cDNA-derived amino acid sequence of acetoacetyl-CoA synthetase from rat liver[1]

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Abstract In order to examine the primary structure of acetoacetyl-CoA synthetase (acetoacetate-CoA ligase, EC 6.2.1.16; AA-CoA synthetase), the cDNA clone encoding this enzyme has been isolated from the cDNA library which was prepared from the liver of rat fed a diet supplemented with 4% cholestyramine and 0.4% pravastatin for 4 days. Nucleotide sequence analysis of cloned cDNA revealed that AA-CoA synthetase of rat liver contains an open reading frame of 2019 nucleotides, and the deduced amino acid sequence (672 amino acid residues) bears 25.0 and 38.9% homologies with acetyl-CoA synthetases of *Saccharomyces cerevisiae* and *Archaeoglobus fulgidus*, respectively.

Key words: Acetoacetyl-CoA synthetase; cDNA; Rat; Liver; Ketone body

1. Introduction

Acetoacetyl-CoA synthetase (acetoacetate-CoA ligase, EC 6.2.1.16; AA-CoA synthetase) is a novel cytosolic acetoacetate-activating enzyme purified for the first time from *Zoogloea ramigera* I-16-M, a bacterium isolated from activated sludge [1] and rat liver [2] by us. Although cytosolic activation of acetoacetate to its CoA ester was first observed in pigeon liver homogenate by Stern and Ochoa [3] in 1951, the enzyme responsible for this reaction had not been identified until we purified AA-CoA synthetase as a discrete acetoacetate-specific ligase.

In mammals, AA-CoA synthetase is present in the cytosolic fractions of various tissues [4–7], especially lipogenic ones, and acetoacetate is known to be effectively incorporated into cholesterol and fatty acids [8]. These results indicate that AA-CoA synthetase may supply acetyl units to cytosolic compartment for cholesterol and/or fatty acid biosynthesis. Furthermore, the activity [4] and content [5] of AA-CoA synthetase is enhanced in rat liver by the administration of hypocholesterolemic agents, such as cholestyramine and ML-236B (compactin), suggesting that this enzyme is involved in cholesterol biosynthesis. However, the physiological role of AA-CoA synthetase is not fully understood. To clarify the function of AA-CoA synthetase in mammalian cells and to confirm discreteness of the enzyme, it is essential to isolate the cDNA clones for this protein. In this study we report the cloning of cDNA encoding AA-CoA synthetase from the cDNA library obtained from the liver of rat fed a diet supplemented with pravastatin and cholestyramine.

2. Materials and methods

2.1. Dietary experiment

Female rats of the Sprague-Dawley (SD) strain (6 weeks old, 130–150 g) were from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). They were fed a standard diet (Mouse Flat, Oriental Yeast Co., Ltd., Japan) supplemented with 0.4% pravastatin and 4% cholestyramine, which were kindly donated by Sankyo Co. Ltd. (Tokyo, Japan) and Bristol-Myers Squibb K.K. (Tokyo, Japan), respectively.

2.2. Enzyme assay and determination of enzyme protein concentration

AA-CoA synthetase activity was assayed as described previously [2]. AA-CoA synthetase protein concentration was estimated by enzyme immunoassay using anti-rat AA-CoA synthetase antiserum of rabbit [5] as described previously [9].

2.3. Protein digestion and separation of peptides

Purified enzyme [2] was lyophilized and dialyzed against the buffers suitable for each of the following peptidases. The enzyme was digested with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), chymotrypsin (Wako), or trypsin (Sigma, St. Louis, MO, USA) under the optimum condition as described in the manufacturers' instructions. The resulting peptides were separated by a μ blotter system (PE Applied Biosystems, Foster City, CA, USA) using a linear gradient of 0–80% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 5 μl/min. Absorbance was monitored at 210 nm.

2.4. Amino acid sequencing

Peptide fragments were sequenced by the automated Edman degradation using an amino acid sequencer Procise cLC (PE Applied Biosystems).

2.5. cDNA library construction

The several preliminary trials to obtain cDNA clones for AA-CoA synthetase from the liver of the rat fed a normal diet were unsuccessful possibly because the content of AA-CoA synthetase mRNA in the liver was very low. Therefore, total RNA was obtained by the acid guanidinium-phenol-chloroform method [10] from liver of female SD rat fed a diet supplemented with 0.4% pravastatin plus 4% cholestyramine for 4 days (see Section 3). Poly(A)$^+$ RNA was separated from the total RNA using Oligotex(dT)30-Super (Takara Shuzo Co. Ltd.,

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[1] The nucleotide sequence reported in this paper has been submitted to the DDBJ database under accession number AB026291.

