THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 262, No. 21, Issue of July 25, pp. 10104-10108, 1987
Printed in U.S.A.

Molecular Cloning and Nucleotide Sequence of cDNA Encoding the Entire Precursor of Rat Liver Medium Chain Acyl Coenzyme A Dehydrogenase*

(Received for publication, March 26, 1987)

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cDNA encoding the precursor of rat liver medium chain acyl-CoA dehydrogenase (EC 1.3.99.3) was cloned and sequenced. The longest cDNA insert isolated was 1866 bases in length. This cDNA encodes the entire protein of 421-amino acids including a 25-amino acid leader peptide and a 396-amino acid mature polypeptide. The identity of the medium chain acyl-CoA dehydrogenase clone was confirmed by matching the amino acid sequence predicted from the cDNA to the NH_2-terminal and nine internal tryptic peptide sequences derived from pure rat liver medium chain acyl-CoA dehydrogenase. The calculated molecular masses of the precursor medium chain acyl-CoA dehydrogenase, the mature medium chain acyl-CoA dehydrogenase, and the leader peptide are 46,600, 43,700, and 2,900 daltons, respectively. The leader peptide contains five basic amino acids and only one acidic amino acid; thus, it is positively charged, overall. Cysteine residues are unevenly distributed in the mature portion of the protein; five of six are found within the NH_2-terminal half of the polypeptide. Comparison of medium chain acyl-CoA dehydrogenase sequence to other flavoproteins and enzymes which act on coenzyme A ester substrates did not lead to unambiguous identification of a possible FAD-binding site nor a coenzyme A-binding domain. The sequencing of other homologous acyl-CoA dehydrogenases will be informative in this regard.

Medium chain acyl-CoA dehydrogenase (EC 1.3.99.3) is one of the five known acyl-CoA dehydrogenases. It acts on a wide spectrum of straight chain acyl-coenzyme A esters of medium chain length (1) and plays a crucial role in fatty acid oxidation. Other enzymes in this group are short chain acyl-CoA, long chain acyl-CoA, isovaleryl-CoA, and 2-methyl-branched chain acyl-CoA dehydrogenases (1–3). All of them are mitochondrial flavoproteins and share many biochemical and molecular features, differing only in length and configuration of their acyl-CoA substrates. Each acyl-CoA dehydrogenase is a homotetramer, containing 1 mol of noncovalently bound FAD per subunit. The subunit molecular mass as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis ranges from 41 kDa for short chain acyl-CoA to 45 kDa for medium chain acyl-CoA and long chain acyl-CoA dehydrogenases. These five enzymes are synthesized in the cytosol as precursors that are a few kilodaltons larger than their ultimate forms, imported into mitochondria, and proteolytically cleaved to their mature forms (4).

The shared features of acyl-CoA dehydrogenases in their molecular properties and reaction mechanisms (4–6) are consistent with the hypothesis that these enzymes are closely related in evolution. They share significant homology in their sequences (7) and may have been derived from a common ancestral gene. Relatively small overlaps of their substrate specificities (1–3) are indicative of accurate construction of structures surrounding their active sites. Thus, the determination of primary structure of acyl-CoA dehydrogenases would provide valuable information for understanding the structure-function relationship, as well as their phylogenic interrelationship.

Hereditary deficiency of medium chain acyl-CoA dehydrogenase in man causes intolerance to prolonged fasting and recurrent hypoglycemic coma, which occasionally leads to the patient's death (8). These episodes are accompanied by medium chain dicarboxylic aciduria and increased octanoylcarnitine excretion. Since it was first described less than 5 years ago, more than 50 patients with this disorder have been reported,[1] indicating a relatively high incidence of medium chain acyl-CoA dehydrogenase deficiency. Cloning and sequencing of medium chain acyl-CoA dehydrogenase cDNA are the prerequisites for the study of the molecular basis of this disease.

