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7α-Dehydroxylation of Bile Acids by Resting Cells of a *Eubacterium lentum*-Like Intestinal Anaerobe, Strain c-25

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7α-Dehydroxylation of cholic acid and chenodeoxycholic acid by whole cells of strain c-25, a *Eubacterium lentum*-like intestinal anaerobe, was studied. 7α-Dehydroxylase activity was observed only in whole cells grown in the presence of the primary bile acid (cholic acid or chenodeoxycholic acid). Chenodeoxycholic acid was twice as effective as cholic acid as an inducer. Although cells grown in the presence of chenodeoxycholic acid had no significant substrate specificity for the two primary bile acids, cells grown in the presence of cholic acid showed two times greater activity against cholic acid than chenodeoxycholic acid. Exposure of cell suspensions to atmospheric oxygen resulted in little loss of the 7α-dehydroxylase activity. The induced enzyme had an optimal pH range of 7.3 to 7.7. Although adding flavin mononucleotide to the growth medium significantly increased the 7α-dehydroxylation of bile acids without an increase in cell growth, inhibition of the enzyme activity was observed in the resting cell system when flavin mononucleotide was included in the reaction mixture.

The physiologically most important transformation of bile acids in the gut is 7α-dehydroxylation. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are converted through this reaction into deoxycholic acid (DCA) and lithocholic acid (LCA), respectively (9, 22). Despite numerous attempts, only a few strains of intestinal anaerobic bacteria have so far been reported to have 7α-dehydroxylase activity (4–8, 10, 12, 13, 28). Furthermore, the enzyme activity in resting cells has been found only in *Clostridium leptum* (23, 24), *Eubacterium* sp. strain VPI 12708 (15, 29), *Clostridium bifermentans* (2), and an unidentified, gram-positive, nonsporeforming anaerobic bacterium, strain HD-17 (18). Although most fecal bile acids are 7α-dehydroxylated, the above organisms usually show only feeble activity in in vitro cultures. The pH and redox potential of the growth medium and the composition of the medium and anaerobic environment have been reported to be important in the 7α-dehydroxylation of bile acids (1, 3, 18, 21). White et al. (26) stated that NAD^+ is the cofactor stimulating the 7α-dehydroxylase activity in cell extracts of *Eubacterium* sp. strain VPI 12708. However, available information concerning factors responsible for the prevalence of 7α-dehydroxylation in vivo is far from sufficient.

We recently reported that adding flavins to the growth medium specifically enhances the 7α-dehydroxylation by growing cultures of a *Eubacterium lentum*-like intestinal anaerobe, strain c-25 (19). To gain some insight into the mechanism of this enhancement, this study determined the effect of flavin mononucleotide (FMN) on the 7α-dehydroxylation by resting cells of strain c-25. Additionally, we investigated the inducibility and oxygen stability of the enzyme in cells.

MATERIALS AND METHODS

Bacterial strain. Strain c-25 is an *E. lentum*-like intestinal anaerobe previously isolated from human feces in our laboratory (13) and has been characterized further (11). The strain possesses both 7α-dehydroxylase and 7α-hydroxysteroid dehydrogenase activities (11, 13), like most of the 7α-dehydroxylating organisms previously reported (3, 4, 6, 10, 21). The organism was maintained in GAM semisolid agar (25) (Nissui Pharmaceutical Co.) in a tightly stoppered tube at 4°C, and the inoculum came from an overnight culture in GAM broth.

Preparation of washed whole cells. An 800-ml quantity of freshly boiled and cooled modified peptone-yeast extract broth (13, 14) containing 150 μg of bile acid per ml was inoculated with 40 ml of the inoculum culture. After 21 h of incubation at 37°C in an anaerobic jar under nitrogen, the culture was harvested by centrifuging at 6,000 × *g* for 30 min at 4°C. The cells were washed three times with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium thioglycolate. The final sediment was resuspended in an appropriate amount of the same buffer solution. All these procedures were carried out under ordinary atmospheric conditions.

Assay of 7α-dehydroxylase activity in washed whole cells. The standard reaction mixture consisted of 3.0 ml of 0.1 M sodium phosphate buffer (pH 7.5), 0.1% sodium thioglycolate, 500 μg of bile acid, and 1.0 ml of washed cell suspension (1.0 mg of whole-cell protein). The mixture was placed in a small test tube and held in a water bath at 37°C without agitation under atmospheric conditions. The reaction was stopped after the desired period of incubation by adding three drops of 6 N HCl. Protein concentration was estimated by the method of Lowry et al. (17) after alkali solubilization (1 N NaOH) of whole cells.

