CURRENT MICROBIOLOGY Vol. 30 (1995), pp. 259–263

Current Microbiology
An International Journal

Expression in *Escherichia coli* and Characterization of a Bile Acid-Inducible 3α-Hydroxysteroid Dehydrogenase from *Eubacterium* sp. Strain VPI 12708

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Abstract. We have previously cloned and sequenced three members of a bile acid-inducible gene family from *Eubacterium* sp. strain VPI 12708 that encode 27,000-M_r polypeptides. Two copies of these genes (*baiA1* and *baiA3*) are identical, while the third copy (*baiA2*) encodes a polypeptide sharing 92% amino acid identity with the *baiA1* and *baiA3* gene products. We have overexpressed the *baiA1* gene in *Escherichia coli* and analyzed the expressed activity. Thin-layer chromatography of ^{14}C-labeled bile acid products from reactions using cell-free extracts revealed a 3α-hydroxysteroid dehydrogenase activity for the BaiA1 protein. The BaiA1 protein could utilize both NAD^+ and $NADP^+$, and the preferred steroid substrate was the cholyl-coenzyme A conjugate rather than free cholic acid. These results show that the BaiA proteins are novel 3α-hydroxysteroid dehydrogenases.

The intestinal bacterium *Eubacterium* sp. strain VPI 12708 possesses a bile acid 7α-dehydroxylation activity that is induced by culturing in the presence of primary bile acids [21]. Bile acid 7α-dehydroxylation has been proposed to proceed by a multistep pathway in which the bile acid is first linked to coenzyme A (CoA) upon entering the cell (Fig. 1) [7, 15]. The bile acid-CoA conjugate then undergoes a two-step oxidation followed by loss of the 7α-hydroxy group in a dehydration step. A three-step reduction then yields the 7-dehydroxylated product.

Eubacterium sp. strain VPI 12708 synthesizes several new polypeptides following cholic acid induction [18, 22]. Several of these inducible polypeptides have been purified, and the genes encoding them have been cloned and sequenced [7, 10, 14, 23, 24].

One of the purified polypeptides, a 27,000 M_r species, is encoded by two identical genes (*baiA1* and *baiA3*) [7, 12]. A third member of this gene family (*baiA2*) [24] shares 81% nucleotide sequence identity with the *baiA1* and *baiA3* genes, while the polypeptide encoded by the *baiA2* gene shares 92% amino acid sequence identity with the other two 27,000-M_r polypeptides (Fig. 2). The *baiA2* gene resides on a large bile acid-inducible operon containing at least nine open reading frames [9, 14], while the other two gene copies (*baiA1* and *baiA3*) are transcribed as monocistronic mRNA species and have been cloned and sequenced from separate chromosomal DNA fragments [7, 12, 24]. All three *baiA* genes are bile acid inducible and have highly conserved promoter regions [14].

Most of the enzymes involved in bile acid 7α-dehydroxylation are thought to be encoded from the large bile acid-inducible operon. The *baiB* gene has been shown to encode a bile acid-coenzyme A ligase [15], and the *baiH* gene encodes a NADH-flavin oxidoreductase [10]. The 27,000-M_r polypeptides have previously been shown to have significant homology with the superfamily of short-chain alcohol dehydrogenases [7, 12]. We therefore hypothesized that the *baiA* genes encoded 3α-hydroxysteroid dehydrogenases [14]. In this study we overexpressed the *baiA1*/*baiA3* gene and analyzed the catalytic activities.

Materials and Methods

Subcloning and expression of the *baiA1* gene in *E. coli*. The *baiA1* gene from *Eubacterium* sp. strain VPI 12708 was previously cloned

