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Cyclodextrins as Hosts for Poorly Water-Soluble Compounds in Enzyme Catalysis

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ABSTRACT

The capability of cyclodextrins to enhance greatly the solubility in water of poorly water-soluble substances makes them an ideal alternative for investigating the expression of enzyme activity with such substrates in aqueous solution. This capability is demonstrated by using soybean lipoxygenase with linoleic acid/β-cyclodextrin and diethylstilbestrol/γ-cyclodextrin, and cholesterol oxidase with cholesterol/methyl-β-cyclodextrin.

Index Entries: Cyclodextrins; linoleic acid; diethylstilbestrol; cholesterol; lipoxygenase; cholesterol oxidase.

Abbreviations: α-CD, alpha-cyclodextrin; β-CD, beta-cyclodextrin; mβ-CD, methyl-beta-cyclodextrin; hpβ-CD, 2-hydroxypropyl-propyl-beta-cyclodextrin; malβ-CD, maltosyl-beta-cyclodextrin; γCD, gamma-cyclodextrin; LA, linoleic acid; CMC, critical micelle concentration; DES, diethylstilbestrol.

INTRODUCTION

The catalytic efficiency and specificity of enzymes with respect to both the substrate and the catalyzed reaction are their most important properties for use in biocatalysis. However, their use is very restricted

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owing to the low water solubility of substrates that are of interest, since enzymes hardly support the presence of solvents that are suitable for substrate solubilization.

One approach to the problem consists of transferring active enzymes from their home aqueous medium to a water-inmiscible organic medium, where hydrophobic substrates easily dissolve. For this, enzymes have been added directly to the organic medium either as a lyophilized powder (*1*) or modified with polyethylen glycol (*2*). Reverse micelles have also been used as carriers of the enzyme in the organic medium, with a high catalytic yield (*3–5*). Biphasic systems have also proven to be suitable systems for biocatalysis (*6*). However, these approaches have serious limitations regarding catalytic efficiency when the enzyme is used directly in organic solvent, product recovery in the case of microemulsions, and the enzyme stability in biphasic systems. In addition, complicated equations are required to describe the enzyme kinetics (*7*).

An alternative approach is to bring the substrate to the aqueous medium, in which the enzyme is efficient and stable. Large quantities of poorly water-soluble compounds can be solubilized in detergent micelles, but not all detergents are harmless to the enzyme (*8*), and just a few are sufficiently characterized or pure enough to be employed in enzyme assays (*9*).

One new system that seems promising is the CD-aided solubilization of poorly water-soluble substrates (*10*). CDs are natural, water-soluble cyclic oligosaccharides, composed of six, seven, or eight glucose units and named α-, β-, and γ-CD, respectively. Substitution of the free—OH groups gives rise to a great variety of CD derivatives, such as mβ-CD, hpβ-CD, and malβ-CD (*11*). CDs are capable of forming molecular inclusion complexes with poorly water-soluble compounds in aqueous solutions, thus greatly enhancing their solubility in water (*12,13*). These compounds occupy the hydrophobic cavity of CD and behave as water-soluble substrates. From the biocatalysis point of view, this opens new opportunities for the general use of enzymes regardless of the hydrophobic nature of their substrates. Here we show three reactions in which these possibilities are demonstrated: the lipoxygenase-catalyzed dioxygenation of linoleic acid at low pH, the lipoxygenase-catalyzed hydroperoxidation of DES, a stilbene derivative, and the cholesterol oxidase-catalyzed oxidation and isomerization of cholesterol. In all three cases, the reaction can be easily followed by UV-visible spectroscopy by virtue of the transparency of the solutions and the low absorption of the CDs.

MATERIALS AND METHODS

Linoleic acid (LA) was purchased from Cayman Chemical Co. (Paris, France). Cholesterol was from Fluka (Buchs, Switzerland), and *Brevibacterium sterolicum* cholesterol oxidase, β-CD, γ-CD, DES, and lipoxygenase (type V) from soybean were obtained from Sigma (Madrid, Spain). Methyl-

β-cyclodextrin (mβ-CD) and maltosyl-β-cyclodextrin (malβ-CD) were purchased from Ensuiko Sugar Refining, Co. (Tokyo, Japan) and 2-hydroxypropyl-β-cyclodextrin was from RBI (Natik, MA). All the other chemicals used were of the highest purity.

