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Selective inhibition of lignoceroyl-CoA synthetase by adenosine 5′-alkylphosphates

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Abstract Structural analogs of adenosine 5′-acylphosphates, which are intermediates of the reaction catalyzed by acyl-CoA synthetases, were synthesized by condensing primary alcohols with AMP to examine the inhibitory effects on the lignoceroyl-CoA and palmitoyl-CoA synthetase activities. Hexadecyl, octadecyl, eicosyl, docosyl and tetracosyl esters of AMP were remarkably potent inhibitors of the lignoceroyl-CoA formation. On the other hand, the eicosyl, docosyl or tetracosyl esters of AMP did not behave as significant inhibitors of the palmitoyl-CoA formation at the concentration at which the two other shorter chain analogs were effective. Namely, these longer alkyl esters of AMP have selective inhibitory effects on the lignoceroyl-CoA synthetase activity. The K_i value of adenosine 5′-tetracosylphosphate, the most potent inhibitor, was about one tenth lower than the K_m value for the substrate lignoceric acid. Furthermore, the results support the notion that lignoceroyl-CoA synthetase is distinct from palmitoyl-CoA synthetase.

Key words: Lignoceroyl-CoA synthetase; Palmitoyl-CoA synthetase; Adenosine 5′-alkylphosphate; Inhibitor

1. Introduction

The first step in the metabolism of carboxylic acids is the formation of CoA esters by a reaction involving the appropriate acyl-CoA synthetase. Studies on the reaction mechanism of the formation of acetyl-CoA have shown that the reaction involves a two-step process [1]. ATP combines with free enzyme and is followed by acetate. The first product, pyrophosphate, is released and an enzyme-bound intermediate, adenosine 5′-acylphosphate, is formed. Subsequently, the binding of CoA and release of acetyl-CoA and AMP occur. It is generally accepted that the mechanism for the long-chain acyl- CoA synthetase reaction is similar to that for acetyl-CoA synthetase, in which adenosine 5′-long-chain-acylphosphate is an intermediate [2].

Aminoalkyl adenylates, which are analogs of intermediates of aminoacyl-tRNA formation, are selective inhibitors of aminoacyl-tRNA synthetases [3]. It is reasonable to assume that long-chain alkyl esters of AMP which are structural analogs of adenosine 5′-acylphosphates inhibit long-chain acyl-CoA synthetase, and their inhibitory effects on palmitoyl-CoA and lignoceroyl-CoA synthetases are dependent upon their chain length. Moreover, if these compounds have potent and selective inhibitory effects, they could be applied to affinity labeling or affinity chromatography as ligands with which to study acyl-CoA synthetase proteins. In this article we report the synthesis of adenosine 5′-long-chain-alkylphosphates and the inhibition of palmitoyl-CoA and lignoceroyl- CoA synthetases by these AMP esters.

2. Materials and methods

2.1. Materials

[1-^{14}C]Lignoceric acid and [1-^{14}C]palmitic acid were synthesized from tricosyl bromide and pentadecyl bromide, respectively, using [1-^{14}C]KCN (51.9 mCi/mmol, NEN, USA) according to the procedure described previously [4]. Heptakis(2,6-di-*O*-methyl)-*β*-cyclodextrin (dimethyl-*β*-cyclodextrin) was purchased from Wako Pure Chemical Industries, Ltd. ATP (Na salt) and AMP (free acid) were obtained from Boehringer Mannheim Biochemicals, and CoA (Li salt) from Sigma. Microsomal fractions were prepared from rat (Wistar, male, 7- to 9-week-old) and chicken livers (60-day-old broilers) as previously described [5].

2.2. Measurement of fatty acyl-CoA synthetase activity

Fatty acyl-CoA synthetase activity was assayed as described previously [5]. Adenosine 5′-alkylphosphates, which were synthesized as mentioned below, were dissolved in a solution of dimethyl-*β*-cyclodextrin by sonication together with a 1-^{14}C-labeled fatty acid. The reaction mixture (200 *μ*l) consisted of 1-^{14}C-labeled fatty acid (5 *μ*M), Tris-HCl buffer, pH 8.0 (100 mM), $MgCl_2$ (10 mM), ATP (3 mM), CoA (150 *μ*M), dithiothreitol (2 mM), KCl (100 mM) and microsomes (3 *μ*g of protein for lignoceroyl-CoA synthetase activity and 0.1 *μ*g of protein for palmitoyl-CoA synthetase activity). Incubations were carried out at 37°C for 5 min. Protein was determined by means of the bicinchoninic acid method [6].