We have recently described the isolation of partial rat and human medium chain acyl-CoA dehydrogenase cDNA clones (9). Extensive search by screening several rat and human cDNA libraries did not yield a full length cDNA clone from either source. We have, therefore, prepared a rat cDNA library highly enriched for medium chain acyl-CoA dehydrogenase sequences using immunopurified medium chain acyl-CoA dehydrogenase mRNA. We now report a successful isolation and sequence of rat cDNA encoding the entire medium chain acyl-CoA dehydrogenase protein.

* This work was supported by National Institutes of Health Research Grants DK 17453, DK 38154, DK 09527, and March-of-Dimes Grant I-378. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J02791.

‡ Supported, in part, by an intramural James Hudson Brown-Alexander B. Coxe fellowship from Yale University School of Medicine.

§ Supported by a postdoctoral fellowship from the Muscular Dystrophy Association.

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[1] P. M. Coates, personal communication.

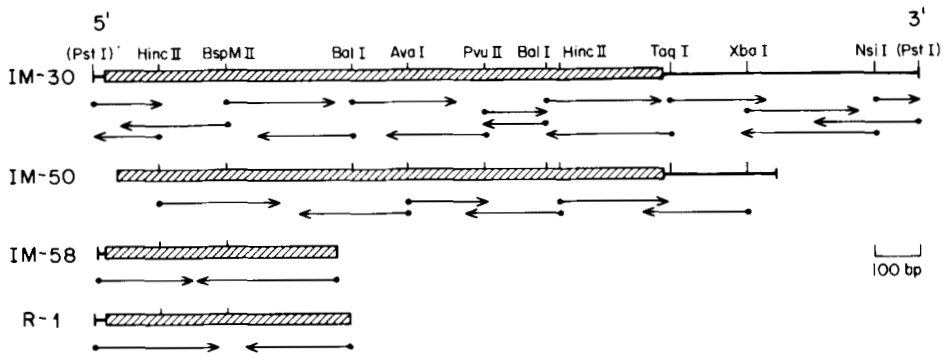

FIG. 1. **Medium chain acyl-CoA dehydrogenase cDNA clones and sequencing strategy.** IM-30, IM-50, and IM-58 are new medium chain acyl-CoA dehydrogenase cDNA clones isolated from the enriched library. R-1 is a previously isolated medium chain acyl-CoA dehydrogenase clone (9). The *hatched section* of the cDNA inserts indicates coding regions. The *straight, heavy lines* indicate 5'- and 3'-noncoding regions. cDNAs were digested with various restriction enzymes as shown here, subcloned into plasmid pGEM-2, and sequenced using T7 and SP6 promoter primers. The *arrows* indicate the direction and extent of sequencing.

EXPERIMENTAL PROCEDURES

Construction of a cDNA Library Enriched for Medium Chain Acyl-CoA Dehydrogenase cDNA—mRNA coding for medium chain acyl-CoA dehydrogenase precursor was immunopurified from rat liver polysomes as previously described (10, 11). The purified mRNA was used to construct a cDNA library enriched for medium chain acyl-CoA dehydrogenase cDNAs according to the method previously described (11) with modification; DNA ligase was omitted from the reaction for second-strand synthesis, and the reaction mixture was further incubated with T4 DNA polymerase at 37 °C for 10 min. The dC-tailed double-stranded DNA was annealed with *Pst*I-cut, dG-tailed pBR 322 (Bethesda Research Laboratories) according to a recently modified protocol.[2] Briefly, the double-stranded DNA and vector were mixed at 1:10 (wt/wt) ratio at a final DNA concentration of 2 μg/ml in 10 mM Tris·HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA buffer. The mixture was incubated 60 min at 57 °C, 50 min at 42 °C, 20 min at 22 °C, and then kept on ice. *Escherichia coli* of DH5 strain were prepared for transformation according to Simanis (12). A total of 25 ml of DH5 competent cells (12 aliquots, ~3.4×10^9 cells/aliquot) was transformed with 4.5 μg (2.2 ml) of the annealed DNA. The library was plated on 36 plates (ø 150 mm). A total of 100,000 independent colonies was obtained.