Preparations of Complexes

LA–β-CD complex solution was prepared by dissolving β-CD in buffer, pH 6.3, containing 1% v/v EtOH, followed by mixing with the fatty acid dispersed in the same buffer until the turbidity completely disappeared. The samples were flushed with N_2 to prevent oxidation of LA during preparation. Complexes of cholesterol and mβ-CD or DES and γ-CD were formed by adding the appropriate amount of solid cholesterol or DES to a 10-m*M* CD solution in buffer and stirring until total dissolution; 0.1*M* phosphate buffer was used throughout, and all solutions were filtered through 0.2-μm filters.

Solubility of DES in CDs

An excess of solid DES was added to a 15-m*M* CD solution in buffer, and stirred in an incubator at 25°C for 2 d. The nondissolved material was separated by centrifugation and the clear supernatant was filtered through 0.2-μm filters. The concentration of DES was determined by its absorbance at 238 nm according to Nozawa et al. (*14*).

Enzyme Assays

Lipoxygenase activity was assayed by monitoring the increase in absorbance at 234 nm (ϵ_{234} = 25,000 M^{-1}/cm) of the forming hydroperoxides. Measurements were made in a Uvikon 940 spectrophotometer from Kontron Instruments at 25°C, equipped with thermostated cells. The transformation of cholesterol into Δ^4-cholesten-3-one, and DES into DES quinone was followed by scanning spectrophotometry in the UV-VIS region in a Hewlett-Packard 8452A diode array spectrophotometer equipped with a Peltier thermostat. The reactions were started by adding 5 μL of enzyme solution to 1 mL of complex preparation.

RESULTS AND DISCUSSION

Lipoxygenase-Catalyzed Dioxigenation of LA

Lipoxygenase (EC 1.13.11.12) catalyzes the oxidation of unsaturated fatty acids containing cis,cis-1,4-pentadiene system. When using LA as substrate, soybean lipoxygenase yields principally the 13-hydroperoxide product and a minor amount of 9-hydroperoxide, but these isomers give similar UV spectra. Thus, the dioxygenase activity can be followed by monitoring the absorbance at 234 nm (*15*).

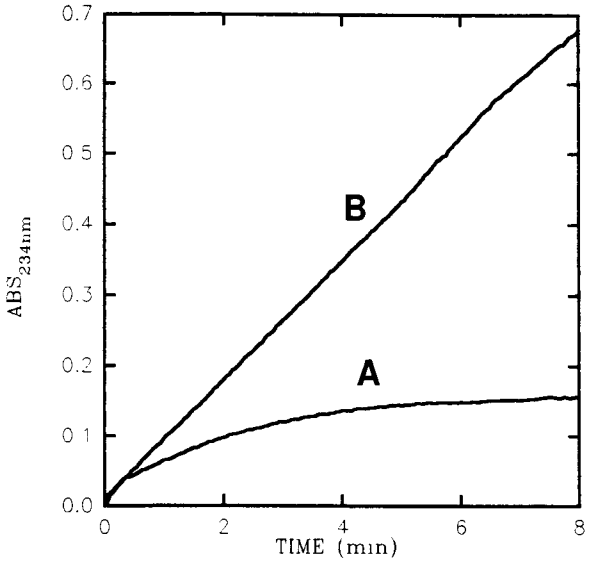

Fig. 1. Time-course of the soybean lipoxygenase-catalyzed dioxygenation of LA in the absence (**A**) and presence (**B**) of β-CD at pH 6.3. In (A) a 7-μ*M* LA concentration was used. In (B) the medium contained 1 m*M* β-CD and 217 μ*M* LA was used, which yields 7 μ*M* free LA according to the equilibrium constants given in Table 1.