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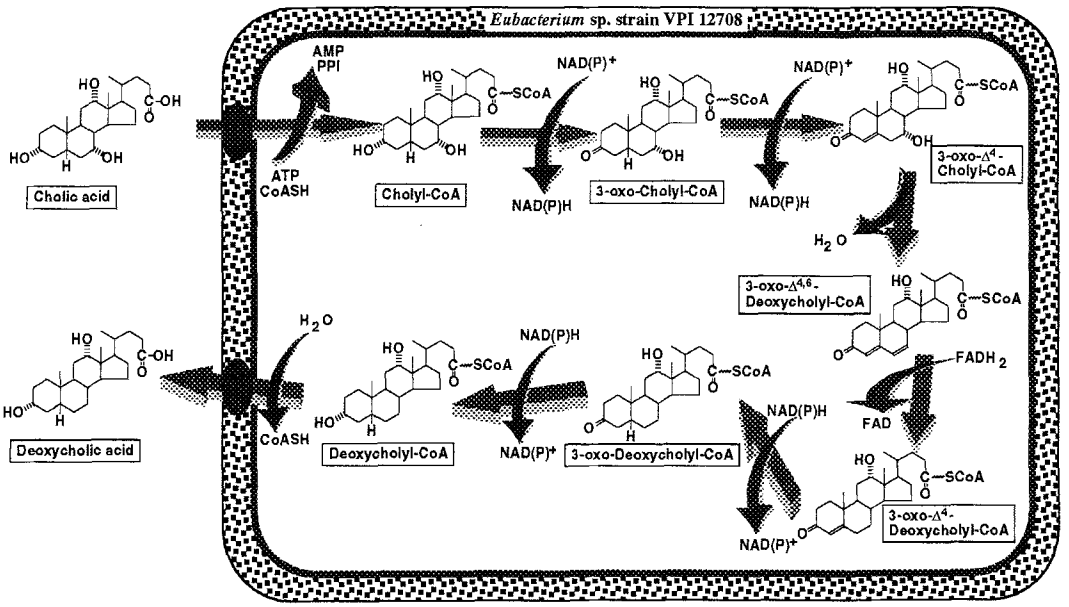

Fig. 1. Proposed pathway for bile acid 7α-dehydroxylation in *Eubacterium* sp. VPI 12708.

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                    **  * *
BaiA1,3     1 MKLVQDKITIITGGTRGIGFAAAKLFIENGAKVSIFGETQEEVDTALAQL 50
              |.|||||:|||||||||||||||||:||:|||||||||||||||||||||
BaiA2       1 MNLVQDKVTIITGGTRGIGFAAAKIFIDNGAKVSIFGETQEEVDTALAQL 50

                      *                   *
BaiA1,3    51 KELYPEEEVLGFAPDLTSRDAVMAAVGTVAQKYGRLDVMINNAGITMNSV 100
              |||||||||||||||||||||||||||| ||||||||||||||||||| |.|
BaiA2      51 KELYPEEEVLGFAPDLTSRDAVMAAVGQVAQKYGRLDVMINNAGITSNNV 100

                                                           ●
BaiA1,3   101 FSRVSEEDFKNIMDINVNGVFNGAWSAYQCMKDAKQGVIINTASVTGIYG 150
              |||||||:||:|||||||.|||||||:|||||||||.||||||||||||:|
BaiA2      101 FSRVSEEEFKHIMDINVTGVFNGAWCAYQCMKDAKKGVIINTASVTGIFG 150

                ●  ●                 ○○                  ○
BaiA1,3   151 SLSGIGYPTSKAGVIGLTHGLGREIIRKNIRVVGVAPGVVDTDMTKGLPP 200
              ||||:|||.|||:|||||||||||||||||||||||||||:||||.| ||
BaiA2      151 SLSGVGYPASKASVIGLTHGLGREIIRKNIRVVGVAPGVVNTDMTNGNPP 200

BaiA1,3   201 EILEDYLKTLPMKRMLKPEEIANVYLFLASDLASGITATTISVDGAYRP 249
              ||:|:|||.|||||||.|||||||||||||||||||||:||||||||
BaiA2      201 EIMEGYLKALPMKRMLEPEEIANVYLFLASDLASGITATTVSVDGAYRP 249
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Fig. 2. Amino acid sequence comparison of BaiA1 and BaiA3 polypeptides (BaiA1,3) with BaiA2. Comparison was done with the GCG Gap program (Genetics Computer Group, Madison, Wisconsin). Marked residues are highly conserved among short-chain alcohol dehydrogenases and have been suggested to be involved in $NAD(P)^+$ binding (*) [3, 11, 17, 19] and active site or steroid binding (●) [4, 11, 20, 21]. A possible CoA binding site is also marked (○) [16].

in a pGEM2 vector (Promega, Madison, Wisconsin) and expressed in *E. coli* HMS174(DE3) [20]. This plasmid will be referred to as pGEM2-27K1.