Screening of the cDNA Library—Six-thousand colonies from the enriched cDNA library were screened by colony hybridization (13) using a previously isolated partial rat medium chain acyl-CoA dehydrogenase cDNA clone (R-1, 580 base pairs) as a probe (9). The probe was radiolabeled with [^{32}P]dCTP by the random primer DNA-labeling method (14).

DNA Sequencing—Restriction fragments of medium chain acyl-CoA dehydrogenase cDNAs were subcloned into pGEM2 plasmid (Promega Biotech). Nucleotide sequence was determined directly from double-stranded DNA (15) using the dideoxy-sequencing method (16). T7 or SP6 oligonucleotide primers (Promega Biotech) and either Klenow fragment of *E. coli* polymerase I (Boehringer Mannheim) or avian myeloblastosis virus reverse transcriptase (Life Sciences) were employed. For sequencing GC-rich regions, 7-deaza-dGTP (Boehringer Mannheim) was used to eliminate ambiguities due to band compression (17).

Peptide Separation and Amino Acid Sequence Analysis—Purified rat liver medium chain acyl-CoA dehydrogenase was first carboxymethylated with iodoacetate in 7.5 M guanidinium chloride in order to unambiguously identify cysteine residues. Tryptic peptides were prepared and initially fractionated on a 4.6 mm × 7.5 cm Altex Ultrapore RPSC (C_3) column using 0.1% trifluoroacetic acid and an acetonitrile gradient between 0–60%. In some cases, fractions were rechromatographed under identical conditions except that 0.1% acetate, pH 6.0, was substituted for the trifluoroacetic acid. Amino-terminal sequences of tryptic peptides and of native medium chain acyl-CoA dehydrogenase were determined using a model 470A gas-phase sequencer (Applied Biosystems, Inc.) and an in-line model 120A phenylthiohydantoin analyzer.

In Vitro Transcription/Translation of cDNA—*Pst*I-cut cDNA insert of medium chain acyl-CoA dehydrogenase cDNA clone (IM-30) was subcloned into *Pst*I-site of pGEM-2 transcription vector. *In vitro* transcription of the cDNA was carried out according to the manufacturer's protocol using T7 RNA polymerase (Promega Biotech). The synthesized mRNA was translated *in vitro* employing the rabbit reticulocyte lysate translation system (9), and the translation products were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Sequence Comparison Analysis—The computer programs of the University of Wisconsin Genetics Computer Group, including Bestfit, Compare, and Gap, were used.

RESULTS AND DISCUSSION

Isolation of Medium Chain Acyl-CoA Dehydrogenase cDNA Clones—Several nanograms of immunopurified medium chain acyl-CoA dehydrogenase mRNA were isolated from 2,200 A_{260} units of polysomes using approximately 60 g of rat liver. This mRNA was used to prepare a cDNA library that contained 100,000 recombinants. A small portion of the enriched cDNA library (6,000 colonies) was screened using a previously isolated rat medium chain acyl-CoA dehydrogenase cDNA (R-1), which encoded the amino-terminal portion of the protein. Fifty-eight positive clones were identified. The size of cDNA inserts ranged from 150 to 1,866 base pairs. Restriction enzyme patterns of these cDNA clones indicated that the largest clone (IM-30) extended further than any other clones at either 5' or 3' ends with an exception of R-1, which appeared to share the same 5' end. Four clones (IM-30, IM-50, IM-58, and R-1) were subjected to nucleotide-sequencing analysis according to the strategy shown in Fig. 1.

Nucleotide Sequence of Medium Chain Acyl-CoA Dehydrogenase—Nucleotide sequence of medium chain acyl-CoA dehydrogenase cDNA, based on the sequence of IM-30, is shown in Fig. 2. The putative initiation codon ATG at position 1 is contained within the sequence GCATCATGG. This is in good agreement with the consensus sequence for eukaryotic initiation sites proposed by Kozak (18), suggesting that this ATG codes for the amino-terminal methionine of the precursor medium chain acyl-CoA dehydrogenase.

