Enzyme-Caytalyzed Oxidation of Cholesterol in Supercritical Carbon Dioxide

Supercritical carbon dioxide was studied as a solvent for the enzymatic oxidation of cholesterol with molecular oxygen. Enzymes isolated from *Streptomyces* sp., *Norcardia* sp., *Pseudomonas* sp., and *Gloeocysticum chrysocreas* are active in supercritical carbon dioxide.

For the oxidation of cholesterol to cholest-4-ene-3-one (via cholesterol oxidase from *Gloeocysticum chrysocreas*, turnovers are almost two orders of magnitude higher than those found in aqueous solutions; initial rates of the reaction catalyzed by *Streptomyces* sp. in supercritical carbon dioxide are comparable to or greater than those found in aqueous solutions. Cholesterol oxidase from *Gloeocysticum chrysocreas* is stable in supercritical carbon dioxide at 100 bar and 35°C, while the enzyme from *Streptomyces* sp. is not. A trace amount of water is necessary for catalysis in carbon dioxide.

The rate of oxidation is increased markedly by addition of small amounts of tert-butanol and iso-butanol, moderately by ethanol, slightly by acetone and *n*-butanol, and not at all by addition of methanol. Solubility data cannot explain these observations.

EPR spectroscopy reveals no large conformational changes in the enzyme from *Gloeocysticum chrysocreas* as a function of carbon dioxide pressure or cosolvent addition. However, EPR spectroscopy indicates that aggregation of cholesterol molecules in supercritical carbon dioxide is strongly affected by changes in pressure or cosolvent content. The degree of cholesterol aggregation correlates well with observed rate enhancements.

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Introduction

Enzymes possess a number of desirable catalytic properties: among these are a high degree of specificity (both for the reactant and for the reaction which is catalyzed) and the ability to catalyze reactions at temperatures only slightly above room temperature. Despite their unique catalytic properties, enzymes are not widely employed in industrial chemical processes, partly because of the perceived inability of enzymes to function in nonaqueous solvents, which would permit a wider range of potential substrates to be employed. Recent studies show that not only can enzymes exhibit higher activity in mixtures of water and organic solvents than in pure water (Butler, 1979), but some enzymes are active in organic solvents containing as little as 0.02% water (Zaks and Klibanov, 1985). This activity should not be surprising since, even in their native cellular environment, enzymes function in a microenvironment containing various proteins, lipids, and electrolytes.

Besides providing a medium, in which enzymes may function better catalytically, nonaqueous solvents may offer two significant engineering advantages over aqueous solvents: 1. reactants may be more soluble in nonaqueous solvents and 2. downstream product recovery may often be simplified.

Supercritical fluids provide a particularly interesting class of solvents which may be used for enzyme-catalyzed reactions. The same advantages which make supercritical fluids attractive extractants also make them attractive solvents for reactions. Among these are high diffusivities and low viscosities relative to those for liquids, and the ability to cause large changes in solvent power and dielectric constant through small changes in temperature and pressure. Therefore, supercritical solvents may allow better control of reaction rates and selectivities and may make

possible simplified product recovery. As pointed out by Larson and King (1986), precipitation from a supercritical fluid may result in very small crystalline particles, providing an alternative to mechanical milling.

Because of the heat-labile nature of enzymes and many biochemicals of practical interest, the choice of supercritical solvent is limited to those with a critical temperature near or below room temperature. These solvents include ethane (T_c = 32.3°C), ethylene (9.5°C), nitrous oxide (36.5°C), trifluoromethane (25.9°C), and carbon dioxide (31.1°C). Supercritical carbon dioxide is the solvent of greatest interest because of its low toxicity, nonflammability, and low cost. Since proteins are insoluble in supercritical carbon dioxide, enzyme recovery is relatively simple.