Abbreviations: AA-CoA, acetoacetyl-CoA; SD, Sprague-Dawley; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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Japan), and cDNA library was constructed from poly(A)$^+$ RNA according to the method of Gluber and Hoffman [11] using the vector λZipLox (GIBCO BRL, Grand Island, NY, USA).

2.6. 3′-RACE method

Rapid amplification of cDNA ends (RACE) [12] was done using a Takara 3′-Full RACE Core Set (Takara Shuzo). First-strand cDNA was synthesized using oligo dT-3 sites adaptor primer and 1 μg of total RNA as a template. Second-strand cDNA was synthesized using the oligonucleotide (5′-CTCGGATCCCTCAAYGGCAAGAAAGTGGA-3′, 29-mer), where Y is C or T. The 20-mer of this 29-mer oligonucleotide was derived from the sequences of EST clones R48370 (human) and W12611 (mouse) (see Section 3) and the sequence of *Bam*HI restriction site, and three extra nucleotides were added to its 5′-end. The amplified DNA by PCR was digested with *Bam*HI, and inserted to the *Bam*HI site of the pUC118 plasmid.

2.7. DNA sequencing

The sequencing reaction was performed by dideoxynucleotide chain termination method using a Bigdye terminator cycle sequencing FS ready reaction kit (PE Applied Biosystems). The reaction mixture was analyzed with a 377 automated DNA sequencer (PE Applied Biosystems). The deleted clones for sequencing of the entire region of the cDNA were prepared using a Kilo sequence deletion kit (Takara Shuzo).

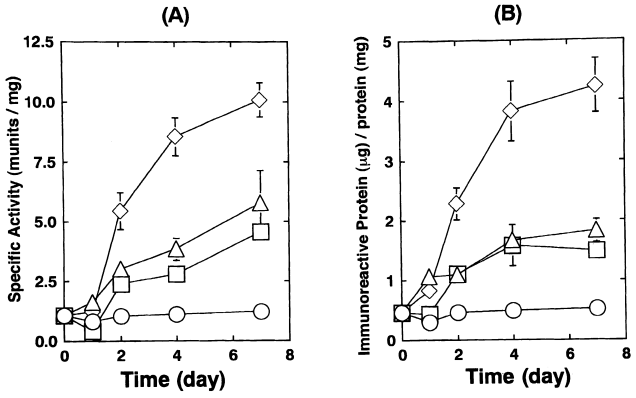

Fig. 1. Time course of the induction of AA-CoA synthetase by hypocholesterolemic agents. Female rats (6 weeks old) were fed with the standard diet (○), or with the standard diet containing 0.4% pravastatin (△), 4% cholestyramine (□), or 0.4% pravastatin and 4% cholestyramine (◇). (A) AA-CoA synthetase activity and (B) AA-CoA synthetase protein content in the liver supernatant fraction were measured as described in Section 2. Each point represents the mean ± S.E. of three experiments.

3. Results and discussion

3.1. Induction of rat liver AA-CoA synthetase

Since this enzyme is known to be induced by hypocholesterolemic agent [4,5], we investigated the effect of administration of pravastatin, a new potent 3-hydroxy-3-methylglutaryl-CoA inhibitor clinically used in the treatment of hypercholesterolemia [13], as well as of cholestyramine, an accelerator of bile acid excretion, on the AA-CoA content in rat liver. As shown in Fig. 1, the enzyme content in the liver extract increased about 5.0-, 8.4-, and 9.3-fold on days 2, 4, and 7, respectively, of the start of feeding rats with the diet containing both 4% cholestyramine and 0.4% pravastatin. Thus, cDNA library was constructed using purified mRNAs isolated from livers of rat fed a diet supplemented with 0.4% pravastatin plus 4% cholestyramine for 4 days (see Section 2).