Partial purification of the *baiA1* gene product. For partial purification of BaiA1, *E. coli* strain HMS174(DE3) containing the pGEM2-27K1 plasmid was used to inoculate 3 L of LB medium [1% tryptone (Difco Laboratories, Detroit, Michigan), 1% yeast extract, 0.5% NaCl, pH 7.5], containing 100 μg/ml ampicillin. The cells were grown with shaking at 37°C to a Klett reading of 80 before induction with 0.5 mM isopropylthio-β-D-galactoside (IPTG). After 2 h additional growth, the cells were harvested by centrifugation, suspended in 1/100th volume of distilled water containing 100 μg of DNase I and 20 mM 2-mercaptoethanol (2ME), and disrupted by sonication. The cell lysate was centrifuged at 105,000 *g* for 2 h, and the supernatant fluid was collected and filtered extensively with Centriprep-10 concentrators (Amicon Corp., Danvers, Massachusetts) to remove low-molecular-weight components. The suspension was then applied to a Waters AP-2 DEAE high-performance liquid chromatography (HPLC) column (Millipore Corp., Burlington, Massachusetts). Fractions eluted from a 0 to 500 mM NaCl gradient in 20 mM sodium phosphate buffer (pH 6.5), 20 mM 2-mercaptoethanol were collected (1-min fractions at a flow rate of 3.5 ml/min). Fractions were assayed spectrophotometrically with NAD^+ as described below. Pooled fractions displaying hydroxysteroid dehydrogenase activity in the spectrophotometric assay were used for the subsequent studies. Protein concentrations

were determined with the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, California) [2].

TLC assays. The standard reaction mixture for analysis of 3α-hydroxysteroid dehydrogenase activity by thin-layer chromatography (TLC) contained 0.2 mM NAD^+ or $NADP^+$, 10 mM MOPS (morpholinepropane-sulfonic acid; pH 7.0), 10 mM 2ME, 5 mM $MgCl_2$, 2.5 mM ATP, 2.5 mM CoA, 5 μM [24-^{14}C]-cholic acid (0.05 μCi), purified bile acid-CoA ligase [15], and partially purified BaiA1 in a 250-μl volume. Ethyl acetate extractions of acidified reaction mixtures were performed before and after boiling with 1 M NaOH to deconjugate the water-soluble bile acid-CoA conjugates [15]. Extracted bile acids were chromatographed on silica 1B TLC plates (J.T. Baker, Inc., Phillipsburg, New Jersey) with solvent systems S1 or S4 [8]. The plates were dried and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, New York) for 24 h at room temperature before developing the film.

Spectrophotometric assays. Spectrophotometric assays for hydroxysteroid dehydrogenase activity were performed by monitoring reduction of NAD^+ or $NADP^+$ at A_{340} with 1-ml samples containing 0.2 mM NAD^+ or $NADP^+$, 50 mM sodium phosphate, pH 7, 0.1 mM bile acid, and BaiA1. Assays with partially purified BaiA1 and various free and conjugated bile acids were performed with approximately 0.07 U/mg protein where one unit of activity is defined as the amount of enzyme required to reduce 1 μmol of $NADP^+$ per minute under standard assay conditions with cholyl-CoA.

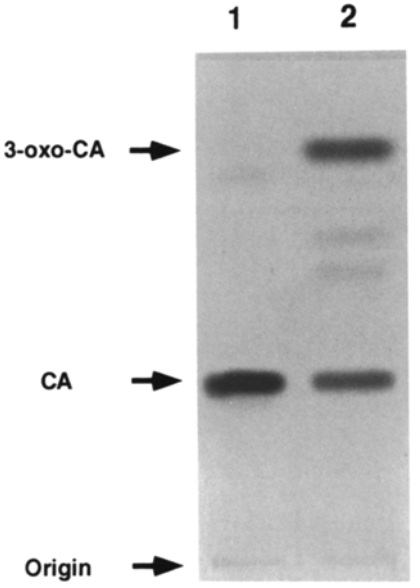

Fig. 3. Thin-layer chromatography of products from reaction of ^{14}C-labeled cholic acid with bile acid-CoA ligase, partially purified BaiA1, and $NADP^+$. Lane 1, first ethyl acetate extraction; lane 2, second ethyl acetate extraction following boiling in 1 M NaOH to deconjugate bile acid-CoA conjugates. CA, cholic acid; 3-oxo-CA, 3-oxo-cholate.