Enzymatic conversion of steroids provides a suitable reaction for studying the possible advantages of a dense carbon-dioxide bioreactor. Steroids are nearly insoluble in water; for example, cholesterol is only soluble to 4.7 μmol/L in pure water; cholesterol forms micelles at 25–40 nanomolar concentrations (Haberland and Reynolds, 1973). At 123 bar, cholesterol is about 50 times more soluble in carbon dioxide (Wong and Johnston, 1986). Cholesterol oxidase (E.C. 1.1.3.6) catalyzes the oxidation of cholesterol by molecular oxygen to cholest-4en-3one, a precursor of interest in the pharmaceutical production of androst-1,4-diene-3,17-dione (Buckland et al., 1975). Further chemical modification can produce such products as estradiol, a component in oral contraceptives.

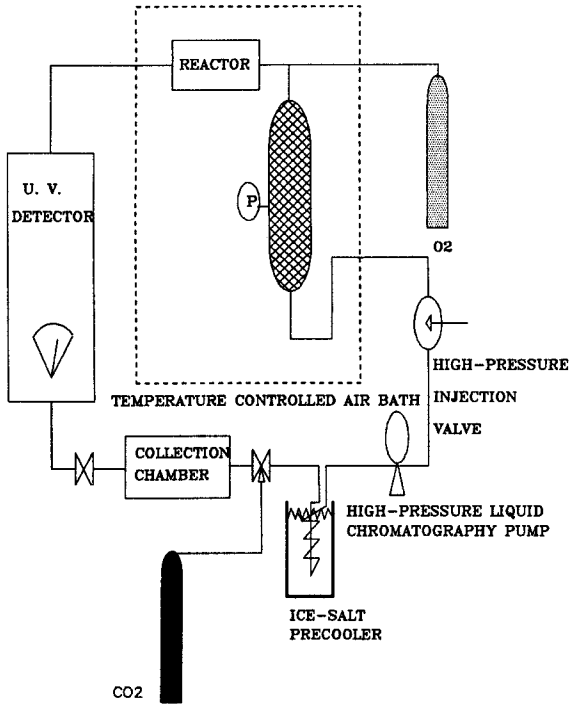

Figure 2. Continuous reactor with on-line UV detection of products.

Experimental Methods

Apparatus

All supercritical-fluid experiments were conducted in the stainless-steel reactor shown schematically in Figures 1 and 2. Figure 1 shows the reactor for batch operation; Figure 2 indicates the scheme employed for continuous operation, with recycle of carbon dioxide and on-line measurement of product concentration by the high-pressure UV detector. The batch-mode reactor has an internal volume of 195 mL; the continuous-mode setup has an internal volume of 203 mL. In both modes of operation, the desired oxygen/carbon dioxide composition is obtained by first pressurizing to a known pressure with oxygen, then pumping in liquid carbon dioxide to the desired final pressure.

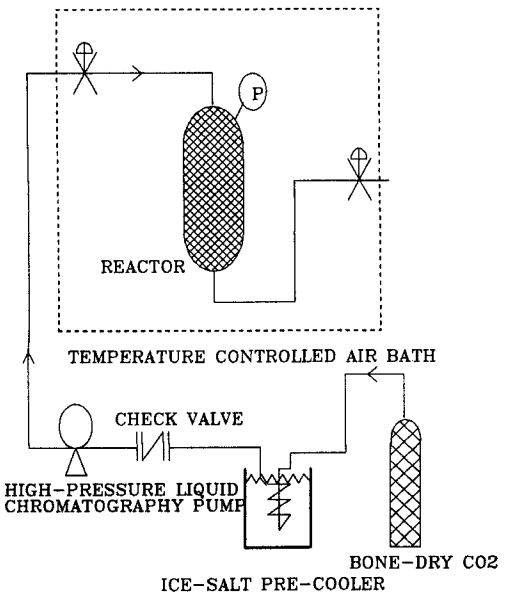

Figure 1. Batch reactor.

Batch-reactor conditions

In the batch reactor were placed 0.0773-g (0.2-mmol) cholesterol, 2.0-mL 0.1 M $NaHCO_3$ pH 9.0, and either 0.036 or 0.072 mg cholesterol oxidase from *Streptomyces* sp. Oxygen was added to 8.3 bar at 22°C; then carbon dioxide was pumped in to 101.3 bar at 35°C.