3.2. 3′-RACE

The purified AA-CoA synthetase was digested with lysyl endopeptidase, chymotrypsin or trypsin, and the peptide fragments obtained were separated. Table 1 shows the eight different amino acid sequences determined by the automated Edman degradation method. EST database was surveyed for the sequence of the peptide 8 (TVEHRGAFSNPESLDLYRDIPELQD) (Table 1). Four EST clones were obtained, which were (1) R48370 yj67e08.r1 *Homo sapiens* cDNA clone 153830 5′, (2) R59020 yg96d03.r1 *H. sapiens* cDNA clone 41440 5′, (3) N71593 yw38c12.r1 *H. sapiens* cDNA clone 254518 5′, and (4) W12611 ma71b12.r1 Soares mouse p3NMF19.5 *Mus musculus* cDNA clone 316127 5′. The oligonucleotide (5′-CTCGGATCCCTCAAYGGCAAGAAAGTGGA-3′, 29-mer) was designed from the nucleotide sequences of EST clones (1) R48370 (human) and (4) W12611 (mouse), and used for synthesizing second-strand of cDNA in the 3′-RACE method. Thus, a cDNA fragment of 1.2 kb length was obtained, and it was confirmed to contain a partial open reading frame, termination codon and 3′-non-coding region of the cDNA. The sequence of peptide 8 (Table 1) was present in the amino acid sequence deduced from the cDNA sequence.

3.3. cDNA for AA-CoA synthetase

From the nucleotide sequence of the 1.2 kb cDNA fragment, two synthetic antisense oligonucleotides, 5′-CTTGGATCCACCGAGTGCGAGCCATTGGT-3′ and 5′-CTTGGATCCGCTGTCCCTAAGTTCCATCA-3′, which were derived from the region after termination codon (TGA) of the 1.2 kb cDNA fragment, were designed. From the nucleotide sequence of the vector λZipLox, two oligonucleotides, 5′-CTTGGATCCCACACAGGAAACAGCTATGA-3′ and 5′-CTTGGATCCCCATGATTACGCCAAGCTCT-3′, were also designed. The specific cDNA fragments amplified on the whole cDNA library with these four oligonucleotides were

Table 1
Codon and nucleotide location of peptide fragments of AA-CoA synthetase determined by protein microsequencing

Peptide fragment	Amino acid sequence	Codon location	Nucleotide location
Peptide 1	IADVPEWF	87–94	367–390
Peptide 2	VVLIPYV	236–242	814–834
Peptide 3	FLDDFLASGT	258–267	880–909
Peptide 4	VDRIGITI	370–377	1216–1239
Peptide 5	GIVMLGR	533–539	1705–1725
Peptide 6	NIVEAFDEVE	558–567	1780–1809
Peptide 7	VVLFLK	583–588	1855–1872
Peptide 8[a]	TVEHRGAFSNPESLDLYRDIPELQD	647–671	2047–2121

Amino acids are listed by their single letter designation, with their amino acid position (codon location) and nucleotide location on the AA-CoA synthetase cDNA.
[a]Peptide sequence used for library screening.