Reduction of FMN. Three milliliters of 0.1 M sodium phosphate buffer (pH 7.5) containing 5.3 μmol of FMN, 0.1% sodium thioglycolate, 500 μg of bile acid, and 1.4 μmol of EDTA was illuminated at a distance of 7 cm by a 40-W tungsten lamp. Chemical reduction of FMN was performed with sodium dithionite. An 8-μmol quantity of sodium dithionite was added to 3.0 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 2 μmol of FMN, 0.1% sodium thioglycolate, and 500 μg of bile acid. Photochemical and chemical reduction of FMN was performed in a Thunberg tube under anaerobic conditions, which were maintained by

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several cycles of vacuum degassing and replacement with oxygen-free nitrogen.

Analysis of bile acids by GLC. Free bile acids extracted with ethyl acetate from acidified samples (spent culture medium or incubated reaction mixture) were methylated by the methanol-sulfuric acid procedure and were analyzed by gas-liquid chromatography (GLC) with a 3% QF-1 column as previously described (13). Individual bile acids were identified by relative retention time compared with methyl deoxycholate. Quantities were calculated by measuring peak areas and were expressed as percentages of composition after confirming that the recovery of sample bile acids was compatible with the quantity of the original substrate bile acid.

Bile acids. Bile acids are referred to by abbreviations of their trivial names: CA (3α,7α,12α-trihydroxy-5β-cholanoic acid), CDCA (3α,7α-dihydroxy-5β-cholanoic acid), ursodeoxycholic acid (UDCA) (3α,7β-dihydroxy-5β-cholanoic acid), DCA (3α,12α-dihydroxy-5β-cholanoic acid), LCA (3α-hydroxy-5β-cholanoic acid), and taurocholic acid (TCA) (3α,7α,12α-trihydroxy-5β-cholanoyl taurine).

The free bile acids used as substrates were CDCA (99.9% pure), a gift from Tokyo Tanabe Pharmaceutical Co., and CA (99.1% pure), purchased from Sigma Chemical Co., both of which proved to be pure when tested by GLC. TCA was obtained from Calbiochem-Behring. The methyl esters of bile acids used as GLC references were all obtained from Gasukuro-Kogyo Co.

RESULTS AND DISCUSSION

Relation between cell concentration and 7α-dehydroxylase activity in whole cells. Identification and quantification of the reaction products were performed by GLC as previously described (13). The quantity of the 7α-dehydroxylated bile acids was expressed as the percentage of the total bile acids (percentage of 7α-dehydroxylation). Strain c-25 7α-dehydroxylated both CA and CDCA, yielding DCA and LCA, respectively, and also catalyzed 3α- and 7α-dehydrogenation, giving rise to the formation of 3-oxo and 7-oxo bile acids. Figure 1 shows 7α-dehydroxylase activity in washed whole cells of strain c-25 grown in the presence of CDCA. The enzyme activity was linear with cell protein concentration. The cells dehydroxylated CA and CDCA to similar extents, as reported by Aries and Hill (3) with cell-free extracts of various bacterial species.

Oxygen sensitivity of 7α-dehydroxylase in whole cells. The 7α-dehydroxylating organisms are able to grow only under strict anaerobic conditions (4, 5, 8, 10, 13, 20), and the enzyme was reported to require an obligatory anaerobic environment for activity because aeration of the reaction mixture completely inhibits the 7α-dehydroxylation of CA (18). Furthermore, Aries and Hill (3) stated that the enzyme is labile even in cell-bound form. In contrast, less than 10% loss of activity was observed even after 1 h of exposure of a cell suspension to atmospheric oxygen (Fig. 2). Thus, the 7α-dehydroxylase in whole cells of strain c-25 was fairly oxygen stable, unlike the situation reported by Aries and Hill. However, using ultrasonic oscillation to rupture the cells resulted in complete loss of the enzyme activity. Similar inactivation of the enzyme was also reported by Stellwag and Hylemon (24) for *C. leptum* cells and by Masuda and Oda (18) for cells of a gram-positive, nonsporeforming anaerobic bacterium, strain HD-17. On the other hand, cell extracts of *Eubacterium* sp. strain VPI 12708 were reported to retain active 7α-dehydroxylase (29). Therefore, the stability of the enzyme may vary from strain to strain.