The reaction was terminated by freezing the reactor in liquid nitrogen until the carbon dioxide solidified. The reactor was opened, and 20-mL methylene chloride were added. The reactor was warmed to room temperature, allowing the carbon dioxide to sublime. The remaining liquid contents of the reactor were centrifuged ten minutes at 10,000 rpm to separate the aqueous and organic phases, and to settle any solids. The organic phase was collected and analyzed by HPLC on a reverse-phase, C-18 column with methanol as the mobile phase. The product, cholest-4en-3one, was quantitatively detected by UV absorbance at 242 nm.

Continuous-reactor conditions

The 150-mL saturation chamber, Figure 2, was filled with loosely-packed glass wool. One gram of cholesterol was dissolved in 25 mL of methylene chloride. The solution was poured into the saturation chamber, which was warmed to 50°C to boil off methylene chloride and to deposit the cholesterol onto the glass wool. The chamber was placed under vacuum to assure the removal of all methylene chloride.

The packed-bed reactor consisted of enzymes immobilized on

glass beads (described below) placed in a stainless-steel tube, 10 cm × 0.25 cm ID. The tube ends were plugged with glass-wool stoppers to contain the glass beads.

After 10-mL water were added to the saturation chamber (unless otherwise indicated, water-saturated solutions were always used), the reactor was sealed. Then 8.2-atm oxygen at 35°C were added, followed by carbon dioxide to 100 atm at 35°C. Carbon dioxide was continuously recycled. Downstream of the UV detector, an expansion valve was adjusted to maintain the pressure between the valve and the pump at approximately 60 atm to assure that products and unreacted cholesterol precipitated from the solution in the product-collection vessel. Located between the pump and the saturation chamber, a high-pressure injection valve with a 1.0-mL sample loop provided capability to inject cosolvents during the reaction.

Product concentrations were measured on line by UV absorption at 242 nm. (At 242 nm, cholesterol does not absorb, while cholest-4-ene-3-one absorbs strongly. The extinction coefficient of 4-cholesten-3-one in CO_2 at 100 bar is 12.4 $cm^2/\mu mol$.) On-line UV measurements were confirmed at the end of each run by rinsing the product collection vessel with methylene chloride and injecting the solution onto an HPLC (RP-18 column, methanol mobile phase), where the total production of the run could be measured by UV absorbance at 242 nm.

Immobilization of enzymes

Five grams of 50-micron diameter glass beads were activated by refluxing 24 hours in a mixture of 10% v/v 3-aminopropyltriethoxysilane in acetone. Excess 3-aminopropyltriethoxysilane was removed first by washing with acetone, followed by washing with 50-mM phosphate buffer, pH 6.5. The glass beads were next stirred in a solution of 2.5% v/v glutaraldehyde in phosphate buffer for two hours and then washed again with buffer. Nine-mg enzyme in 5 mL of phosphate buffer were poured onto the glass beads and allowed to react for four hours with occasional stirring. Uncoupled enzyme was washed away from the beads with phosphate buffer, and the beads were stored at 5°C in 50-mM phosphate buffer, pH 6.5 with 0.1% sodium azide added as a preservative.

A second, simpler technique for enzyme immobilization was also used. Enzyme was dissolved in 50 mM phosphate and slowly dried at room temperature onto the surface of 50-mm glass beads using the technique of Hammond et al. (1985). This technique provided higher enzyme loading and higher enzymatic activity per gram of glass beads than covalent attachment, but was also mechanically more fragile.

Measurement of enzyme activity in aqueous solution

Cholesterol-oxidase activity in aqueous solutions was measured in stirred glass reactors at 35°C. To solubilize cholesterol, 5% v/v of either DMSO or isopropyl alcohol were added to a 50-mM phosphate buffer, pH 7.0. Cholesterol was added to make a 1-mM solution. Reactions were initiated by adding cholesterol oxidase dissolved in the above solution without cholesterol. Product formation was monitored by taking 10-μL samples at regular intervals and injecting 2-μL of the sample onto an HPLC. Products were detected by U.V. absorbance at 242 nm, and concentrations were calculated using cholest-4-en-3-one standards. In water with no solubilizing agent present, no reaction could be detected after three days.