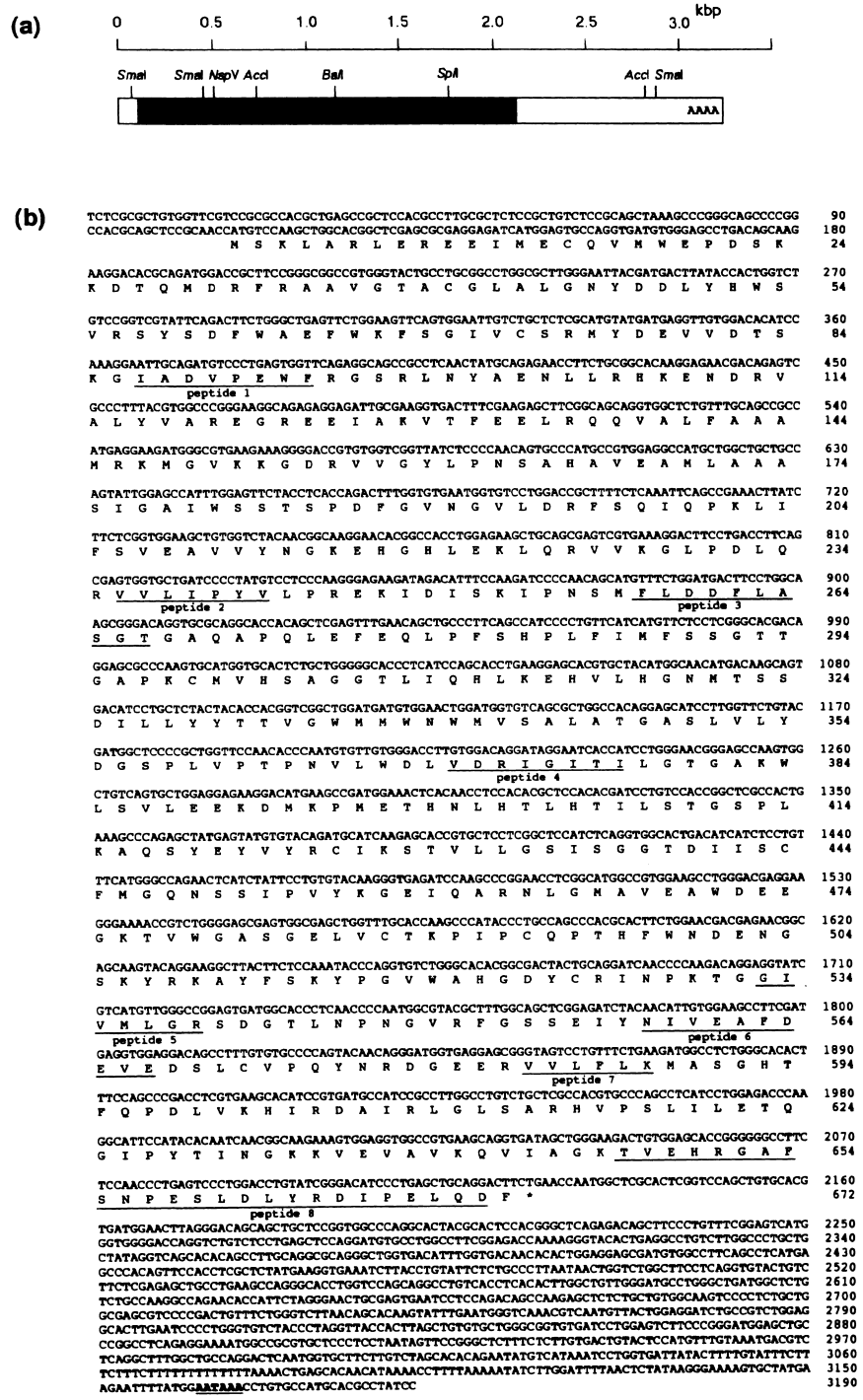

Fig. 2. Structure of the cDNA. (a) Restriction map of the cDNA. The solid box represents the open reading frame. (b) Nucleotide sequences and amino acid sequences of AA-CoA synthetase. The amino acid sequences determined by protein microsequencing (Table 1) and putative poly(A)$^+$ addition signal are underlined.

digested with *Bam*HI, inserted to the *Bam*HI site of pUC118, and transferred to *Escherichia coli* JM109. Thirteen clones encoding the open reading frame of AA-CoA synthetase were obtained, and two of them contained the initiation codon. Fig. 2a shows the restriction map of the entire cDNA clone. Although the N-terminal amino acid sequence of the purified AA-CoA synthetase has not been clarified so far because of N-terminal blocking of the enzyme, the ATG codon at nucleotide 109 in the translation frame was tentatively concluded to be the initiator codon since the Kozak's consensus sequence for initiation of translation in vertebrates [14] was conserved at nucleotides 101 to 111. These clones had a coding region for a protein of 672 amino acid residues with a calculated molecular weight of 75 039 Da (Fig. 2b). All peptide sequences (Table 1) analyzed using the purified AA-CoA synthetase were seen in the deduced amino acid sequence of this protein (Fig. 2b).

