrates are not only capable of partitioning between the organic continuous phase and the aqueous core but also into the interface composed of the surfactant. In the case of cholesterol, a substantial amount of the substrate is likely to be present in the interface and this partitioning of the substrate at the interface accounts for the variation in the value of $K_{m,app}$ with surfactant concentration. However, the "triphasic" model which considers the enzyme to be present entirely within the water pool cannot explain the observed variation in the pseudo-first-order rate constant $k_{cat,app}$ with surfactant concentration. A simple modification to this kinetic model (discussed in the following section) allowing for the partitioning of the enzyme into the interfacial domain and/or the noncompetitive inhibition of the enzyme by the surfactant can explain the effect of surfactant concentration on $k_{cat,app}$.

Modified Kinetic Model for Enzymatic Reactions in Reverse Micelles. The model discussed below is based on the following three considerations. Firstly, we assume that the reverse micellar system is composed of three microdomains: the micellar core which includes both the free water and the water bound to the surfactant head groups, the interfacial domain which is constituted of the surfactant molecules and the organic solvent continuous phase. Secondly, we assume that the enzyme is not confined to the micellar core but is also partitioned into the interface. This is consistent with the fact that the enzyme used in the present work, viz., cholesterol oxidase from *Norcardia* has a bimodal amphipathic structure with separate hydrophilic and hydrophobic domains linked by a proteinase-sensitive region. It is this hydrophobic anchor region that facilitates binding to hydrophobic domains of detergent micelles (Cheetham et al., 1982). Thirdly, we assume that no reaction takes place in the interface since the active site is not conformationally accessible to the substrate in this region. Thus, the enzyme within the interfacial domain is inactive.

On the basis of these assumptions, we establish relationships between the Michaelis–Menten kinetic constants in the water pool and the apparent or experimentally measured kinetic constants in the reverse micelles. In the equations below, the subscripts "wp" refers to the micellar core, "I" to the micellar interface, "org" to the organic continuous phase, and "t" to the overall or total system. θ refers to the volume fractions of various domains in the reverse micellar system. Since the enzyme is present both in the micellar core and the interface, a mass balance on the enzyme yields

$$E_{o,t} = E_{o,wp}\theta_{wp} + E_{o,I}\theta_I \tag{2}$$

From a substrate mass balance, we can write

$$S_{o,t} = S_{o,wp}\theta_{wp} + S_{o,I}\theta_I + S_{o,org}\theta_{org} \tag{3}$$

The overall reaction velocity can be written similar to eq 1 but in terms of the apparent kinetic constants and the overall concentrations based on the total system volume as

$$v_t = \frac{k_{cat,app}E_{o,t}S_{o,t}}{K_{m,app} + S_{o,t}} \tag{4}$$

Introducing eqs 2 and 3 in eq 4, we get

$$v_t = \frac{k_{cat,app}[E_{o,wp}\theta_{wp} + E_{o,I}\theta_I][S_{o,wp}\theta_{wp} + S_{o,I}\theta_I + S_{o,org}\theta_{org}]}{K_{m,app} + [S_{o,wp}\theta_{wp}0 + S_{o,I}\theta_I + S_{o,org}\theta_{org}]} \tag{5}$$

Since the reaction is assumed to be confined to the water pool, we can also write

$$v_t = v_{wp}\theta_{wp} = \frac{k_{cat,wp}E_{o,wp}S_{o,wp}}{K_{m,wp} + S_{o,wp}}\theta_{wp} \tag{6}$$

where v_{wp} is the reaction velocity in the aqueous core of the micelle. Comparing eqs 5 and 6, the apparent and true reaction rate constants are related as follows:

$$k_{cat,app} = \frac{k_{cat,wp}}{1 + \dfrac{E_{o,I}\theta_I}{E_{o,wp}\theta_{wp}}} = \frac{k_{cat,wp}}{1 + p[AOT]} \tag{7}$$

Since the molar ratio R of water-to-surfactant is maintained constant at different surfactant concentrations, the ratio of the volume fraction of the interface to that of the water pool is a constant throughout the study. Since the partitioning of the enzyme into the interfacial region is assumed to occur as a consequence of interactions with the surfactant, the concentration of the enzyme in the interfacial region can be taken proportional to the total concentration of AOT in the system. Thus, the second equality in eq 7 is written with p as a constant of proportionality. The effect of surfactant concentration on $k_{cat,app}$ thus originates from the partitioning of the enzyme into the surfactant interface where it is not active.