Determination of the stability of cholesterol oxidase

Stability of cholesterol oxidase at 40°C was determined by placing enzyme immobilized on glass beads in either an aqueous solution with 1% v/v isopropanol, 1-mM cholesterol, and 50-mM phosphate, pH 7.0, or in carbon dioxide with 1-mM cholesterol and 10% O_2 at 103.4 bar. After a measured time, the enzyme-containing beads were removed from their respective media and washed with a solution of 50-mM phosphate with 1% isopropanol. Finally, they were assayed for activity at 25°C in a 1-mM cholesterol solution with 1% isopropanol, 50-mM phosphate, pH 7.0.

Spin-labeling of cholesterol oxidase from Gloeocysticum chrysocreas

Twenty-five mg cholesterol oxidase from *Gloeocysticum chrysocreas* were dissolved in 5 mm of 50-mM phosphate buffer, pH 7.0. 12 mg 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid N-hydroxysuccinimide ester were dissolved in 0.5 mL isopropanol and added to the cholesterol oxidase solution. The mixture was allowed to react for 24 hours at room temperature. Unreacted spin label was removed by 24 hours of dialysis against a 50-mM phosphate buffer containing 5% v/v isopropanol, then by 24 more hours of dialysis against a phosphate buffer without isopropanol. The final dialysis solution contained 0.1% sodium azide as a preservative. Spin-labeled enzyme was concentrated by ultrafiltration before use.

Materials

Cholesterol (Sigma chromatographic grade), glutaraldehyde, 3-aminopropyltriethoxysilane, and cholesterol oxidases from *Norcardia* sp., *Pseudomonas* sp., and *Streptomyces* sp. were from Sigma Chemical. Chromatographic standards of cholest-4-en-3-one and cholest-5-ene-3-one were purchased from Aldrich Chemicals. Cholesterol oxidase from *Gloeocysticum chrysocreas* was from the Chemical Dynamics Corp. Oxygen and bone-dry carbon dioxide were from Matheson Gas Products Corp. 2,2,5,5-tetramethyl-1,3-pyrrolin-1-oxyl-3 carboxylic acid *N*-hydroxy succinimide ester was from Kodak Laboratory Chemicals. 3-Doxyl-5-α-cholestane was from Aldrich Chemicals. All chemicals were used without further purification. Enzyme purity was checked by gel electrophoresis; enzymes were used after dialysis against a 50-mM phosphate buffer, pH 7.0.

Results and Discussion

Figure 3 shows the batch conversion of cholesterol as a function of time in supercritical carbon dioxide at 101 bar, using cholesterol oxidase from *Streptomyces* sp. Each point represents one experiment. Control experiments were run under identical conditions but omitting enzyme; possible solid-phase reactions were checked by mixing powdered enzyme and cholesterol in the reactor, without addition of CO_2. In each case, no reaction products could be detected after 24 hours.

At all concentrations of enzyme tested, the extent of conversion reached only about 12–14%. The equilibrium for this reaction lies far towards cholest-4en-3one; in other experiments, 100% conversion was reached using cholesterol oxidase from *Pseudomonas*.

At 40°C, cholesterol oxidase from *Streptomyces* sp. is stable in neither aqueous solution nor in supercritical carbon dioxide.

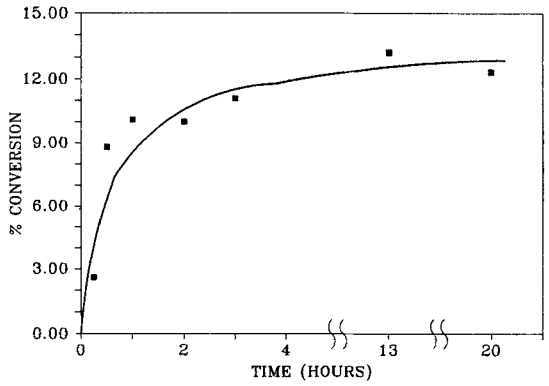

Figure 3. Batch reaction kinetics of cholesterol oxidation with cholesterol oxidase from *Streptomyces* in supercritical CO_2 at 308 K, 101 bar.