2.3. Preparation of adenosine 5′-alkylphosphates

Adenosine 5′-alkylphosphates were prepared by a procedure involving condensation of long-chain alcohols with AMP. AMP (0.29 mmol) and alcohol (2.9 mmol) were dissolved in 5 ml of dry pyridine, and tri-*n*-butylamine (0.96 mmol) was added. Dicyclohexylcarbodiimide (1.45 mmol) was added and the mixture was stirred for 12–15 h at 100°C. After cooling, the mixture was concentrated in vacuo. The product was purified by chromatography on a silica gel column eluted with a stepwise gradient of chloroform and methanol (chloroform alone to 1:1 (v/v) mixture), and finally by preparative thin layer chromatography developed with chloroform/methanol/water/acetic acid (65:35:8:1, v/v) using pre-coated plates (Merck, PLC plates silica gel 60 F_{254}). Whereas adenosine 5′-hexadecylphosphate and adenosine 5′-octadecylphosphate have been synthesized using peracetylated AMP [7], we synthesized them according to the present procedure in yields of 50.5 and 50.4%, respectively. The three other esters synthesized are novel compounds: adenosine 5′-eicosylphosphate, adenosine 5′-docosylphosphate and adenosine 5′-tetracosylphosphate. The synthesized AMP esters were all free of contamination as confirmed by thin-layer chromatography on silica gel plates (Merck, pre-coated TLC plates silica gel 60) using three solvent systems, *n*-butanol/acetic acid/water (5:2:3, v/v), chloroform/methanol/water/acetic acid (65:35:8:1, v/v), and 2-propanol/28% NH_3 (9:1, v/v). Composition analyses were performed using a high resolution mass spectrometer (Hitachi M-2500). Proton NMR spectra (200 MHz) were measured in DMSO-d_6 using a JEOL FX200 spectrometer

Adenosine 5′-eicosylphosphate: 61.7% yield. High resolution SIMS *m/z* calcd. for $C_{30}H_{53}N_5O_7P$ (M^+-1): 626.3680. Found: 626.3690. NMR (DMSO-d_6, TMS, ppm) 0.85 (t, 3H, J = 6.84 Hz, CH_3), 1.04–1.5

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(m, 39H, CH_2), 3.36 (br s, 2H, 2',3'-OH), 3.59 (q, 2H, J = 6.35 Hz, CH_2-O-P), 3.79 (br s, 2H, 5'-CH), 4.01 (br d, 1H, J = 3.42 Hz, 4'-OH), 4.19 (t, 1H, J = 4.15 Hz, 3'-OH), 4.56 (t, 1H, J = 5.37 Hz, 2'-CH), 5.90 (d, 1H, J = 5.37 Hz, 1'-CH), 7.24 (br s, 2H, -NH_2), 8.13 (s, 1H, adenine ring 2-H), 8.43 (s, 1H, adenine ring 8-H).

Adenosine 5'-docosylphosphate: 58.0% yield. High resolution SIMS m/z calcd. for $C_{32}H_{57}N_5O_7P$ (M^+-1): 654.3993. Found: 654.4018. NMR (DMSO-d_6, TMS, ppm) 0.85 (t, 3H, J = 6.59 Hz, CH_3), 0.96–1.5 (m, 43H, CH_2), 3.38 (br s, 2H, 2',3'-OH), 3.59 (q, 2H, J = 6.35 Hz, CH_2-O-P), 3.79 (br s, 2H, 5'-CH), 4.00 (br d, 1H, J = 3.66 Hz, 4'-OH), 4.19 (t, 1H, J = 3.90 Hz, 3'-OH), 4.56 (t, 1H, J = 5.37 Hz, 2'-CH), 5.90 (d, 1H, J = 5.37 Hz, 1'-CH), 7.24 (br s, 2H, -NH_2), 8.13 (s, 1H, adenine ring 2-H), 8.43 (s, 1H, adenine ring 8-H).