The true and apparent values of the Michaelis constant K_m are related as

$$\frac{K_{m,app}}{\theta_{wp} + P_I\theta_I + P_{org}\theta_{org}} = K_{m,wp} \tag{8}$$

Here, P_I is the partition coefficient of the substrate between the interface and the water pool and P_{org} is the partition coefficient of the substrate between the organic continuous phase and the water pool. Both partition coefficients are expressed as ratios of molar concentrations.

$$P_I = \frac{S_{o,I}}{S_{o,wp}} \qquad P_{org} = \frac{S_{o,org}}{S_{o,wp}} \tag{9}$$

Equation 8 reveals how $K_{m,app}$ depends on the surfactant concentration through θ_I.

Predicting the Dependence of $k_{cat,app}$ on the AOT Concentration. To test the validity of the modified kinetic model, the experimentally determined $k_{cat,app}$ for AOT reverse micelles at various AOT concentrations have been fitted to eq 7. The kinetic constants as a function of the AOT concentration are those listed in Table 1. From a correlation of these data to eq 7 shown in Figure 7, we get a value for $k_{cat,wp}$ of about 9.3 s^{-1}. This is comparable to the value of $k_{cat,wp}$ obtained from experimental measurements in the aqueous medium

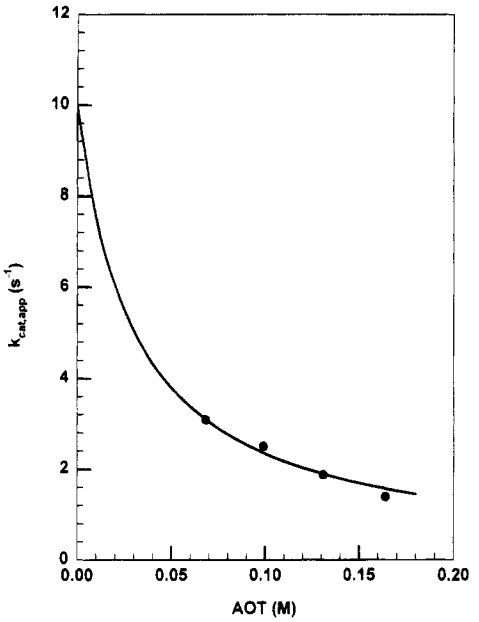

Figure 7. Dependence of the apparent pseudo first-order rate constant $k_{cat,app}$ on the AOT concentration for the oxidation of cholesterol catalyzed by cholesterol oxidase. The points are experimental data and the line represents the correlation based on eq 7.

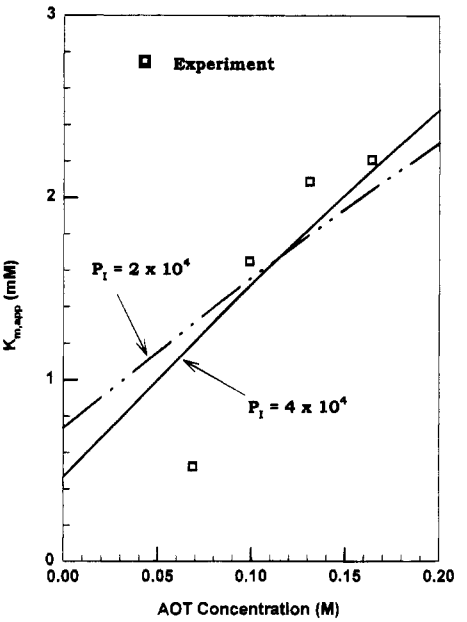

Figure 8. Dependence of the Michaelis–Menten constant $K_{m,app}$ on the AOT concentration for the oxidation of cholesterol catalyzed by cholesterol oxidase. The points are experimental data and the line represents the correlation based on eq 8.