In the sequences of four medium chain acyl-CoA dehydrogenase clones, four nucleotide substitutions were noted. The sequence of IM-50 contained an A rather than a G at position +469 (changing Ala to Thr), a T rather than a G at +1027 (Glu to Stop codon), and a C rather than a T at +1383 (noncoding region). At the first position, the peptide sequence data assigned Ala, ascertaining the sequence obtained using IM-30. The second change in IM-50 produced a termination codon. This alteration was most likely due to a reverse transcription error because the peptide sequence data assigned Glu at this position, confirming the sequence found in IM-30

[2] J. P. Kraus, and Y. Matsubara, unpublished.

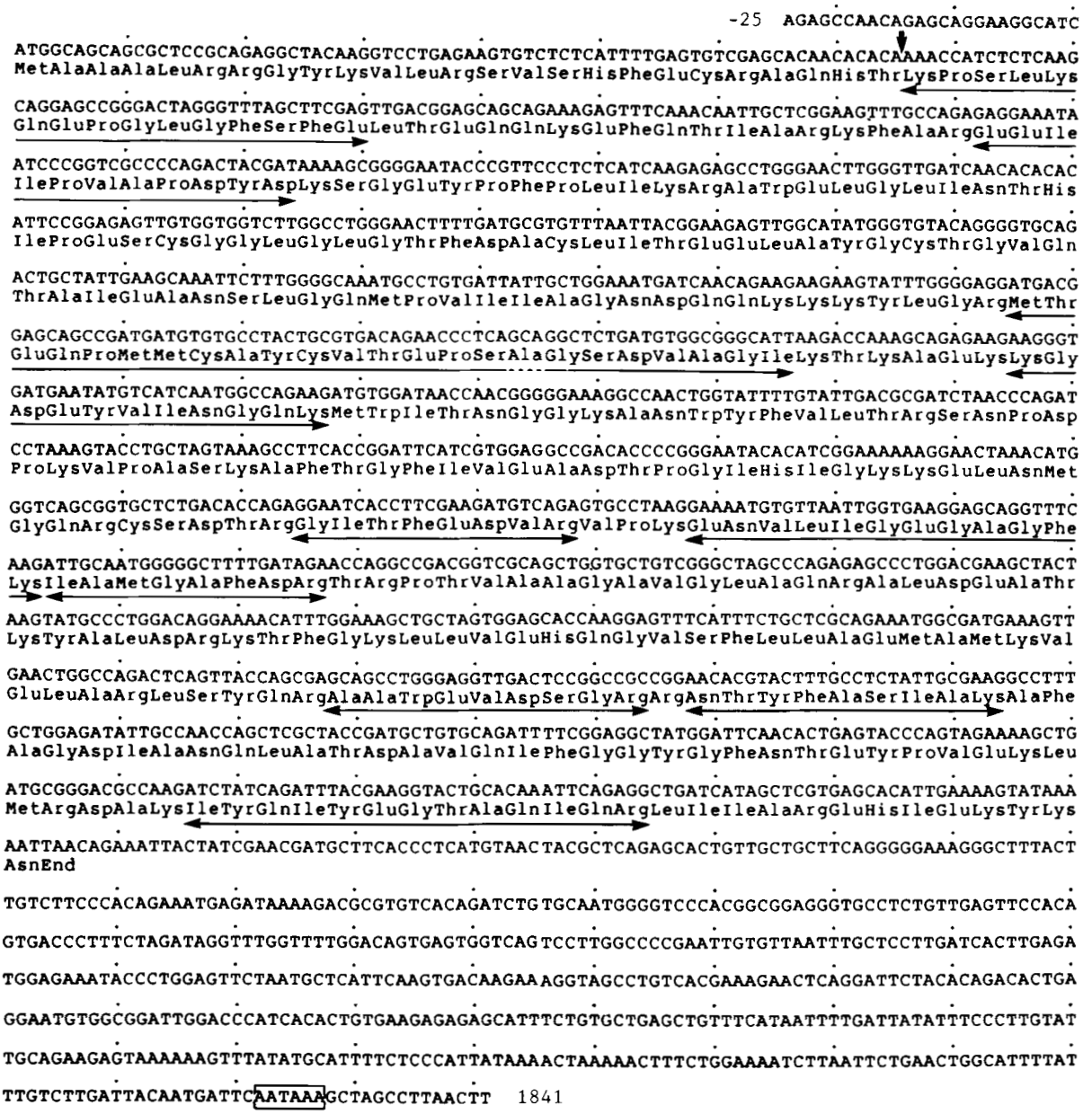

FIG. 2. **Nucleotide sequence of medium chain acyl-CoA dehydrogenase cDNA and deduced amino acid sequence of rat liver medium chain acyl-CoA dehydrogenase precursor.** The positive numbers of amino acids and nucleotides start at the first residue of the coding sequence. Nucleotide sequence at position −25 was taken from R-1, because IM-30 starts at position −24. *Underlined* amino acid sequences matched those obtained from the NH_2-terminal sequence of mature enzyme (amino acid residues 26–40) and its internal tryptic peptides. Amino acid residue 165 (Ala, *underlined* with a *broken line*) was not identified by protein sequencing. The *downward arrow* between residues 25 and 26 indicates the cleavage site between the leader peptide and the mature protein. A possible polyadenylation signal in the 3′-untranslated region is *boxed*.

(Fig. 2). A similar error has been reported in cDNAs encoding a catalytic subunit of cAMP-dependent protein kinase (19). R-1 contained a silent change of C→T at +555, which may represent a polymorphism.