Adenosine 5'-tetracosylphosphate: 52.3% yield. High resolution SIMS m/z calcd. for $C_{34}H_{61}N_5O_7P$ (M^+-1): 682.4305. Found: 682.4311. NMR (DMSO-d_6, TMS, ppm) 0.85 (t, 3H, J = 6.83 Hz, CH_3), 1.04–1.52 (m, 47H, CH_2), 3.28 (br s, 2H, 2',3'-OH), 3.61 (q, 2H, J = 6.59 Hz, CH2-O-P), 3.83 (m, 2H, 5'-CH), 4.02 (br d, 1H, J = 3.66 Hz, 4'-OH), 4.22 (t, 1H, J = 3.91 Hz, 3'-OH), 4.55 (t, 1H, J = 5.13 Hz, 2'-CH), 5.91 (d, 1H, J = 5.37 Hz, 1'-CH), 7.02 (br s, 2H, -NH_2), 8.13 (s, 1H, adenine ring 2-H), 8.40 (s, 1H, adenine ring 8-H).

2.4. Data analysis

Kinetic data were processed by using the computer program 'micro-RESEARCHER II' on a Hitachi EWS4800/60.

3. Results

The chemical structures of the synthesized adenosine 5'-alkylphosphates are shown in Fig. 1. Adenosine 5'-hexadecylphosphate (C_{16}-O-AMP) and adenosine 5'-tetracosylphosphate (C_{24}-O-AMP) are structurally analogous to the intermediates of palmitoyl-CoA and lignoceroyl-CoA formation, adenosine 5'-hexadecanoylphophate and adenosine 5'-tetracosanoylphosphate, respectively. The inhibitory effects of the five synthetic analogs on rat liver microsomal lignoceroyl-CoA synthetase activity were examined by varying their concentrations at a fixed concentration (5 μM) of the fatty acid substrate in the presence of saturating amounts of ATP, CoA and Mg ions as described in section 2.

The results indicated that all these esters, despite their alkyl chain lengths, inhibited the lignoceroyl-CoA formation to a similar extent (75–80% inhibition) at a concentration of 1 μM (Fig. 2). At a concentration of 5–10 μM, the extent of inhibition was over 90% (data not shown).

A difference in inhibitory effects was observed at a lower concentration of the AMP esters as shown in Fig. 2. The analog C_{24}-O-AMP, of which the alkyl chain is the same length as that of the substrate, lignoceric acid, and C_{22}-O-AMP were more effective at 0.2 μM, where the inhibition was 49 and 43%, respectively.

The inhibitory effects on palmitoyl-CoA synthetase activity

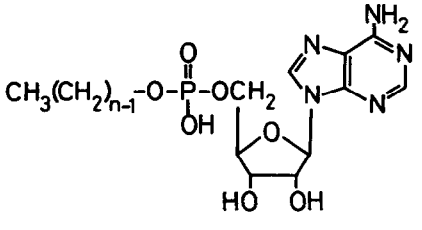

Fig. 1. The series of adenosien 5'-alkylphosphates studied here. C_{16}-O-AMP where n = 16; C_{18}-O-AMP where n = 18; C_{20}-O-AMP where n = 20; C_{22}-O-AMP where n = 22; C_{24}-O-AMP where n = 24. These AMP esters were synthesized by condensation of AMP and primary alcohols in sufficient yields according to the procedure described in section 2.