(though in the presence of some detergents), thus supporting the model. The parameter p which is a proportionality constant is found to have a value of 32.11, if the concentration of AOT in the correlation is expressed in units of M. Thus the modified model predicts a decrease in $k_{cat,app}$ with increasing AOT concentration. Only in the limit of zero surfactant concentration the prediction $k_{cat,app} = k_{cat,wp}$ provided by the "biphasic" and "triphasic" models are satisfied.

Predicting the Dependence of $K_{m,app}$ on the AOT Concentration. The apparent and true Michaelis equilibrium constants $K_{m,app}$ and $K_{m,wp}$ are related by eq 8. In this equation, θ_{wp}, θ_{org}, and θ_I are the micellar, organic continuous phase, and interface volume fractions, respectively, that are computed from a knowledge of the AOT concentration and of R. The partition coefficient P_I corresponds to the partitioning of cholesterol between the surfactant domain and the water pool. It can be estimated from studies of solubilization of cholesterol in micellar solutions and knowing that the aqueous phase solubility of cholesterol is 4.7 μM. No direct measurements of the solubilization of cholesterol in AOT micelles are presently available. From our earlier study of the solubilization of hydrophobic substances in aqueous micellar solutions, we estimate that P_I for cholesterol lies in the range of 2×10^4 to 5×10^4 (see Appendix for details). A value of $P_{org} = 619$ is estimated for the partition coefficient of cholesterol between the organic continuous phase and the micellar core based on the experimentally determined solubility of 2.91 mM cholesterol in isooctane. To test the validity of the modified kinetic model, the experimentally determined $K_{m,app}$ for AOT reverse micelles at various AOT concentrations have been fitted to eq 8 using the values for the partition coefficients estimated as above (Figure 8). The two correlation lines shown in the figure correspond to two different estimates of P_I since an unique value is not available from direct measurements. The correlation yields $K_{m,wp} = 0.0012$ mM when $P_I = 2 \times 10^4$ and $K_{m,wp} = 0.000\,92$ mM when $P_I = 4 \times 10^4$. The fitted $K_{m,wp}$ values are consistent with the estimates of 0.001 mM in phosphate buffer containing no detergent (specified by the manufacturer), and 0.0025 mM obtained in the presence of 2% 2-propanol (Aleksandrovskii, 1988). One may note that the kinetic constants measured in aqueous solutions are influenced by the presence of the detergents added and therefore, the magnitude of $K_{m,app}$ listed in Table 1 for the aqueous media are much larger than this fitted value of $K_{m,wp}$.

Role of Surfactant AOT as a Noncompetitive Inhibitor. Equation 7 suggests that the influence of the surfactant AOT on the enzymatic oxidation of cholesterol to cholestenone can be explained on the basis of an alternate model which assumes that the surfactant is a noncompetitive inhibitor of the enzyme. For purely noncompetitive inhibition by the surfactant, the second equality of eq 7 is applicable (Bailey and Ollis, 1986) with the parameter p being the association equilibrium constant for enzyme–surfactant binding. The noncompetitive inhibition of cholesterol oxidase activity by the surfactant is more directly revealed by the experimental activity data in aqueous buffer at different concentrations of molecularly dissolved surfactant AOT (Figure 9). Since the concentration of cholesterol in these experiments is considerably larger than the value of K_m, the enzyme activity is proportional to the pseudo-first-order rate constant k_{cat}. The concentration of AOT in the buffer is less than the critical micelle concentration of AOT in water and, hence, the change in enzyme activity with AOT concentration is not affected by the complications of micelle formation in the buffer. From Figure 9, it is apparent that the enzyme activity decreases with increasing surfactant concentration, qualitatively consistent with eq 7. The surfactant AOT in this case does behave as a noncompetitive inhibitor for the cholesterol oxidation.