Figure 1 shows the soybean lipoxygenase-catalyzed dioxigenation of LA both in the absence and in the presence of β-CD, at pH 6.3. In such conditions without β-CD, the reaction medium becomes turbid above 9 μ*M* LA owing to the formation of large aggregates of fatty acid. At lower concentrations (those usually used for lipoxygenase assay at neutral pH), the medium is transparent, but the spectrophotometric method is less sensitive and the substrate consumption is so fast that initial velocity determinations are very inaccurate, as can be seen in Fig. 1A. In the presence of β-CD a much larger amount of LA can be solubilized, keeping the medium homogenous and transparent. In addition, the consumption of substrate is negligible with respect to its concentration, exhibiting a long steady-state phase as seen in Fig. 1B.

Fatty acids in aqueous solution are very insoluble, especially at pH lower than its pK_a, forming a dispersed, turbid oil phase above its CMC (*16*). When β-CD is present, the turbidity disappears, indicating that the oily aggregates have been dissolved. We previously demonstrated that this is owing to inclusion complex formation and that CMC increases by such an effect of β-CD. Likewise, the analysis of the dependence of CMC on β-CD concentration led to the proposal of a stoichiometry of 1:2 (LA:β-CD) and an equilibrium characterized by two equilibrium constants, K_1 and K_2 (*10*). Such a physicochemical characterization is the basis of the correct use of inclusion complexes in biocatalysis, because it permits the calculation of the concentration of every species in the reaction medium: free, complexed 1:1, and complexed 1:2.

Table 1
Interaction Between LA and β-CD According to López-Nicolás et al. (*10*)

$$\beta\text{-CD} + \text{LA} \underset{}{\overset{K_1}{\rightleftharpoons}} \beta\text{-CD} - \text{LA} \underset{+\ \beta\text{CD}}{\overset{K_2}{\rightleftharpoons}} \beta\text{-CD}_2 - \text{LA}$$

$$(\text{LA})_f = [(\text{LA})_t / 1 + K_1(\text{CD})_t + K_1K_2(\text{CD})_t^2]$$

$$K_1 = 11.2\ \text{m}M^{-1}{}^a \qquad K_2 = 1.7\ \text{m}M^{-1}{}^a$$

[a] At pH 6.3 and 25°C.

By doing so, the total concentration of LA at a given concentration of β-CD was calculated so that free concentration of LA was the same as that assayed in the absence of β-CD. The equilibrium constants and the formula used are shown in Table 1. As shown in Fig. 1, initial velocities in both cases were the same, but although the substrate is quickly exhausted in the absence of β-CD, the reaction progresses for a longer time at the same rate. The constancy of the reaction rate at a constant free concentration of LA indicates that lipoxygenase is only able to use that pool of substrate, without noticing the existence of the LA complexed with CD. On the other hand, the fact that the reaction progresses linearly for a long time in the presence of CD indicates that the complexed substrate is being utilized by the enzyme, although not in a direct way. The most likely mechanism is that as free LA is transformed into product, it is replaced by fresh LA from complexes, which dissociate according to the equilibrium described in Table 1, thus keeping the concentration of free LA practically constant.

Lipoxygenase-Catalyzed Hydroperoxidation of DES

In addition to its dioxygenase O_2-dependent activity, soybean lipoxygenase has been shown to have a hydroperoxidase activity by which a series of compounds, such as phenols, catechols, hydrazin, hydroxylamine, and hydrazide derivatives, can be oxidized in the presence of peroxides, such as LA hydroperoxide or H_2O_2 (*17,18*).

DES is an inexpensive stilbene derivative with an estrogenic activity comparable to that of the natural strogen, estradiol-17β (*19*). It has been used as a food additive for livestock and, in women, as a postcoital contraceptive. However, after clinical usage, it has been shown to be carcinogenic in animals (*20*), and its use as an abortifactant in humans has been linked to the appearance of genital tract tumors in women whose mothers were treated with the drug (*21*). The mechanisms underlying DES carcinogenicity are as yet unresolved, but have been thought to be related to its peroxidatic oxidation to DES quinone, which covalently binds to DNA (22). Even though several in vivo studies of peroxidase-catalyzed metabolism of [^{14}C] DES derivatives have been carried out, little is known about its in vitro kinetic oxidation because of its poor solubility in water,