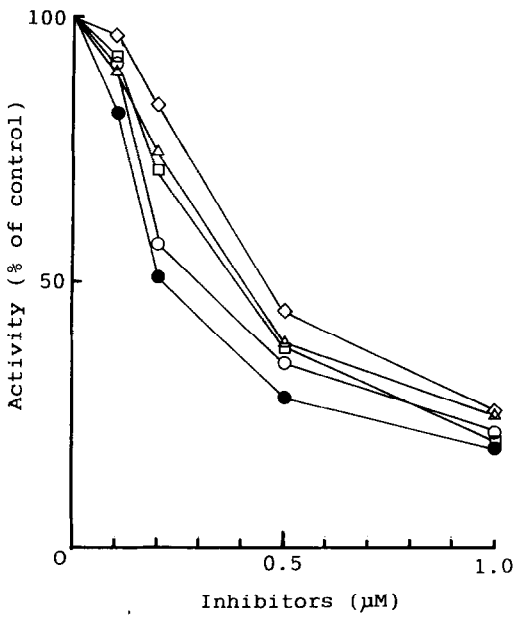

Fig. 2. The inhibition of rat liver microsomal lignoceroyl-CoA synthetase activity by adenosine 5'-alkylphosphates. The enzyeme acitivity was measured by varying the concentration of AMP esters under the conditions described in section 2 and the inhibitory effects were expressed as the percentage of the residual activities to the control. □. C_{16}-O-AMP; ◇, C_{18}-O-AMP; △, C_{20}-O-AMP; ○, C_{22}-O-AMP; ●, C_{24}-O-AMP.

were also examined under the same conditions except for the use of palmitic acid as a substrate. Fig. 3 shows distinct inhibitory effects on palmitoyl-CoA formation. C_{16}-O-AMP and C_{18}-O-AMP affected the formation of palmitoyl-CoA causing 64 and 50% inhibition at a concentration of 1 μM, respectively, although the inhibitory effects were less than those upon lignoceroyl-CoA formation. Ninety per cent inhibition occurred in the presence of 10 μM C_{16}-O-AMP or 20 μM C_{18}-O-AMP. However, the longer chain alkyl esters did not cause significant inhibition at the concentrations at which the C_{16}- and C_{18}-analogs were effective.

Focusing upon the effects of the longer alkyl chain esters C_{20}-O-AMP, C_{22}-O-AMP and C_{24}-O-AMP, the results shown in Fig. 2 and Fig. 3 show that these AMP esters have marked inhibitory effects upon lignoceroyl- CoA synthetase, but not upon palmitoyl-CoA synthetase activity. Thus, the two synthetase activities were distinguishable by means of the AMP esters. Similar results were also obtained using chicken liver microsomal enzymes (data not shown).

The kinetics of the inhibition of the rat liver microsomal lignoceroyl-CoA synthetase activity were examined. The K_m value for lignoceric acid was 0.72 ± 0.07 μM (mean value of three independent experiments), which was derived from Lineweaver–Burk plots, whereas the estimation from Dixon plots gave 0.062 ± 0.027 μM as the K_i value for C_{24}-O-AMP, and 0.11 ± 0.04 μM for C_{16}-O- AMP (mean value of two independent experiments). The nature of this inhibition was examined by varying the lignoceric acid concentration using C_{24}-O-AMP and C_{16}- O-AMP as inhibitors. As shown in Fig. 4, inhibition by C_{24}-O-AMP and C_{16}-O-AMP was mixed and non-competitive, respectively, with respect to lignoceric acid. These results suggest that these AMP esters bind at both the

ATP-AMP and fatty acid sites of the enzyme, when combined with those obtained for the inhibition of acetyl-CoA synthetase by methyl or ethyl esters of AMP, which are noncompetitive inhibitors [8].

4. Discussion

The results reported here indicate that structural analogs of adenosine 5′-acylphosphates, in which the mixed anhydride bond has been replaced by an ester linkage, behave as inhibitors of lignoceroyl-CoA synthetase and palmitoyl-CoA synthetase. An inhibitory effect on lignoceroyl-CoA synthetase of C_{16}-O-AMP (Fig. 2) was not predicted, because Wanders et al. have reported that the addition of palmitic acid into the assay mixture did not inhibit lignoceroyl-CoA formation [9]. On the other hand, the corresponding alcohol, 1-hexadecanol, was not highly inhibitory, that is, the residual activity was 86–87% of the control at a concentration of 5 mM (data not shown). We reported that AMP itself inhibited lignoceroyl-CoA formation at a concentration three orders of magnitude higher than that of C_{16}-O-AMP [5]. Thus, the introduction of a long-chain alkyl group into the structure of AMP remarkably increases its participation in binding to the active site of the enzyme. In addition, the results showed that the mixed anhydride linkage of the intermediate is not essential for binding to the active site. An AMP ester with the same alkyl chain length as lignoceric acid did not demonstrate inhibitory effect on the palmitoyl-CoA synthetase activity (Fig. 3). This is consistent with the findings that the formation of palmitoyl-CoA from palmitic acid is not affected by lignoceric acid [9]. These results indicated that the size of the active site of palmitoyl-CoA synthetase is not suitable for binding C_{24}-O-AMP.