This inhibitory participation of AOT in cholesterol oxidation is analogous to the effect of surfactants on other enzymes such as α-chymotrypsin, acid phosphatase, and lipase. It has been observed that the surfactant AOT competitively inhibits the enzyme α-chymotrypsin in aqueous phase at or around the critical micelle concentration and also in reverse micelles

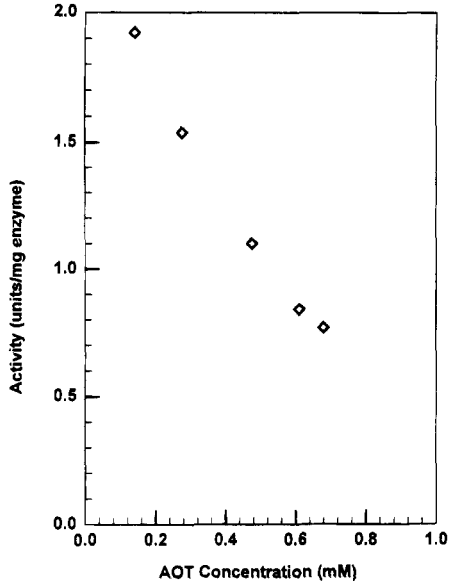

Figure 9. Activity of the enzyme in the aqueous phase as a function of the concentration of AOT in the aqueous buffer. No micellar aggregates are present, and the surfactant is present in the monomeric form.

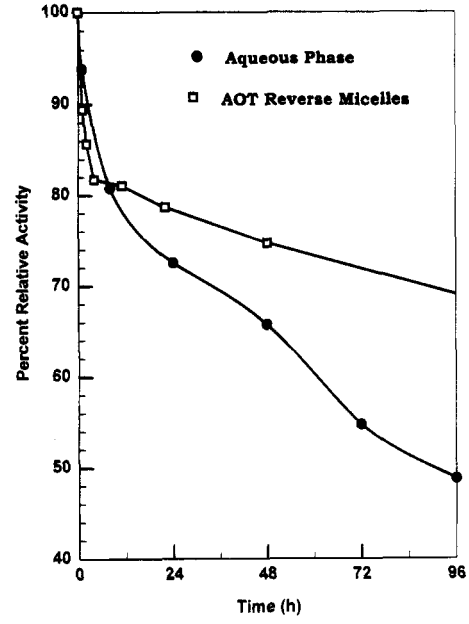

Figure 10. Percent relative activity of cholesterol oxidase in 0.05 M Tris-HCl buffer at pH = 7.5 and in 0.068 M AOT–isooctane reverse micelles at pH = 5 and R = 8, as a function of time.

(Fletcher et al., 1984). Similarly, studies conducted by O'Connor and Walde (1986) on the effect of AOT concentration on the catalytic rate constants of esterase activity of human milk lipase using 4-nitrophenyl propionate as substrate revealed that there was a decrease in k_{cat} and an increase in K_m for the enzymatic reaction in reverse micelles as the concentration of AOT is increased. The authors interpreted this behavior as being due to mixed inhibition by the surfactant. The enzyme acid phosphatase which exhibits superactivity in AOT–isooctane reverse micelles has also been reported to be noncompetitively inhibited by the surfactant AOT (Levashov et al., 1986). It is believed that the sulfo group in the polar portion of the AOT molecule is capable of blocking the active site of the enzyme. These studies which demonstrate that the surfactant is not a passive component of the reaction mixture, but may be actively involved in the enzymatic reaction are significant to the exploitation of reverse micelles as microheterogeneous reaction media.