Northern Blot Analysis—The size of rat medium chain acyl-CoA dehydrogenase mRNA is estimated to be 2.2 kilobases (9). The cDNA clone IM-30 is, therefore, approximately 300 base pairs shorter than the full length cDNA. If one takes a poly(A) tail of 200 nucleotides into account, the total of nucleotides that is missing from the noncoding region at the 5′ and 3′ ends of this clone is about 100 bases. A possible polyadenylation signal (20) is indicated in Fig. 2.

In Vitro Transcription/Translation of cDNA—cDNA insert of IM-30 was subcloned into pGEM-2 transcription vector. The transcription of the cDNA, followed by *in vitro* translation, produced several polypeptides of different sizes, all immunoprecipitable with anti-medium chain acyl-CoA dehydrogenase antibody (Fig. 3). The largest polypeptide was identical in size to rat medium chain acyl-CoA dehydrogenase precursor on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the ATG at +1 is the initiator codon for the medium chain acyl-CoA dehydrogenase precursor. The smaller polypeptides were probably derived from initiation of translation at different internal methionine codons.

Amino Acid Sequence of Medium Chain Acyl-CoA Dehydrogenase—The sequence of an amino-terminal peptide and

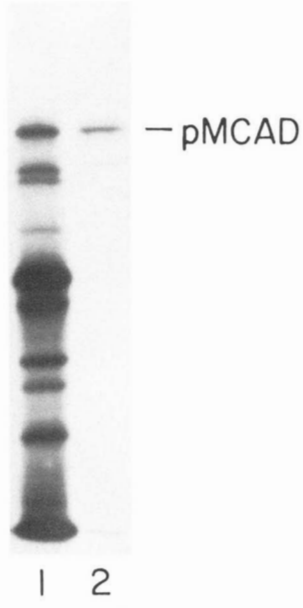

FIG. 3. ***In vitro* transcription and translation of IM-30.** *Lane 1,* translation products of mRNA transcribed from IM-30 cDNA after immunoprecipitation with anti-medium chain acyl-CoA dehydrogenase antiserum; *lane 2,* translation products of total rat liver mRNA followed by immunoprecipitation with anti-medium chain acyl-CoA dehydrogenase antiserum, indicating the position of precursor medium chain acyl-CoA dehydrogenase (*pMCAD*).