Lignoceroyl-CoA synthetase is coupled to β-oxidation in peroxisomes [10] and to the biosynthetic system of sphingolipids in microsomes [11], although the existence of lignoceroyl-CoA synthetase remains ambiguous. Both lignoceroyl-CoA formation and palmitoyl-CoA formation may be catalyzed by a single enzyme [12], or by two distinct enzymes [13–15]. The results obtained here indicated that the active site of lignoceroyl-CoA synthetase is different from that of palmitoyl-CoA synthetase as mentioned above, supporting the latter idea. Moreover, since the AMP esters with very-long-chain alkyl groups have potent and selective inhibitory effects on the lignoceroyl-CoA synthetase activity, they may be used as ligands for affinity chromatography or affinity labeling for purification or elucidation of lignoceroyl-CoA synthetase.

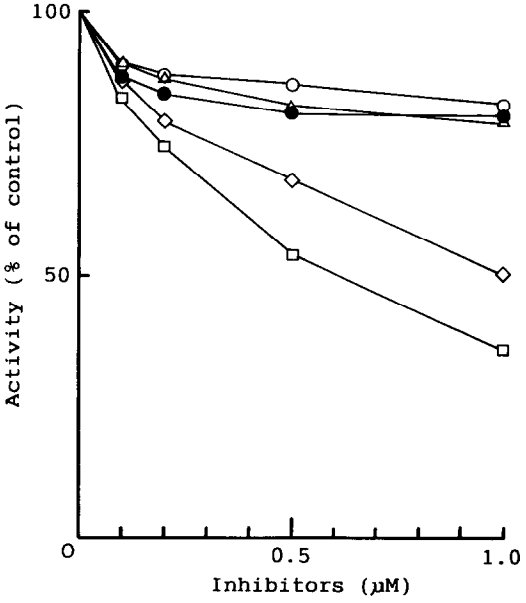

Fig. 3. The inhibition of rat liver mocrosomal palmitoyl-CoA synthetase activity by adenosine 5′-alkylphosphates. Inhibitory effects were measured in a similar manner as Fig. 2. □, C_{16}-O-AMP; ◇, C_{18}-O-AMP; △, C_{20}-O-AMP; ○, C_{22}-O-AMP; ●, C_{24}-O-AMP.

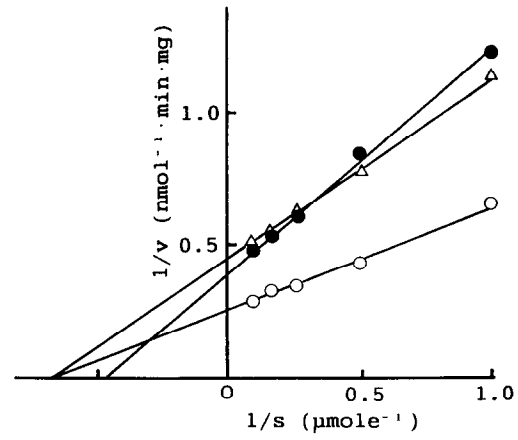

Fig. 4. Lineweaver–Burk plots of the inhibition of lignoceroyl-CoA synthetase activity by C_{24}-O-AMP and C_{16}-O-AMP. Incubation conditions were those described in section 2, except that varying amount of lignoceric acid were used in the absence of an inhibitor (○), and in the presence of 0.5 μM C_{24}-O-AMP (●) or 0.5 μM C_{16}-O-AMP (△). Each plots represents the mean value of triplicate determinations.

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