Enzyme concentration 1.8×10^{-4} mg/mL, starting cholesterol concentration 1 mM.

A plot of relative activity v. time, Figure 4, shows a distinct break in the slope at about 1 hour, probably indicating a two-step deactivation process. The observed plateau in cholesterol cholesterol conversion of 12–14% may be explained by this thermal deactivation. During the first 1.5 hours, the relative activity measured in the stability experiment decreased by a factor of 5, while the apparent first-order rate constant in the batch reactor decreased from an initial value of 0.7 h^{-1} to about 0.1 h^{-1}.

On the other hand, cholesterol oxidase from *Gloeocysticum chrysocreas* is much less thermally labile than the oxidase derived from *Streptomyces* sp. As previously shown elsewhere (Randolph et al., 1988), cholesterol oxidase from *Gloeocysticum chrysocreas,* when immobilized on glass beads, shows no apparent denaturation after three days in cholesterol-saturated carbon dioxide with 10% oxygen at 101.3 bar and 35°C. Throughout the experiment, reactor residence time was maintained at 13 seconds, and approximately 70% of the cholesterol was converted to cholest-4-ene-3-one.

To examine possible mass-transfer limitations in the packed-bed reactor, cholesterol-saturated carbon dioxide was pumped through a 10-cm × 0.25-cm packed bed filled with *Streptomyces* sp.-derived cholesterol oxidase immobilized on 50-mm glass beads. Flow rates varied from 100 to 460 mL/h; as shown in Figure 5, the rate of cholest-4en-3one production was linear with flow rate. It appears that diffusion of cholesterol to the enzyme is not a rate-limiting step in this range of flow rates; had the reaction been mass-transfer-limited, the conversion should have been exponentially decreasing with increasing flow rate, rather than the observed conversion.

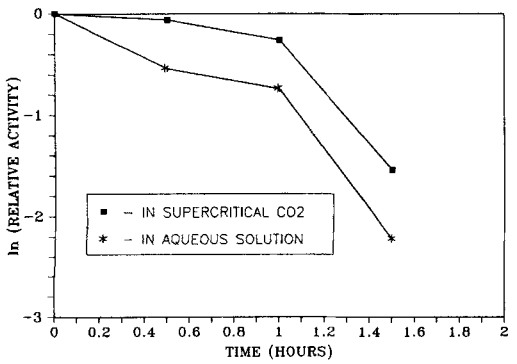

Figure 4. Thermal denaturation of cholesterol oxidase from *Streptomyces* sp. in supercritical carbon dioxide (101 bar) and water (50-mM phosphate, pH 7.0) at 40°C.

Assay for activity conducted in a 1-mM cholesterol solution in 50-mM phosphate buffer with 1% isopropanol added as a solubilizing agent.

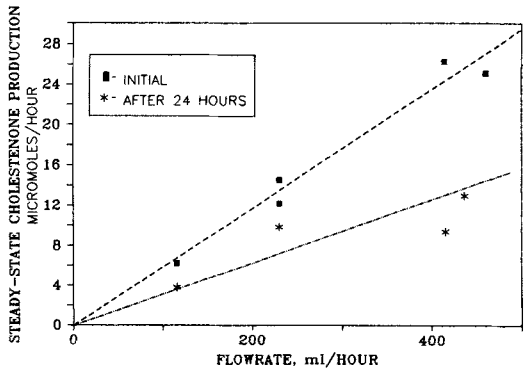

Figure 5. Effect of carbon dioxide flow rate on packed-bed kinetics of cholesterol oxidation using cholesterol oxidase from *Streptomyces.*

In the range 80–100 bar, the rate of cholesterol oxidation catalyzed by cholesterol oxidase increases monotonically with pressure, Figure 6. The rapid increase of reaction rate with pressure in this region parallels both the increase in cholesterol solubility and increasing cholesterol aggregation.