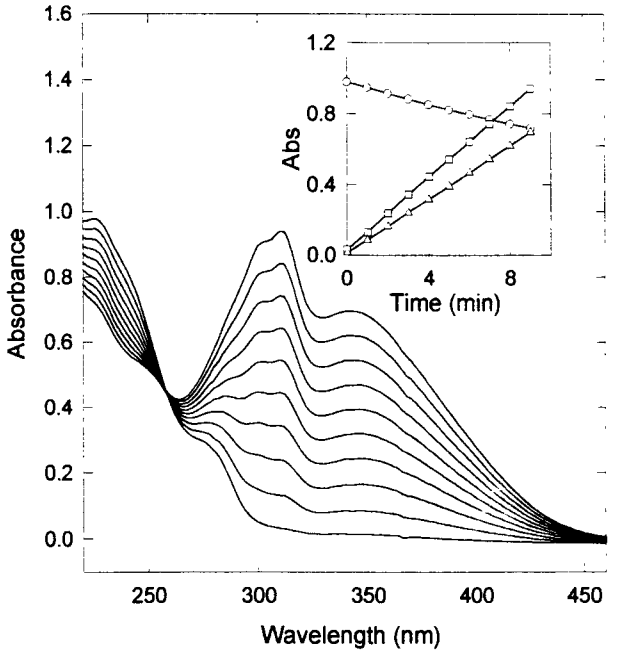

Fig. 2. Oxidation of DES by soybean lipoxygenase with H_2O_2 in γ-CD. The reaction medium contained 0.15 μ*M* lipoxygenase, 0.13 m*M* H_2O_2, 76 μ*M* DES, 1.5 m*M* γ-CD in 0.1*M* phosphate buffer, pH 7.4. Spectra were recorded every minute. Inset: Time-course of the reaction monitored at 226 nm (○), 310 nm (□), and 340 nm (Δ).

Table 2
Solubility Enhancement of DES
in Aqueous Medium by Complexation with CDs

Medium	DES, μg/mL[a]	Solubility enhancement, *x*-fold
Water	11.5	1
α-CD	31.2	2.7
β-CD	31.2	2.7
hpβ-CD	1502	131
mβ-CD	1525	133
malβ-CD	1666	145
γ-CD	2042	177

[a]Solubility in 15 m*M* CD at 25°C.

the use of very low DES concentration, or organic solvent (ethanol) being needed to detect enzymatic activity (22).

As mentioned before, the solubility of poorly water-soluble compounds can be greatly enhanced with the aid of CD, in the case of DES up to 177 times (Table 2). Only modified CDs (hpβ-CD, mβ-CD, malβ-CD) or

the bulkiest CD (γ-CD) produced a considerable increase in solubility, which was greater in γ-CD (177 times). When this DES/γ-CD complex was incubated with LOX and H_2O_2, an increase in the 290–400 nm region was observed. The yellow color corresponded with the DES-quinone, which has two maxima at 310 and 345 nm as previously described (*23*). In CD medium, this oxidation shows a clearly defined isosbestic point, which implies that DES has been transformed into DES-quinone and that only these two species were present in the solution. As can be seen in Fig. 2 (inset), the decrease in DES (λ = 226 nm) and the concomitant increase in DES-quinone, with the two maxima (λ = 310 and λ = 340 nm), occurs at a constant rate. In water or water/organic solvent mixtures, this isosbestic point is not maintained as long as in CD medium, because the DES-quinone quickly undergoes nonenzymatic transformations (tautomerization) into isodienestrol (*23*). This result is an indication of the stabilizing effect of CD on highly reactive species.