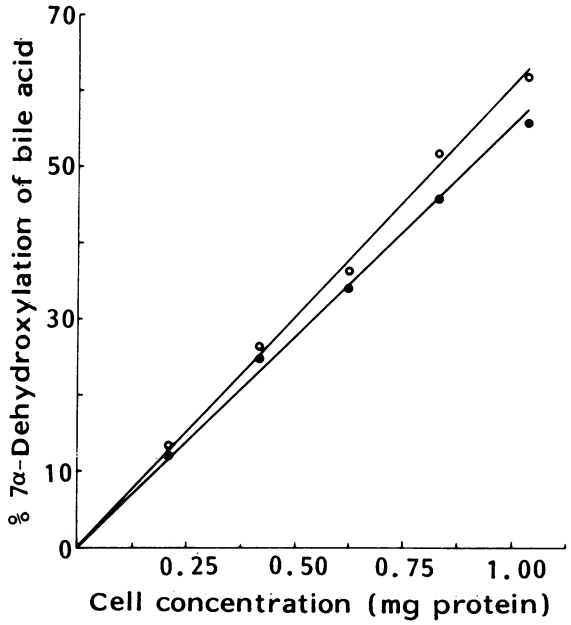

FIG. 1. 7α-Dehydroxylase activity as a function of protein concentration of *E. lentum*-like intestinal anaerobe, strain c-25, grown in the presence of CDCA. Cells at the indicated concentration were incubated with 500 μg of CA (●) or CDCA (○) for 6 h at pH 7.5.

Optimum pH for 7α-dehydroxylation. The enzyme activity had an optimum pH of 7.3 to 7.7 (Fig. 3), which is more alkaline than the value of 6.5 to 7.0 for *C. leptum* cells (24) or the value of pH 7.0 for *Eubacterium* sp. strain VPI 12708 cells (15). It appears that the pH optima against CA and CDCA are different (Fig. 3). These results suggest the presence of two distinct enzymes, one specific for CA and the other specific for CDCA.

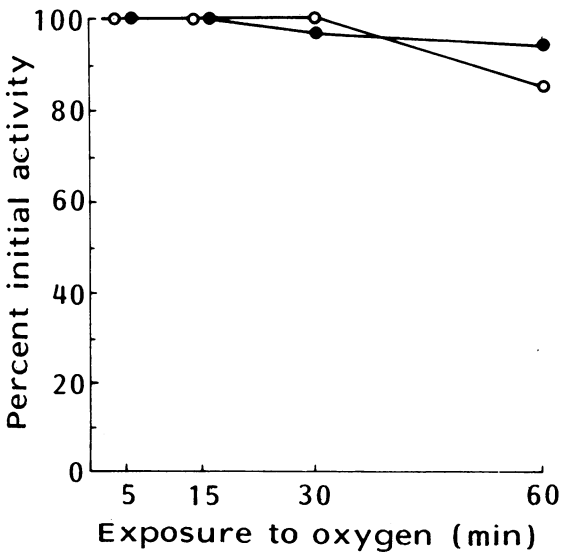

FIG. 2. Effect of O_2 exposure on 7α-dehydroxylase activity in whole cells. Whole cells grown in the presence of CDCA were shaken in an L-shaped tube. After the indicated period, exposure to air was stopped by three cycles of vacuum degassing and replacement with pure nitrogen in a Thunberg tube. The standard assay was used to determine the activity against CA (●) and CDCA (○).

Induction of the enzyme. The enzyme activity was observed only in whole cells grown in the presence of 7α-hydroxy bile acids (Fig. 4). In agreement with White et al. (29), who reported that the induction of the enzyme is specific for the bile acids containing a free carboxyl group at C-24 in cells of *Eubacterium* sp., adding conjugated 7α-hydroxy bile acid to the growth medium did not induce production of the enzyme. In this regard, the gram-positive nonsporeforming anaerobic bacterium strain HD-17 is unique in that conjugated CA is a more powerful inducer than free CA (18). Complete inducibility of 7α-dehydroxylase was also reported by Masuda and Oda (18) for strain HD-17 cells. In contrast, cells of *Eubacterium* sp. grown in the absence of an inducer were reported to have low but measurable amounts of the enzyme activity, although adding CA to the growth medium caused a 46-fold increase in the activity (29). White et al. (29) also stated that CA induces three- to fivefold higher activity than does CDCA in cells of *Eubacterium* sp. Furthermore, Masuda and Oda (18) reported that CDCA is capable of inducing much less enzyme activity than CA in whole cells of strain HD-17. In contrast with these findings, CDCA is twice as effective as CA as an inducer in strain c-25 (Fig. 4).

