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Chemical synthesis of bile acid acyl-adenylates and formation by a rat liver microsomal fraction

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ABSTRACT

In mammals, unconjugated bile acids formed in the intestine by bacterial deconjugation are reconjugated (*N*-acylamidated) with taurine or glycine during hepatocyte transport. Activation of the carboxyl group of bile acids to form acyl-adenylates is a likely key intermediate step in bile acid *N*-acylamidation. To gain more insight into the process of bile acid adenylate formation, we first synthesized the adenylates of five common, natural bile acids (cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic, and lithocholic acid), and confirmed their structure by proton NMR. We then investigated adenylate formation by subcellular fractions of rat liver (microsomes, mitochondria, cytosol) using a newly developed LC method for quantifying adenylate formation. The highest activity was observed in the microsomal fraction. The reaction required Mg^{2+} and its optimum pH was about pH 7.0. In term of maximum velocity (V_{max}) and the Michaelis constant (K_m), the catalytic efficiency of the enzyme under the conditions used was highest with cholic acid of the bile acids tested. The formation of cholyl-adenylate was strongly inhibited by lithocholic and deoxycholic acid, as well as by palmitic acid; ibuprofen and valproic acid were weak inhibitors. In cholestatic disease, such adenylate formation might lead to subsequent bile acid conjugation with glutathione or proteins.

1. Introduction

During their enterohepatic circulation, conjugated (*N*-acylamidated) bile acids are deconjugated by bacterial enzymes in the distal intestine. The liberated unconjugated bile acids are returned to the liver and undergo efficient reconjugation (*N*-acylamidation) with glycine or taurine with the result that virtually all bile acids secreted into bile are in amidated form. The magnitude of such re-amidation may exceed the amidation occurring in bile acid biosynthesis. The amidation of newly synthesized bile acids and re-amidation of bile acids returning from the intestine are important steps in bile acid metabolism, as conjugation of bile acids is essential for efficient canalicular secretion and micellar solubilization of dietary lipids in the small intestine [1,2].

For amidation, bile acids must be first activated to their coenzyme A (CoA) thioesters. This activation is catalyzed by bile acid:CoA ligase (BAL) that is distinct from the enzyme(s) that activate fatty acids: however, the reaction mechanism is analogous to that of fatty acyl-CoA ligase. The mechanism was first studied by Berg [3] who proposed that the ligation reaction of acetate proceeds by the two-step reaction sequence with the mixed 5′-adenylic acid-carbonyl anhydride (acyl-AMP) as an obligatory enzyme-bound intermediate, where ATP combines with the free enzyme which then combine with acetate. The first product, pyrophosphate is released and an enzyme-bound intermediate, acyl-AMP, is formed. Subsequently, the binding of CoA and release of acetyl-CoA and AMP occur. Formation of such acyl-AMP derivatives is now considered to occur as the first step in multiple pathways in which the carboxyl group is activated. These include the linkage of biotin to apocarboxylase to form holocarboxylase [4], amino acid activation in tRNA acylation [5], the metabolism of valproic acid [6] and ibuprofen [7] in rat liver mitochondrial fraction, nonribosomal

Abbreviations: Cholyl-adenylate (CA-AMP), cholyl adenosine 5′-phosphate diester; Deoxycholyl-adenylate (DCA-AMP), deoxycholyl adenosine 5′-phosphate diester; Ursodeoxycholyl-adenylate (UDCA-AMP), ursodeoxycholyl adenosine 5′-phosphate diester; Lithocholyl-adenylate (LCA-AMP), lithocholyl adenosine 5′-phosphate diester; 12-Oxolithocholyl-adenylate (12-oxoLCA-AMP), 12-oxolithocholyl adenosine 5′-phosphate diester; Valproyl-adenylate (valproyl-AMP), valproyl adenosine 5′-phosphate diester; Ibuprofenyl-adenylate (ibuprofenyl-AMP), ibuprofenyl adenosine 5′-phosphate diester; AMP, adenosine 5′-monophosphate; ATP, adenosine-5′-triphosphate; Palmitic acid, hexadecanoic acid; Valproic acid, 2-propylpentanoic acid; Ibuprofen, 2-[4-(2-methylpropyl)phenyl]propanoic acid.

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synthesis of peptides [8] during actinomycin [9] or tyrocidine [10] biosynthesis, 4-chlorobenzoate dehydrogenation by *Pseudomonas* [11], formation of alkyl aldehyde in bioluminescence in *Photobacterium* [12], and propionate catabolism in *Salmonella enterica* [13]. It has also been demonstrated that cholic acid (CA) is converted to its acyl-AMP by cell free extracts of *Eubacterium* [14]. Recently, we also reported that during the activation of CA to the CoA thioester in rat liver microsomes, cholyl-adenylate (CA-AMP) is first formed by the reaction which is likely to be catalyzed by the BAL [15,16]. Such bile acid adenylates spontaneously reacted with taurine [15], histone [17], and glutathione (GSH) [18] in a phosphate buffer solution under nonenzymatic conditions. We also reported that bile acids can be transformed into their GSH conjugates via their acyl-linked intermediary metabolites catalyzed by glutathione *S*-transferase; such GSH conjugates are then excreted into the bile [19]. These findings indicated a greater reactivity of the acyl-AMP toward amino and thiol groups. It has also been shown that bile acid acyl-AMPs are reactive intermediates capable of generating covalently bound bile acid adducts with peptides [20] and proteins [17,20] by nucleophilic displacement of the 5′-adenylic acid by the free amino groups of lysine residues. Formation of adducts in which bile acids are covalently bound cellular proteins [21] and to histones [22] has been considered to occur in cholestasis and colon cancer.