Effect of Water Content on Reaction Rate

It has been proposed that enzymes may need at least a shell of water to function catalytically (Zaks and Klibanov, 1985). Water serves a structural purpose in many enzymes, helping to maintain a correct conformation through hydrogen bonding and by providing a medium of suitable dielectric constant. When damp immobilized enzyme in the packed bed reactor was exposed to bone-dry carbon dioxide (passed over a molecular sieve to insure dryness), the enzyme quickly lost its activity. Loss of activity, however, appeared to be reversible, because activity was quickly regained when 1% v/v water was injected into the system, Figure 7.

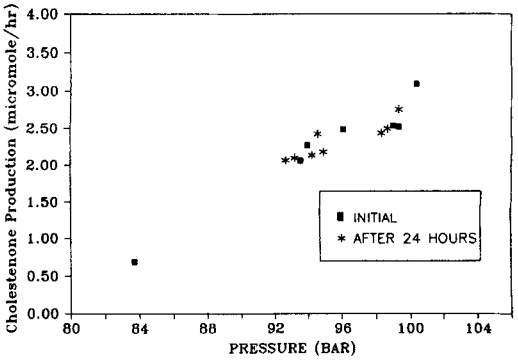

Figure 6. Effect of pressure on the rate of cholesterol oxidation in supercritical carbon dioxide.

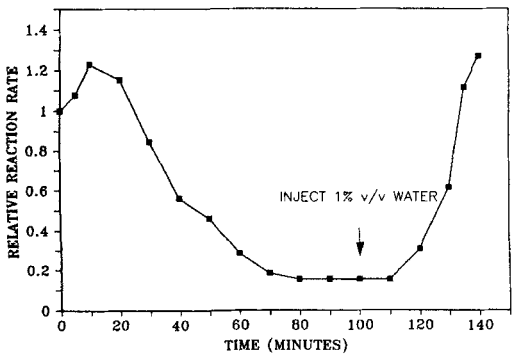

Figure 7. Effect of water concentration on the rate of enzymatic oxidation of cholesterol in supercritical carbon dioxide using cholesterol oxidase from *Gloeocysticum chrysocreas*.

Cosolvent Effects on Enzymatic Oxidation Rate

Addition of small amounts (<5 mol %) of polar cosolvents has been shown to increase dramatically the solubility of cholesterol in supercritical carbon dioxide (Wong and Johnston, 1986). It is probable that enzyme activity in carbon dioxide is also affected by addition of such cosolvents, but the effect cannot be predicted. Increased substrate solubility is likely to raise the reaction rate; however, if the cosolvent competes with water in the "shell" surrounding the enzyme, it may be detrimental to the enzyme's conformational stability or it may cause steric hindrance at the enzyme's active site.

Cosolvents were added to a solution of cholesterol in carbon dioxide (with 10% oxygen) to investigate their effect on the rate of cholesterol oxidation. Cosolvents were injected in increments of 0.5% v/v. The rate of reaction was allowed to stabilize at a new steady state before the next increment of cosolvent was added. Table 1 gives a comparison of the activity of cholesterol oxidase from *Gloeocysticum chrysocreas* in aqueous solutions and in solutions of water-saturated carbon dioxide with various cosolvents added. Activities are expressed as turnover numbers or moles product produced per mole enzyme per second. Enzyme molarity was calculated based on a molecular weight of 58,000 (supplied by the Chemical Dynamics Corp.), and represents the total protein rather than the active protein. Aqueous turnover numbers were measured in the stirred glass vessel described earlier; turnover numbers from carbon dioxide and carbon dioxide/cosolvent mixtures were measured in the continuous flow apparatus.