Storage Stability of Cholesterol Oxidase in AOT Reverse Micelles. For the long-term use of enzymes in a continuous reactor, the economic feasibility of the process may hinge on the useful lifetime of the enzyme biocatalyst, making this a very important factor in the commercial exploitation of reverse micelles for enzymatic biosynthesis. Many enzymes have been found to retain their activity in reverse micelles for reasonable lengths of time. A notable example is that of α-chymotrypsin which has been shown to retain its activity for up to several months in AOT–octane reverse micelles (Levashov et al., 1981).

The enzyme deactivation data are usually obtained by exposing the enzyme to the denaturation conditions in the absence of the substrate. For the present case, this means keeping the enzyme solubilized in the reverse micelles without contacting the substrate. At specified intervals of time, the initial activity of the enzyme is determined by adding the substrate to the enzyme under standard conditions. The activity retention of the enzyme obtained in this manner is the storage stability of the enzyme in the reverse micelle medium.

We have investigated the storage stability of cholesterol oxidase in anionic reverse micelles composed of 0.068 M AOT–isooctane–buffer at the optimum condition of pH = 5 and R = 8 at 25 °C. The enzyme is found to retain 79% of its activity after 24 h and 70% after 96 h of being immobilized in the reverse micelles (Figure 10). The enzyme retained 62% of its initial activity even one week after being encapsulated in the reverse micelles. For comparison, the activity retention of the enzyme in the aqueous buffer at optimum conditions is also shown in the figure. It can be seen that cholesterol oxidase is even more stable in AOT–isooctane reverse micelles than in the aqueous solution. One plausible explanation for this increased stability could be the restrictive environment of the surfactant shell which forces the enzyme to retain its rigid, biologically active conformation and slows down its deactivation. This appreciable activity of cholesterol oxidase and other enzymes in reverse micelles suggests that the reverse micelles constitute a potentially useful media for enzymatic bioconversions.

The deviation of the inactivation of cholesterol oxidase in reverse micelles from first-order kinetics observed in Figure 10 deserves mention. This deviation may be due to the existence of a complex associative–dissociative process of deactivation in reverse micelles involving a number of steps in series or parallel. This phenomenon has been observed for other proteins such as glucose-6-phosphate dehydrogenase in mixed surfactant reverse micelles (Eremin and Metelitsa, 1986).

Preparative Conversion of Cholesterol to Cholestenone. We have investigated the preparative conversion of cholesterol to cholestenone in reverse micelles composed of 0.068 M AOT in isooctane, at the optimum condition of R = 8 and pH = 5. The concentration of the substrate cholesterol in the reaction mixture was 3 mM. The reaction volume was 20 cm^3 and contained 1.16 mg of cholesterol oxidase and 2 mg of catalase. By using high-performance liquid chromatography to analyze the concentration of the product cholestenone at different intervals of time, it was shown that complete conversion of cholesterol could be achieved within a period of 30 min (Figure 11). The figure also

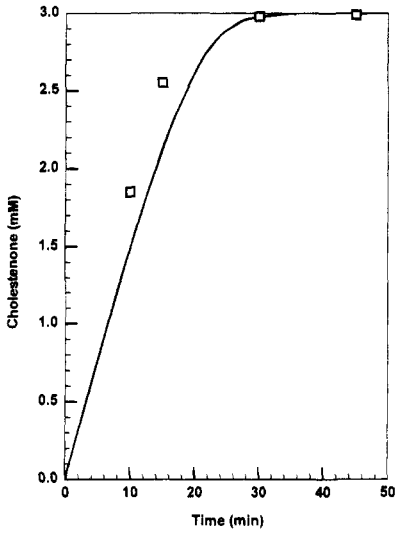

Figure 11. Preparative conversion of cholesterol to cholestenone in 0.068 M AOT–isooctane reverse micelles at $R = 8$ and pH = 5. Points are experimental data; the continuous line is the theoretical prediction.