Results

Overexpression of the *baiA1* gene and partial purification of the gene product. The *baiA1* gene was previously cloned in plasmid pGEM2-27K1 [20]. Polyacrylamide-gel electrophoresis and Western blots of extracts from cells transformed with the pGEM2-27K1 plasmids revealed the 27,000-M_r *baiA1* gene product [20]. The BaiA1 protein was partially purified by DEAE-HPLC, and pooled fractions displaying hydroxysteroid dehydrogenase activity in the spectrophotometric assay (see Materials and Methods) were used for all subsequent studies.

TLC assays. Reactions were performed with ^{14}C-labeled bile acids, purified bile acid-CoA ligase, and partially purified BaiA1. Ethyl acetate-extracted bile acids were separated by TLC. Results with ^{14}C-cholic acid (Fig. 3) showed accumulation of a steroid product from the second ethyl acetate extraction (conjugated bile acids) which comigrated with authentic 3-oxo-cholate. This suggests that 3-oxo-cholyl-CoA has accumulated and that cholyl-CoA is the substrate for the reaction. Similar reactions performed with extracts from *E. coli* strains not containing the cloned *baiA1* gene showed no accumulation of the 3-oxo-cholate product. Further assays revealed that the 3-oxo-cholate product could be extracted from the TLC plate and converted to cholic acid with commercial 3α-hydroxysteroid dehydrogenase, or reconjugated with the bile acid-CoA ligase and converted, in the reverse reaction, to cholyl-CoA by the BaiA1 protein. As with cholic acid, the BaiA proteins had very little activity against the unconjugated 3-oxo-cholate. It was also determined that either NAD^+ or $NADP^+$ could be utilized in the conversion of cholyl-CoA to 3-oxo-cholyl-CoA. The relative efficacy of NAD^+ and $NADP^+$ in the reaction could not be determined because of interference by the NAD^+-requiring 7α-hydroxysteroid dehydrogenase from *E. coli* [25], which copurified with the BaiA1 protein.

Spectrophotometric assays. Spectrophotometric assays, performed with $NADP^+$, also showed that cholyl-CoA was the preferred substrate for the BaiA proteins. Deoxycholyl-CoA had a relative substrate activity of 75% compared with cholyl-CoA. No activity was detected with cholate, chenodeoxycholate, deoxycholate, glycocholate, taurocholate, ursodeoxycholate, or no bile acid. No activity was detected with dialyzed extracts from *E. coli* strains not containing the cloned *baiA1* gene. The proposed intermediates 3-oxo-cholyl-CoA and 3-oxo-deoxycholyl-CoA (Fig. 1) could not be studied by spectrophotometric assay in the reductive direction because of difficulties in obtaining sufficient quantities of these compounds.

Amino acid sequence analysis. The BaiA polypeptides were analyzed for potential CoA binding sites. Matsubara et al. [16] analyzed several proteins with CoA-containing substrates and suggested a consensus CoA binding site that had two valine residues separated from an aspartate residue by 7 to 11 amino acids. Both BaiA polypeptides were found to have potential CoA binding sites spanning residues 182 to 193 (Fig. 2). Several other amino acids that are conserved among members of the short-chain alcohol dehydrogenase superfamily have been reported to be involved in $NAD(P)^+$ or substrate binding and are shown in Fig. 2.