Table 1. Turnover numbers for the oxidation of cholesterol with cholesterol oxidase from *Gloeocysticum chrysocreas* at 35°C

Solvent	Turnover No., s^{-1}
Water	Not Measurable
Water (5 mM phosphate, pH 7.0) + 5% v/v Isopropanol (Burns and Roberts, 1981)	1
Carbon dioxide, 100 Atm	75
Carbon dioxide + 2.0% v/v methanol	62
Carbon dioxide + 2.0% v/v ethanol	165
Carbon dioxide + 2.0% v/v tert-butanol	274
Carbon dioxide + 2.0% v/v iso-butanol	238
Carbon dioxide + 2.0% v/v acetone	86
Carbon dioxide + 2.0% v/v *n*-butanol (extrapolated)	100

All turnover numbers measured at saturation with cholesterol and under conditions where reaction is zeroth order in oxygen concentration.

An enhancement in the cholesterol-oxidation rate of nearly two orders of magnitude is observed in supercritical carbon dioxide with respect to the rate in water with isopropanol added as a solubilizing agent. Rates in buffered water without cosolvent are too small to permit measurement; no product could be detected after three days of reaction.

Addition of cosolvents causes varied effects on the rate of reaction. For instance, methanol appears to have a slightly negative effect on the rate of cholesterol oxidation. After each injection of an additional 0.5% methanol, the oxidation rate first experiences a large, transient decrease, then recovers (after about ten minutes) to a steady-state level which is about the same as the starting level. The transient reaction-rate decreases caused by methanol addition, as well as the lack of steady-state increases may be at least partially explained by the inhibitory effect of methanol on the enzyme. On the other hand, each addition of ethanol causes an increase in reaction rate.

N-butanol addition causes a rate increase that is intermediate between the effect of methanol and ethanol. A modest reaction-rate increase of about 30% is seen after the addition of 20% v/v *n*-butanol. Each *n*-butanol injection causes about the same increase in reaction rate. The effect of two branched butanols, iso-butanol and tert-butanol is different from the other alcohols. Large rate enhancements are seen with each addition of these cosolvents; after 2.5 v/v tert-butanol is added, the reaction rate more than quadruples. The enhancement is most dramatic for the addition of the first 1% tert-butanol; smaller increases are observed after subsequent injections.

Addition of the aprotic cosolvent acetone does not raise the reaction rate appreciably, although acetone causes about an order of magnitude increase in cholesterol solubility (Wong and Johnston, 1986). Reaction rate increases with addition of acetone roughly parallel those caused by *n*-butanol addition. In a manner similar to that of methanol, acetone addition causes small, transient, negative effects on the rate of reaction before a steady-state is reached at a higher reaction rate.

While accurate solubility data are not available for all of the cosolvents studied here, it is apparent from data available for mixtures of CO_2 and methanol, ethanol, and acetone (Wong and Johnston, 1986) that solubility effects alone cannot explain the varying degrees of rate enhancement with cosolvent addition. All three cosolvents increase solubility by about an order of magnitude, but observed reaction rates are not proportional to cholesterol concentration. The smaller-molecular-weight cosolvents (methanol, acetone, and ethanol) cause the largest increases in solubility, but only ethanol causes substantial increases in reaction rates. The butanols are expected to cause smaller solubility increases, but the two branched butanols (isobutanol and tert-butanol) cause large reaction-rate increases. This unexpected result is discussed later.

EPR Studies of Enzyme Conformation

The effect of cosolvents on reaction rates may be due to a change in the conformation of the enzyme caused by interaction with the cosolvent. To test for possible conformational changes, spin-labeled cholesterol oxidase from *Gloeocysticum chryso-*

creas was studied using high-pressure EPR spectroscopy. There is no significant change in the EPR spectrum when cosolvents tert-butanol or methanol are added. The lack of the major changes in these EPR spectra with addition of cosolvent suggests that reaction-rate variations cannot be explained by large conformational changes in the enzyme (Randolph et al., 1988).

Aggregation of Cholesterol in Supercritical Carbon Dioxide

Another possible explanation for the varying effects of cosolvents on enzymatic reaction rates is the formation of cholesterol aggregates. Since cholesterol is strongly self-associating in aqueous solution (Haberland and Reynolds, 1973), the formation of aggregates in other solvent systems might be expected. Also, cosolvents are known to promote micelle formation in some systems (Luisi and Magid, 1986). Cholesterol aggregation in carbon dioxide and carbon dioxide-cosolvent mixtures was demonstrated previously, using EPR spectroscopy and a spin-labeled derivative of cholesterol, 3-doxyl-5-α-cholestane (Randolph et al., 1988).