(a)

CS (313)	R	V	V	P	G	Y	G	H	A	V	L	P	K	T	D	P
ECH (321)	S	V	V	A	V	E	S	D	P	-	-	K	N	L	D	A
ACO (224)	F	V	V	P	L	R	D	S	-	-	-	N	H	-	D	L
βPCC (306)	T	V	V	P	L	E	S	S	K	A	Y	N	M	L	D	I
MCAD (283)	T	V	A	A	G	A	V	G	L	A	Q	R	A	L	D	E

(b)

CS (313)	R	V	V	P	G	Y	G	H	A	V	L	R	K	T	D	P
ECH (321)	S	V	V	A	V	E	S	D	P	-	-	K	N	L	D	A
ACO (224)	F	V	V	P	L	R	D	S	-	-	-	N	H	-	D	L
βPCC (306)	T	V	V	P	L	E	S	S	K	A	Y	N	M	L	D	I
MCAD (167)	S	D	V	A	G	I	K	T	K	A	E	K	K	G	D	E

FIG. 4. **Alignment of homologous sequences among five CoA-binding enzymes.** The first amino acid residue in each sequence is *numbered* in *parentheses.* Amino acid residues which are shared by at least two sequences are *boxed. CS,* porcine citrate synthase (33); *ECH,* rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (35); *ACO,* two acyl-CoA oxidases from peroxisomes of yeast *Candida tropicalis* (36); *β-PCC,* β-subunit of rat propionyl-CoA carboxylase (34). *MCAD,* medium chain acyl-CoA dehydrogenase.

those of nine internal tryptic peptides (total of 119 residues) from rat liver medium chain acyl-CoA dehydrogenase perfectly matched the amino acid sequence deduced from the DNA sequence (Fig. 2), confirming the identity of the cDNA clones and the fidelity of the reading frame. Comparison of the NH_2-terminal sequence data of pure mature medium chain acyl-CoA dehydrogenase to the predicted sequence of the precursor (Fig. 2) indicates that the peptide consisting of amino acid residues 1–25 is the leader sequence for targeting this protein toward mitochondria (4). The calculated molecular weight of precursor medium chain acyl-CoA dehydrogenase, mature medium chain acyl-CoA dehydrogenase, and the leader peptide was 46,600, 43,700, and 2,900, respectively. Our previous estimates of the molecular weight of mature rat medium chain acyl-CoA dehydrogenase and the size of its leader peptide were 45,000 and 4,000, respectively, by gel electrophoresis (1, 4). The difference between the calculated and estimated molecular weight may be explained by aberrant mobility of the precursor protein in gel. The leader peptide contains four arginines, one lysine, and one glutamate. It is, therefore, highly positively charged. The average hydrophobicity of the leader peptide calculated by using a normalized consensus hydrophobicity (21) is −0.19, similar to that observed in 10 other nuclear-coded mitochondrial enzymes (22). The open reading frame in the putative 5′-untranslated region contains three glutamate codons. Should the true initiation codon be further upstream, this would add 3 acidic residues in the amino-terminal portion of the leader peptide, a situation that has never been observed in any other mitochondrial leader peptide (22). Thus, taking all evidence into consideration, we conclude that ATG at +1 serves as the initiator codon.

It is interesting to note that, in the sequence of mature medium chain acyl-CoA dehydrogenase, Cys residues are unevenly distributed. Five of 6 Cys residues are located within the NH_2-terminal half of the protein, 3 of them are located within 22 residues (+95, +106, and +116), and 2 of them are separated only by two amino acids (+156 and +159). The significance of this uneven distribution of Cys is not clear at present.