Cholesterol Oxidase-Catalyzed Oxidation and Isomerization of Cholesterol

Steroid biotransformation for the pharmaceutical industry is an area of great commercial interest, and the versatility of microbial systems in the production of valuable steroid compounds is now well recognized. The products resulting from the microbial degradation of the steroid used as the carbon source very much depend on the type of microorganism, the strain, and the growing conditions (*24*). Enzymes are advantageous over whole cells in that the substrate's conversion can be fully controlled and characterized. In both cases, there is a problem of steroid solubility. CDs have been employed in fermentation broth to improve the availability of cholesterol, sitosterol, and Δ^4-cholesten-3-one for side chain cleavage by *Mycobacterium* sp., such activity increasing by up to threefold (*25*). Cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation of the 3β-hydroxyl group of sterol compounds, which is a key step in the side-chain cleavage of such steroids. The process is usually associated with the subsequent isomerization of the C-5 double bond to the position C-4 (*see 26* for a review). In the case of cholesterol, the reaction product is Δ^4-cholesten-3-one, which displays a broad UV absorption band with a maximum at 240 nm. The low aqueous solubility of cholesterol (4.6 μ*M*; *27*) can be greatly increased in the presence of mβ-CD (ca. 1 m*M* cholesterol in 10 m*M* mβ-CD, 6 m*M* in 30 m*M*, 14 m*M* in 60 m*M*) (*28*) through the formation of inclusion complexes. The low UV absorption of CDs permits the spectroscopic characterization of the transformation of cholesterol into Δ^4-cholesten-3-one even at wavelengths as low as 200 nm. As shown in Fig. 3, two isosbectic points are clearly defined, at 208 and 275 nm. The existence of isosbectic points between cholesterol and Δ^4-cholesten-3-one indicates that

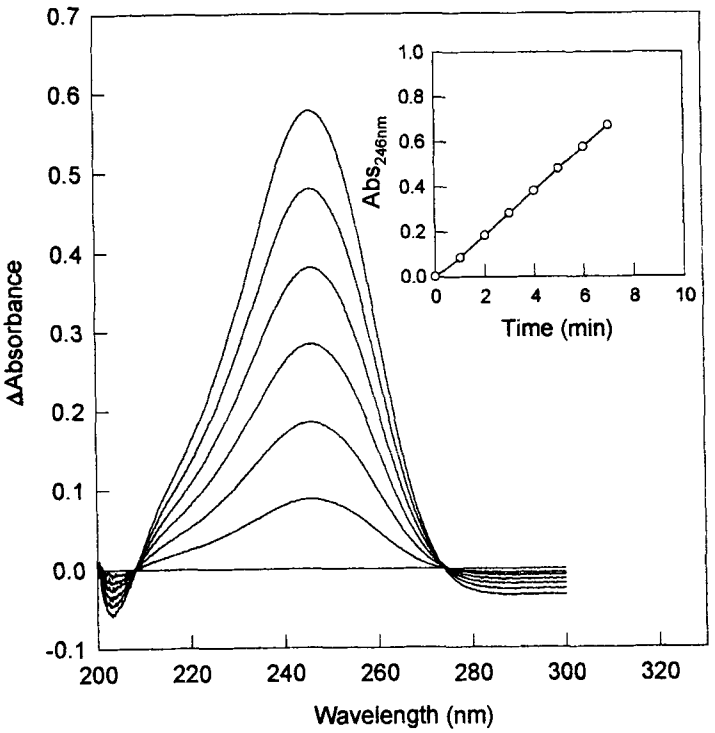

Fig. 3. Difference spectra of cholesterol oxidase-catalyzed oxidation of cholesterol in mβ-CD-containing medium. The reaction medium contained 1 U/mL cholesterol oxidase from *B. sterolicum*, 0.15 m*M* cholesterol, and 5 m*M* mβ-CD in 0.1*M* phosphate buffer, pH 7.4. Spectra were recorded every minute, and substraction was carried out taking the first spectrum as reference in order to emphasize the presence of isosbectic points. Inset: Time-course of the reaction monitored at 246 nm.

the accumulation of the intermediate Δ^5-cholesten-3-one, is practically negligible. The maximum that appears at 240 nm in water–propanol solutions of Δ^4-cholesten-3-one is shifted to 246 nm, indicating some type of interaction, most probably its entrapment in empty mβ-CD molecules. As seen in Fig. 3 (inset), the product accumulates linearly for several minutes, and the reaction can be suitably monitored at the λ_{max} as in the case of LA oxidation by lipoxygenase.

CONCLUDING REMARKS

The present work demonstrates the suitability of CDs to act as carriers of poorly water-soluble compounds for their enzymatic biotransformation. With respect to other experimental approaches, such as lyophilized powdered enzymes in organic solvents and enzymes in reverse micelles or in biphasic systems, CDs present many attractive advantages, such as

enzyme preservation, and ease of medium handling, and of reaction monitorization. Future work in this field will involve the understanding of enzyme kinetics and catalysis in CD media, and is currently under investigation in our laboratory.

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