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RAT LIVER MEDIUM-CHAIN ACYL CoA DEHYDROGENASES DIRECTED BY COMPLEMENTARY DNAs DIFFERING IN THEIR 5'-REGION

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Introduction

Medium-chain acyl CoA dehydrogenase (MCAD) (EC 1.3.99.3) of rat liver is a tetramer of four identical subunits containing one molecule of dissociable FAD per subunit, and is synthesized as a precursor having a leader peptide of 25 amino acid residues. The molecular weights of the precursor and the mature enzyme were estimated to be 46,600 and 43,400 daltons, respectively (1). The leader peptide is presumably indispensable not only for transport across the mitochondrial membrane, but also for establishment of the three-dimensional structure of the catalytically inactive precursor.

We isolated a cDNA encoding this enzyme from cDNA library of rat liver (2) and recently from that of human placenta (3). By manipulating this cDNA, we are able to obtain some derivatives of the enzyme to see the relationship between structure and function of the enzyme. In the present study, we constructed expression plasmids differing at the 5'-region of the cDNA to produce in *Escherichia coli* rat liver enzymes differing in their NH_2-terminal regions.

Results and Discussion

A nearly full-length cDNA for rat liver MCAD containing 1,230 nucleotides of the coding region and 574 nucleotides of the 3'-noncoding region obtained in our previous study (2) was inserted into the *Eco*RI site of plasmid pUC 19. Expression plasmids for MCAD were constructed in two different ways: 1) the recombinant plasmid was cleaved with *Hin*dIII and *Sal*I, repaired with Klenow fragment, and ligated; 2) the recombinant plasmid was cleaved with *Bam*HI and *Sph*I, and then mixed with exonuclease III at 37°C to delete the 5'-region of the cDNA. At 5-second intervals of incubation, aliquots were put into 40 mM sodium acetate buffer (pH 4.5) containing 100 mM NaCl, 2 mM $ZnCl_2$, and 10% glycerol. The deleted plasmids were further digested with mung bean nuclease, repaired with Klenow fragment, and ligated. The deletion mutants were designated as the pRMCADm series.

With these pRMCADm series *E. coli* JM109 cultures were transformed, and six clones judged positive by immunoassay were isolated. By immunoblot analysis, we confirmed that anti-MCAD antibody-reactive proteins were

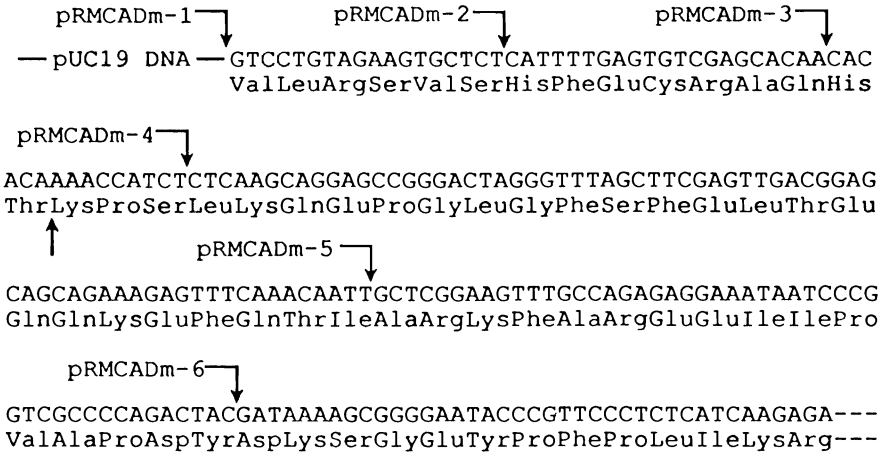

Fig. 1. Nucleotide sequence of 5'-region of expression plasmids pRMCADm-1, 2, 3, 4, 5, and 6. The deduced amino acid sequence is shown under the nucleotide sequence. The downward arrows represent the junction between MCAD gene and pUC 19, and the upward arrow represents the postulated cleavage site between the leader peptide and the mature enzyme (1).

Table 1. MCAD activities in cell-free extracts obtained from *E. coli* carrying pRMCADm-1~6

Clone number	Apparent molecular weight (kDa)	MCAD activity (munit/mg protein)
pRMCADm-1	45	<10
pRMCADm-2	44	74
pRMCADm-3	43	126
pRMCADm-4	43	113
pRMCADm-5	41	14
pRMCADm-6	40	<10

Apparent molecular weights were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. MCAD activity was determined with *n*-octanoyl CoA as substrate. *E. coli* JM109 cells themselves have *n*-octanoyl CoA-dehydrogenating activity, but the specific activity was less than 10 munits/mg protein.

produced in *E. coli* carrying pRMCADm-1~6 grown in the presence of isopropyl-ß-D-thiogalactopyranoside. These expressed proteins with various molecular weights (from 40 to 45 kDa) could be readily extracted from the cells, and more than 90% of the protein produced by each clone was recovered in the supernatant following 10,000 × g centrifugation. Restriction maps of the cDNAs confirmed the sequences of different length in the 5'-region, corresponding to the various molecular weights of protein. Figure 1 shows the nucleotide sequences of the 5'-region of the cDNAs.

Table 1 shows *n*-octanoyl CoA-dehydrogenating activities of cell-free extracts from immuno-positive clones. The extract of *E. coli* carrying pRMCADm-3 showed the highest enzymatic activity, whereas the activities in extracts of *E. coli* carrying pRMCADm-4 and pRMCADm-2 were approx. 90 and 60%, respectively, of the maximum one. The extract of *E. coli* bearing pRMCADm-5 showed only marginal activity, and extracts from the remaining two clones showed no enzymatic activity.

MCADs produced in *E. coli* carrying the expression plasmids pRMCADm-1, pRMCADm-3, and pRMCADm-6 were purified by ammonium sulfate fractionation and column chromatographies with BioGel HPT and BioGel A-5m. The enzymatic properties such as specific activity, K_m, and V_{max} of the MCAD purified from pRMCADm-3-bearing *E. coli* were fairly similar to

those of the enzyme from rat liver mitochodria, and the absorption spectrum of the former coincided with that of the latter. On the other hand, the purified proteins directed by pRMCADm-1 and pRMCADm-6 did not contain FAD and had no enzymatic activity even after the addition of FAD.

From the X-ray diffraction study of pig liver MCAD by Kim and Wu (4), the flavin ring of the coenzyme seems to lie near the NH_2-terminal domain of the apoprotein. The deletion of 42 amino acid residues in the protein directed by pRMCADm-6 means lack of the first helix of the NH_2-terminal portion. Obviously, the absence of this region results in the failure of formation of the functional three-dimensional structure of the mature enzyme, which makes the apoenzyme unable to combine with FAD. The protein directed by pRMCADm-1 contains a substantial portion of the leader peptide, and thus the binding of the flavin coenzyme to the apoprotein is probably disturbed by the leader peptide.

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