Substrate specificity. Although cells grown in the presence of CDCA dehydroxylated CA and CDCA at almost the same rate, cells grown in the presence of CA showed a two-times-higher rate of 7α-dehydroxylation of CA than CDCA (Fig. 4). That is, the enzyme induced by CDCA had no substrate specificity, but the enzyme induced by CA showed substrate specificity for CA as opposed to CDCA. This finding suggests that the enzyme induced by CA may be different from that induced by CDCA. Cells of strain c-25 7-dehydroxylated CDCA but not UDCA. Therefore, the enzyme appears to be a 7α-hydroxy-oriented enzyme, and there does not appear to be 7β-dehydroxylase activity, unlike the findings of White et al. (28), who stated that cells of *Eubacterium* sp. transform both CDCA and UDCA to form LCA and that 7α- and 7β-dehydroxylase activity may be catalyzed by the same enzyme.

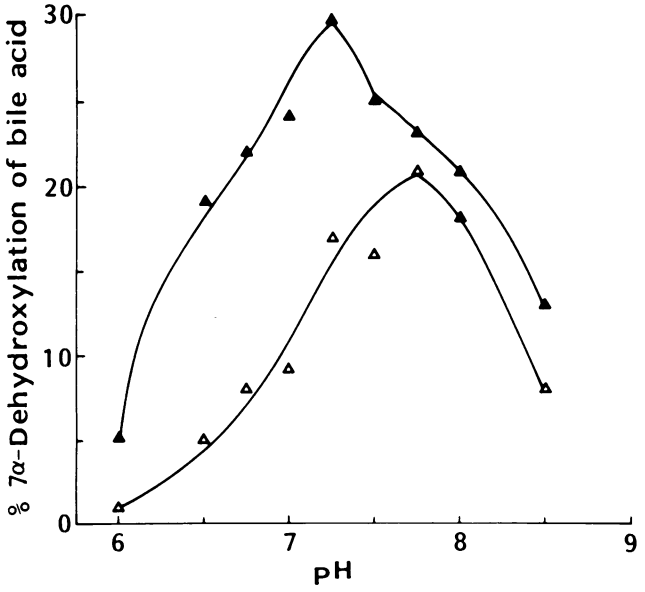

FIG. 3. Effect of pH on 7α-dehydroxylation by whole cells of CA (▲) and CDCA (△). Cells grown in the presence of CA were incubated with either CA or CDCA for 6 h in 75 mM sodium phosphate buffer (pH 6.0 to 8.5).

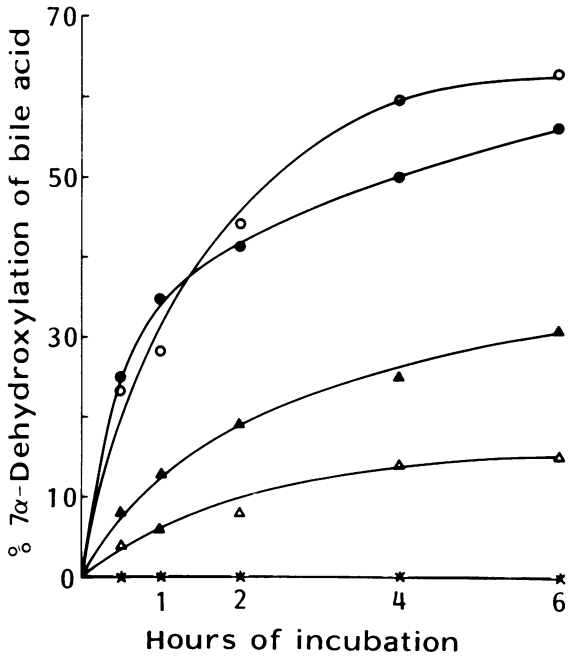

FIG. 4. Time course of 7α-dehydroxylating activity induced by various bile acids. The enzyme activity was determined in cells grown in the presence of various bile acids and either CA or CDCA as substrate at the indicated period. Results are shown for CA-induced cells against CA (▲) and CDCA (△), CDCA-induced cells against CA (●) and CDCA (○), TCA-induced cells against CA (x) and CDCA (x), uninduced cells against CA (x) and CDCA (x), and CDCA-induced cells and CA-induced cells against UDCA (x).