shows that the substrate conversion with time coincides remarkably well with that predicted on the basis of the Michaelis–Menten rate equation using the experimentally determined values of $k_{cat,app} = 3.07\ s^{-1}$ and $K_{m,app} = 0.515$ mM. The predicted substrate concentrations as a function of time plotted in the figure are obtained by integrating eq 1 to get

$$k_{cat}E_o t = S_o - S + K_m \ln \frac{S_o}{S} \qquad (10)$$

In view of the fact that a more than 1000-fold increase in cholesterol solubility can be achieved in this system compared to an aqueous medium of the same volume, the potential of this reaction medium is readily evident.

IV. Conclusions

We have presented a comparative evaluation of reverse micelles composed of the anionic surfactant AOT, the cationic surfactant CTAB, and the nonionic surfactant Triton X-100 as reaction media for the oxidation of cholesterol catalyzed by cholesterol oxidase. The enzyme catalyzes the conversion of cholesterol to cholestenone. Cholesterol oxidase in AOT–isooctane reverse micelles showed the highest activity at the lowest values of R and displayed a shift in its pH profile to the acidic side. This demonstrates that cholesterol oxidase is most active when the water domain reflects the properties of bound water characterized by a lowered polarity. The Michaelis–Menten constants $k_{cat,app}$ and $K_{m,app}$ decreased with increasing R. This variation in $K_{m,app}$ with R is qualitatively consistent with the lower polarity of the water core dominated by bound water which gives rise to an increased solubility of the substrate in that region. The variation in $k_{cat,app}$ with R indicates that there is a difference in the intrinsic activity of the enzyme depending on the relative importance of bound water in the water core of the reverse micelles. There is also the possibility that a change in the conformation of the enzyme could occur upon solubilization into reverse micelles of low water content.

In contrast, cholesterol oxidase in reverse micelles composed of the cationic surfactant CTAB exhibited a shift in its pH profile to the alkaline side and a bell-shaped dependence of its activity on R. In nonionic surfactant Triton X-100 reverse micelles, the monotonic increase in enzymatic activity with R is explained by the absence of electrostatic and ionic hydration effects with the use of nonionic surfactants.

The enzymatic reaction in reverse micelles is seen to follow the classical Michaelis–Menten kinetics. The values of the apparent kinetic constants $k_{cat,app}$ and $K_{m,app}$ differ from the true kinetic constants obtained in the aqueous medium. The apparent Michaelis constant $K_{m,app}$ is 100–1000-fold larger than the true constant in the aqueous medium depending on the surfactant used. The pseudo-first-order rate constant $k_{cat,app}$, on the other hand, is reduced 2–4-fold compared to k_{cat} in the aqueous medium.

The kinetic constants in AOT–isooctane reverse micelles are seen to be strong functions of R, the pH of the water pool and the surfactant concentration in the system. Models which take into account the partitioning of the substrate between the micellar core and organic continuous phase as well as the micellar interface account for the variation in $K_{m,app}$ with surfactant concentration. The variation in $k_{cat,app}$ with surfactant concentration can be described by allowing for the partitioning of the enzyme between the micellar core and the interface and/or the noncompetitive inhibition of the enzyme by the surfactant.

Preparative studies resulted in 100% enzymatic oxidation of cholesterol to cholestenone. The enzyme retained appreciable activity in the reverse micelles and was even more stable in this environment than in the aqueous medium. This demonstrates that reverse micelles can be exploited as effective media for the enzymatic biosynthesis of water-insoluble substrates. One important application of the present study could be the use of reverse micelles-based enzymatic oxidation as a convenient analytical technique for the determination of cholesterol in body fluids.