Addition of cosolvents to supercritical carbon dioxide dramatically affects the degree of cholesterol aggregation. Addition of 3% v/v of the branched butanols iso-butanol and tert-butanol to dilute solutions of spin-labeled cholesterol gives EPR spectra which show high local concentration of spin label. On the other hand, lower local cholesterol concentrations (at the same overall concentration) are observed when the cosolvent is methanol, *n*-butanol, or acetone. Ethanol addition causes local concentrations that are intermediate between those found with addition of methanol and tert-butanol.

A strong correlation exists between the degree of cholesterol aggregation and the enzymatic activity of cholesterol oxidase. The rate increases with increasing *local* cholesterol concentration; addition of branched butanols iso-butanol and tert-butanol promote this high local concentration. Cosolvents methanol, acetone, and *n*-butanol give lower local cholesterol concentrations and correspondingly smaller increases in rates of enzymatic reaction.

There are several possible explanations for the observed increases in enzymatic reaction upon addition of aggregate-promoting cosolvents. Another cholesterol oxidase from *Norcardia rhodocrous* contains a hydrophobic anchor region. This anchor region adds an amphipathic character to the enzymes and enables it to bind to hydrophobic membranes or detergent micelles (Cheetham et al., 1982). Such binding is necessary for full enzymatic activity. While such an anchor region has not been demonstrated on cholesterol oxidase from *Gloeocysticum chrysocreas,* we have shown that cholesterol oxidase from *Gloeocysticum chrysocreas* binds multiple cholesterol molecules in supercritical carbon dioxide (Randolph et al., 1988). Increased hydrophobic surface area due to the formation of larger aggregates may result in stronger binding of the enzyme to cholesterol aggregates. Enzymatic activity may also be increased by more favorable cholesterol orientation or spacing caused by the formation of mixed micelles of cholesterol and cosolvent. Such an increase is observed when cholesterol is placed in mixed micelles of dioctanoylphosphatidylcholine (Ramelmeier et al., 1988). Cosolvent addition might also affect the properties of the multiple cholesterol molecules on the enzyme surface.

Conclusions

Supercritical carbon dioxide is a suitable nonaqueous solvent for the enzymatic oxidation of cholesterol. Cholesterol oxidases isolated from *Streptomyces* sp., *Norcardia* sp., *Pseudomonas,* and *Gloeocysticum chrysocreas* exhibit activity in supercritical carbon dioxide. Stability under supercritical conditions varies according to species, but the oxidase from *Gloeocysticum chrysocreas* shows no loss of activity even after three days of exposure to supercritical carbon dioxide.

Confirming the observations of other workers, at least a trace of water is needed to maintain catalytic activity in dense carbon dioxide.

Reaction rates are comparable to or greater than those found in aqueous solutions. Addition of cosolvents may greatly enhance oxidation rates in supercritical carbon dioxide. The effect of different cosolvents on the rate of oxidation was quite varied. The effect could not be predicted on the basis of a particular cosolvent's effect on cholesterol solubility; for example, addition of methanol, which causes a large increase in cholesterol solubility, causes a slight decrease in reaction rate. On the other hand, the branched butanols iso-butanol and tert-butanol, which are expected to cause smaller solubility increases, yield large (up to 4-fold) increases in reaction rate.

With spin-labeled cholesterol oxidase from *Gloeocysticum chrysocreas,* EPR spectroscopy shows that large changes in the enzyme's conformation do not occur with addition of small amounts of the cosolvents tested. However, the cosolvents do have strong effects on the nature of cholesterol aggregation in supercritical carbon dioxide. As demonstrated by EPR spectroscopy using a spin-labeled-cholesterol derivative, the degree of cholesterol self-association in supercritical carbon dioxide varies with the type of cosolvent added, and there is a strong correlation between the amount of cholesterol aggregation and the rate of enzymatic oxidation.

Acknowledgments

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