Effect of FMN on 7α-dehydroxylase activity in whole cells. Lipsky and Hylemon (16) reported that CA induces 7α-dehydroxylase and NADH:flavin oxidoreductase activities in cell extracts of *Eubacterium* sp. White et al. (26) reported that 7α-dehydroxylase activity increases four- to sixfold in the presence of NAD^+ in cell extracts of *Eubacterium* sp. and that NAD^+ is also required for the reduction of 3α-hydroxy-5β-6-cholen-24-oic acid (Δ^6-intermediate) to LCA. A stimulatory effect on 7α-dehydroxylation by reduced flavin nucleotides was not observed. Furthermore, White et al. (27) stated that adding NAD^+ to the reaction mixture stimulated about twofold the reduction of the Δ^6-intermediate to LCA by cell extracts of *Eubacterium* sp. and that reduced FMN or reduced flavin adenine dinucleotides further stimulated this reduction when added to a reaction

TABLE 1. Effect on 7α-dehydroxylase production of adding FMN to the growth medium[a]

Additive	Bacterial growth (%)[b]	% 7α-Dehydroxylation of CDCA in					
		Growth medium	Whole cells at time (h)				
			0.5	1	2	4	6
None	94	16	30	45	61	63	77
FMN[c]	94	54	38	60	71	68	77

[a] The growth medium was a modified peptone-yeast extract broth containing 150 μg of CDCA per ml.
[b] Percent transmittance of light at 660 nm.
[c] Added at 150 μg/ml.

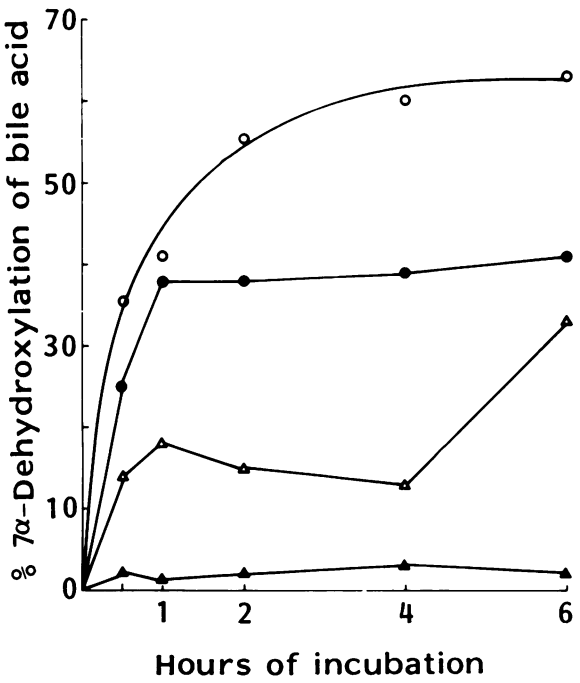

FIG. 5. Effect of adding FMN on the rate of 7α-dehydroxylation of CDCA by whole cells. Cells grown in the presence of CDCA were incubated anaerobically in a Thunberg tube under nitrogen gas. Symbols for substances added: ○, control (no addition); ●, 5.3 μmol of FMN; △, 5.3 μmol of photochemically reduced FMN; ▲, 2 μmol of chemically reduced FMN ($Na_2S_2O_4$).

mixture already containing NAD^+ but FMN or flavin adenine dinucleotides slightly inhibited the reaction. We recently reported (19) that FMN specifically stimulates the 7α-dehydroxylation by anaerobically growing cultures of strain c-25 and that almost complete dehydroxylation was observed with a concentration of FMN equivalent to that of the substrate bile acid. Therefore, we suggested that the shortage of FMN is a cause of the low 7α-dehydroxylase activity in the growth medium. However, resting cells of strain c-25 had almost the same 7α-dehydroxylase activity, irrespective of the presence or absence of FMN in the growth medium, indicating that adding FMN to the growth medium does not affect the production of 7α-dehydroxylase (Table 1). To further study the effect of FMN on 7α-dehydroxylation by resting cells of strain c-25, FMN (oxidized form or reduced form) was individually added to the reaction mixture, and the yield of LCA from CDCA was determined (Fig. 5). Adding FMN or reduced FMN to the reaction mixture unexpectedly inhibited 7α-dehydroxylation. Chemically reduced FMN almost completely inhibited the reaction. However, the mechanism of FMN inhibition of 7α-dehydroxylation by resting cells needs to be studied further.

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