Discussion

The bile acid-inducible *baiA* gene family in *Eubacterium* sp. strain VPI 12708 is comprised of the identical *baiA1* and *baiA3* genes, and the *baiA2* gene which shares 81% nucleotide identity with the other two genes [7, 12, 24]. The 27,000-M_r polypeptides encoded by the genes have previously been shown to belong to the short-chain alcohol/polyol dehydrogenase family of enzymes. Several generally conserved amino acids in this enzyme family are also conserved in the 27,000-M_r polypeptides (Fig. 2). The N-terminal region of the short-chain alcohol/polyol dehydrogenases is thought to contain the $NAD(P)^+$ binding site, while the middle and C-terminal regions are thought to comprise the active site of the enzyme [3, 4, 11, 17, 19].

In this paper, we show that the *baiA1/baiA3* genes encode 3α-hydroxysteroid dehydrogenases. These dehydrogenases are much more active on cholyl-CoA and deoxycholyl-CoA than on the free bile acids. The 7α-dehydroxylation pathway in strain VPI 12708, in which these dehydrogenases are reported to be involved [6, 7], is believed to be initiated by conjugation of cholic acid to CoA by a bile acid-CoA ligase (Fig. 1) [5, 15]. The activity of the 3α-hydroxysteroid dehydrogenases on the cholyl-CoA conjugates reinforces this view.

The presence of a possible CoA binding site in the BaiA amino acid sequences (Fig. 2) also helps to confirm that the enzymes utilize CoA-containing substrates. Three-dimensional structure analysis of 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* has shown that a stretch of residues that align with the possible BaiA CoA binding sites are exposed to the steroid binding pocket, as are the tyrosine and lysine residues analogous to Tyr-157 and Lys-161 in the BaiA polypeptides [11]. The highly conserved tyrosine and lysine residues have been shown to be critical for enzyme activity in *Drosophila* alcohol dehydrogenase [4].

The use of CoA conjugates in the bile acid 7α-dehydroxylation pathway may also be important in intracellular partitioning of 7α-dehydroxylation versus the constitutive 7α-hydroxysteroid dehydrogenase activity [1, 9] and a possibly constitutive 3α-hydroxysteroid dehydrogenase activity [13] in strain VPI 12708. Although the 7α-dehydroxylation pathway appears to progress rapidly without CoA conjugates in cell-free extracts from strain VPI 12708 [13, 22], this reaction may make fortuitous use of the proposed constitutive 3α-hydroxysteroid dehydrogenase. Further studies are needed to help clarify this point.

It is not currently known if bile acid-CoA conjugates are present throughout the entire 7α-dehydroxylation pathway or if the CoA moiety is cleaved during some intermediate step. Current evidence with *Eubacterium* sp. strain VPI 12708 whole cells or cell extracts with ^{14}C-labeled cholic acid suggests that the CoA conjugates persist at least through the 3-oxo-$\Delta^{4,6}$-deoxycholate intermediate (unpublished data). The activity of the BaiA proteins with deoxycholyl-CoA suggests that CoA conjugates may be present throughout the entire pathway (Fig. 1) if the BaiA proteins are active in the reductive arm of the pathway (3-oxo-deoxycholyl-CoA to deoxycholyl-CoA) as well as the oxidative arm (cholyl-CoA to 3-oxo-cholyl CoA). The possibility also exists that a yet-to-be-identified enzyme is responsible for the reductive arm activity.

The possibility that the BaiA1/BaiA3 proteins are active in the oxidative arm of the bile acid 7α-dehydroxylation pathway while the BaiA2 protein is active in the reductive side of the pathway could help explain the apparent gene duplication among the *baiA* genes. However, the high degree of homology between the BaiA proteins (92% identity at the amino acid level) also suggests the possibility that the polypeptide subunits are interchangeable in the tetrameric structure of these dehydrogenases. Further studies with purified BaiA1/BaiA3 and BaiA2 proteins will be required to determine whether there are any catalytic differences within this family of proteins.

In summary we have shown that the *baiA* genes from *Eubacterium* sp. strain VPI 12708 encode novel 3α-hydroxysteroid dehydrogenases. To our knowledge, this is the first report of a bacterial hydroxysteroid dehydrogenase with specific activity on CoA conjugates.

ACKNOWLEDGMENTS

This work was supported by research grants DK40986 and P01-DK38030 from the National Institutes of Health. M.A.L. was supported by training grant F32-DK08755.

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