Acknowledgment

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Nomenclature

Abbreviations for Surfactants

AOT = sodium bis(2-ethylhexyl) sulfosuccinate
CTAB = cetyltrimethylammonium bromide
SDS = sodium dodecyl sulfate
Triton X-100 = isooctylphenoxypolyoxyethanol
Thesit = poly(nonoxyethylenes) lauryl ether

Notations

a = specific activity of enzyme
E_o = concentration of enzyme (sum of both free- and substrate-bound enzyme)
$E_{o,wp}$ = concentration of enzyme in the water pool of reverse micelles
$E_{o,I}$ = concentration of enzyme in the interfacial region of reverse micelles
$E_{o,t}$ = overall concentration of the enzyme in the reverse micelle system
k_{cat} = first-order rate constant
$k_{cat,app}$ = apparent or observed first-order rate constant
$k_{cat,wp}$ = first-order rate constant in the water pool of reverse micelles
K_m = Michaelis dissociation equilibrium constant

$K_{m,app}$ = apparent or observed Michaelis equilibrium constant in the reverse micelle system

$K_{m,wp}$ = Michaelis equilibrium constant in the water pool of reverse micelles

M_E = molecular weight of the enzyme

p = proportionality constant in eq 7

P_I = equilibrium partition coefficient for the substrate between the interfacial region of reverse micelles and the aqueous phase

P_{org} = equilibrium partition coefficient for the substrate between the organic continuous phase and the aqueous phase

R = molar ratio of water-to-surfactant in reverse micelles

S_o = concentration of the substrate

$S_{o,wp}$ = concentration of the substrate in the water pool of reverse micelles

$S_{o,I}$ = concentration of the substrate in the interfacial region of reverse micelles

$S_{o,org}$ = concentration of the substrate in the organic continuous phase of reverse micelle system

$S_{o,t}$ = overall concentration of the substrate in the reverse micelle system

v = reaction velocity

v_t = overall reaction velocity in the reverse micelle system

v_{max} = maximum reaction velocity

v_{wp} = reaction velocity in the water pool of reverse micelles

θ_{wp} = volume fraction of the water pool in the reverse micelle system

θ_{org} = volume fraction of the organic continuous phase in the reverse micelle system

θ_I = volume fraction of the interfacial region in the reverse micelle system

Appendix

Estimation of the Interface–Water Partition Coefficient P_I for Cholesterol. From experimental measurements of the solubilization of hydrophobic substances in aqueous micellar solutions (Chaiko et al., 1984), we have developed correlations for the molar solubilization ratio MSR (moles of solubilizate per mole of surfactant in the micelle) as a function of the molecular volume v (in Å^3) of the solubilizate for three surfactants. For cetylpyridinium chloride, MSR = $1.43 \times 10^6 v^{-2.66}$, for dodecylammonium chloride, MSR = $0.825 \times 10^6 v^{-2.51}$, and for sodium dodecyl sulfate, MSR = $0.539 \times 10^6 v^{-2.58}$. Knowing that cholesterol has a molecular weight of 386.7 and density of about 1.067 g/cm^3 (*CRC Handbook*, 1979), we estimate its molar volume to be 362.4 cm^3 and the molecular volume to be 602 Å^3. Corresponding to this molecular volume, the correlations predict molar solubilization ratios of 0.057, 0.087, and 0.0363, in the three surfactants, respectively. These estimates can be compared to the experimental estimate of 0.05 at 37 °C and 0.028 at 27 °C, reported for the solubilization of cholesterol in a commercial nonionic surfactant (Schott and Sayeed, 1986). We assume that the estimated molar solubilization ratios represent typical values anticipated for the AOT surfactant. Knowing the molecular volume of AOT to be 377 cm^3, we can calculate the solubility of cholesterol in the three micelles to be 0.143, 0.212, and 0.093 M, respectively. Given the aqueous phase solubility of 4.7 μM, the solubilities in the micelles translate into partition coefficient P_I of 3×10^4, 4.5×10^4, and 2×10^4, respectively. The model calculations shown in Figure 8 have been carried out for two differing estimates of P_I since an unique value for cholesterol is not available from direct measurements on AOT micelles.

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