Since the molecular weight of 71 000 Da estimated by SDS–

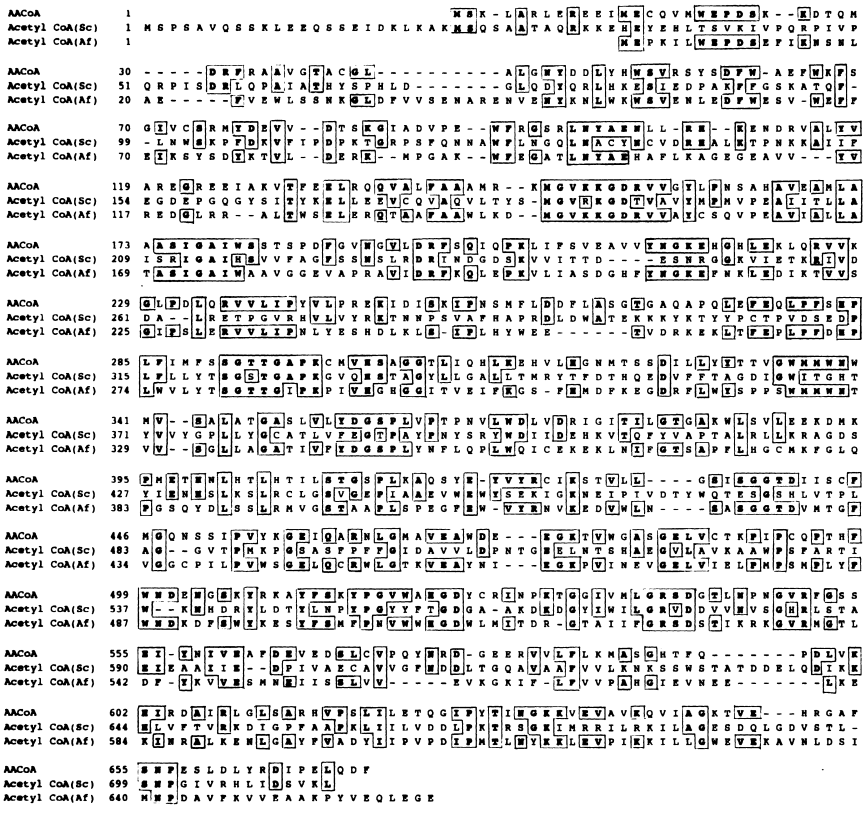

Fig. 3. Homology alignments of AA-CoA synthetase of rat and acetyl-CoA synthetases of *S. cerevisiae* (indicated as Sc) and *A. fulgidus* (indicated as Af). Boxes indicate identical amino acid residues of the sequences.

PAGE [2] is smaller than the calculated molecular weight, it is possible that the translated protein is truncated by post-transcriptional modification. Previous studies showed that alternative translation initiation generated isoforms of acyl-CoA synthetase 3 [15]. We saw four in-frame ATG codons from nucleotides 109 to 195, and supposed that the forth ATG was initiator. Then the calculated molecular weight would be 71 645 Da, which is in good agreement with that estimated by SDS–PAGE. The possibility of the presence of alternative translation or post-transcriptional modification requires further investigation.

3.4. Homology

The amino acid sequence deduced from the open reading frame was searched in protein sequence database [16]. The amino acid sequence of rat liver AA-CoA synthetase showed significantly high homologies with microbial acetyl-CoA synthetases, bearing 25.0% and 38.9% homologies with acetyl-CoA synthetases of *Saccharomyces cerevisiae* [17] (Accession No. X66425) and *Archaeoglobus fulgidus* [18] (Accession No. AE001092), respectively (Fig. 3). When similar amino acids are considered (I = L, V; F = Y, W; K = R, H; D = E), the homologies (similarities) were 34.9 and 50.0%, respectively. It also showed 39.0% homology with *Caenorhabditis elegans* clones (Accession No. U41625), which are supposed to be acetyl-CoA synthetase of *C. elegans* [19]. Compared to bacterial acetyl-CoA synthetase, any long chain or medium chain fatty acyl-CoA synthetase, which has been submitted to the protein sequence database, showed less homology with rat liver acetoacetyl-CoA synthetase (data not shown).

Amino acid sequences of rat AA-CoA synthetase and acetyl-CoA synthetases of *S. cerevisiae* and *A. fulgidus* have AMP-binding motifs ([LIVMFY]-x(2)-[STG]-[STAG]-G-[ST]-[STEI]-[SG]-x[PASLIVM]-[KR]) in the same region (rat AA-CoA synthetase, I287-K298; *S. cerevisiae* acetyl-CoA synthetase L317-K328; *A. fulgidus* acetyl-CoA synthetase V276-K287). The AMP-binding motif is shown in a number of prokaryotic and eukaryotic enzymes which all probably act via an ATP-dependent covalent binding of AMP to their substrate, and share a region of sequence similarity [20–22]. In Fig. 3, WMMWNWM in rat AA-CoA synthetase (W395-M341) is also noticed. Methionine and tryptophan are rarely distributed amino acids in common protein sequence [23], so this region might play an important role for catalysis or its protein structure. Regulation of the AA-CoA synthetase expression under the various physiological conditions is under investigation using cDNA encoding this enzyme.

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