Recently, we identified Rab-3, Rab-12, Rab-16, and M-Ras as proteins that were chemically bound with lithocholic acid (LCA) in the liver of bile duct-ligated rats [23]. Therefore, characterization of the adenylation activity toward bile acid is essential for understanding not only the mechanism of the two-step activation reaction in the amino acid conjugation but also the possible participation of such bile acid adenylates in the formation of bile acid–protein adducts and possibly GSH conjugates. Although BAL has been purified from several species [24–34] and a cDNA-encoding rBAL from a rat liver λZAP II cDNA library has been cloned [35], and extensively characterized by monitoring the final product, acyl-CoA thioester, little work has been done on the formation of bile acid acyl-AMPs as an intermediate in the reaction. In this paper, we report the chemical synthesis of the acyl-adenylates of five common natural bile acids. We then carried out a kinetic characterization of the enzyme activity present in rat liver microsomes that catalyzes the formation of such bile acid acyl-AMPs.

2. Experimental

2.1. Materials

CA, chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). LCA was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and ursodeoxycholic acid (UDCA) was kindly donated by Mitsubishi Tanabe Pharma Co. (Osaka, Japan). 12-Oxolithocholic acid was synthesized in this laboratory by a previously reported method [36]. *rac*-(±)-Ibuprofen and palmitic acid were supplied by Tokyo Chemical Industry, Co. Ltd. Adenosine-5′-triphosphate (ATP) disodium salt and adenosine-5′-monophosphate (AMP) were purchased from Wako Pure Chemical Industries, Ltd. and Sigma–Aldrich Co. (St. Louis, MO, USA), respectively. Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). The potassium salts of bile acids, *rac*-(±)-ibuprofen, and palmitic acid were prepared by passing them through the lipophilic ion exchange gel, carboxymethyl Sephadex LH-20 (K^+ form, 0.90 mequiv./g) [37]. An Oasis HLB cartridge (60 mg, 3 ml) was provided by Waters Co. (Milford, MA, USA) and was successively conditioned with methanol (1 ml) and water (1 ml) prior to use. The dye reagent for the protein assays was purchased from Bio-Rad laboratories (Hercules, CA, USA). All other chemicals and solvents were analytical grade and obtained from Nacalai Tesque, Inc. All glassware used was silanized with trimethylchlorosilane. Water from a Millipore water filtration system (Milli-Q UV Plus) was used to prepare the mobile phase and aqueous solutions described below.

2.2. Apparatus

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 500 MHz with a JEOL JMN-α-500 spectrometer (Tokyo, Japan). Chemical shifts are given as the δ value with tetramethylsilane as the internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; brs, broad singlet). Electrospray ionization mass spectra were obtained by flow injection mode using 50% methanol as a mobile phase with JEOL JMS-700T in the negative ion detection mode. The resolution of the mass spectra was set at 750 and the voltages for the orifice and ring lens were −20 V and −60 V, respectively. The temperatures of the orifice and desolvating plate were 130 °C and 250 °C, respectively.

2.3. Synthesis of bile acid acyl-AMPs

Bile acid acyl-AMPs (Fig. 1) were synthesized employing the carbodiimide method according to a previously reported method [15]. In brief, a solution of *N,N′*-dicyclohexylcarbodiimide in pyridine (0.2 ml) were added to a magnetically stirred solution of bile acid (each 100 mg) and AMP (85 mg) in 75% pyridine; the mixture was stirred at 4 °C for 7 h. The reaction was checked by thin layer chromatography (TLC) using plates coated with silica gel using *n*-butanol–acetic acid–water (5:1:3, v/v/v) as the developing solvent. After centrifugation at 3000 rpm for 5 min to remove the *N*-acylurea derivatives, acetone was added to the supernatant

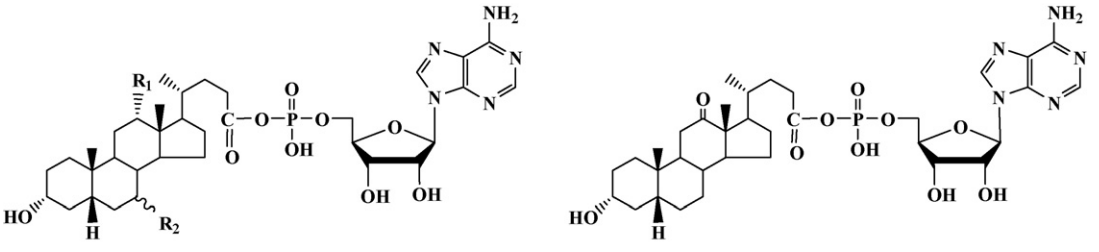

Fig. 1. Chemical structures of bile acid acyl-adenylates.