Functional Domains—Several functional domains of medium chain acyl-CoA dehydrogenase have been proposed from enzymological studies. The presence of CoA-binding site in medium chain acyl-CoA dehydrogenase has been suggested from the studies using substrate analogs such as *S*-alkyl-CoA and other CoA-containing compounds (23). Another possible substrate-binding site which binds hydrocarbon chains of acyl-CoA substrates has also been proposed (23). These substrate-binding sites are probably located in the vicinity of FAD-binding site and the essential cysteine residue inside a crevice, forming the active center (6). At present, these domains of medium chain acyl-CoA dehydrogenase must be deduced from the homology of amino acid sequence to those of enzymes which have been studied by x-ray crystallographic analysis.

The crystallographic analysis of citrate synthase revealed the presence of "adenine recognition loop," the region which binds the adenine ring of acetyl-CoA (24). This is a seven-amino acid portion, starting from Val-314 in porcine citrate synthase. When the β-subunit of rat propionyl-CoA carboxylase (25) and three other CoA-binding enzymes, namely rat peroxisomal bifunctional enzyme (enoyl-CoA hydratase/3-hydroxy-acyl-CoA dehydrogenase) (26) and two yeast acyl-CoA oxidases (27) were compared to the CoA-binding region of citrate synthase, a sequence of limited homology was noted (Fig. 4). There was an absolute conservation of a double valine residue (positions 314, 315 in citrate synthase). An aspartate residue, located after an interval of 7–11 residues, appeared to also be conserved.[3] Comparison analysis by computer indicated that, in the case of rat medium chain acyl-CoA dehydrogenase, there were two potential regions, one starting from Thr-283 (Fig. 4*a*) and the other starting from Ser-167 (Fig. 4*b*), which share four amino acids with the CoA-binding

[3] J. P. Kraus, unpublished.

region of citrate synthase. Homologies of these segments with β-subunit of propionyl-CoA carboxylase are of similar degree (4 or 5 residues). Although one of these segments is a likely candidate, the CoA-binding site in medium chain acyl-CoA dehydrogenase could not be clearly identified.

We found no specific region in the sequence of medium chain acyl-CoA dehydrogenase, which bears clear homology to the FAD-pyrophosphate-binding sites identified or proposed in other FAD-containing enzymes, such as glutathione reductase and lipoamide dehydrogenase (28, 29). The lack of homology between medium chain acyl-CoA dehydrogenase and glutathione reductase/lipoamide dehydrogenase is consistent with the knowledge that in different classes of FAD-containing enzymes the type of FAD-binding site and the surrounding environment are divergent (30). FAD binding in medium chain acyl-CoA dehydrogenase is noncovalent and probably is mediated by hydrogen bonding. On the other hand, FAD in other enzymes, such as glutathione reductase, lipoamide dehydrogenase, and succinic dehydrogenase, is covalently bound to a histidine residue (30). Medium chain acyl-CoA dehydrogenase and other acyl-CoA dehydrogenases require an electron transfer flavoprotein, another flavoprotein with a molecular mass of 59 kDa, as an electron acceptor, while most other flavin-enzymes also contain a binding site for NAD or NADP. It is possible that one of the two potential adenine recognition sites in medium chain acyl-CoA dehydrogenase (Fig. 4) may represent the binding site for FAD-adenine and the other for CoA-adenine. It has been recognized that various nucleotide-binding domains such as those for FAD, GTP, and NAD share a homologous sequence (31).

The attempts to identify various domains in the sequence of medium chain acyl-CoA dehydrogenase thus far did not yield unequivocal identification of any one of them. Medium chain acyl-CoA dehydrogenase is the first to be cloned and sequenced among the five acyl-CoA dehydrogenases. When complete amino acid sequences of the four other acyl-CoA dehydrogenases become available, comparison of medium chain acyl-CoA dehydrogenase with them will reveal homologous domains that are essential for functions commonly shared by these enzymes, as well as divergent domains that determine their specificity.

Acknowledgments—We thank Manju Swaroop for technical advice, Wayne Fenton for critical comments, and Connie Woznick for preparation of this manuscript.

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