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Characterization of Wild-type and an Active Site Mutant of Human Medium Chain Acyl-CoA Dehydrogenase after Expression in *Escherichia coli**

Peter Bross‡, Stefan Engst‡, Arnold W. Strauss§, Daniel P. Kelly§¶, Ihab Rasched‡, and Sandro Ghisla‡

From the ‡Faculty of Biology, University of Konstanz, P. O. Box 5560, D-7750 Konstanz, Federal Republic of Germany and the §Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

The cDNA of human medium chain acyl-CoA dehydrogenase (MCADH) was modified by *in vitro* mutagenesis, and the sequence encoding the mature form of MCADH was introduced into an inducible expression plasmid. We observed synthesis of the protein in *Escherichia coli* cells transformed with this plasmid with measurable MCADH enzyme activity in cell extracts. Glutamic acid 376, which has been proposed by Powell and Thorpe (Powell, P. J., and Thorpe, J. (1988) *Biochemistry* 27, 8022–8028) as an essential residue and the proton-abstracting base at the active site of the enzyme, was mutated to glutamine. After expression in bacteria of this plasmid, the corresponding extracts show no detectable MCADH activity, although mutant MCADH-protein production was detected by protein immunoblots. The mature enzyme and the Gln^{376} mutant were purified to apparent homogeneity. The wild-type enzyme is a yellow protein due to the content of stoichiometric FAD and had a specific activity which is 50% of MCADH purified from pig kidney. The Gln^{376} mutant is devoid of activity (<0.02% that of wild type, expressed enzyme) and is green because of bound CoA persulfide. Properties of the mutant enzyme suggest that the $Glu^{376} \rightarrow Gln$ change specifically affects substrate binding. These results prove that Glu^{376} plays an important role in the initial step of dehydrogenation catalysis.

Medium chain acyl-CoA dehydrogenase (MCADH[1] (also called general acyl-CoA dehydrogenase), EC 1.3.99.3) is a mitochondrial flavoenzyme which catalyzes the initial rate-limiting step in fatty acid β-oxidation. This reaction involves the 2,3-dehydrogenation of acyl-CoA thioesters with formation of the *trans*-2-enoyl-CoA product (1). Its primary substrates are straight chain fatty acids with a length of C_4–C_{16} with an optimum at C_6–C_8. This range of substrate activity overlaps with two related enzymes, short chain acyl-CoA dehydrogenase and long chain acyl-CoA dehydrogenase. These enzymes have been the object of extensive mechanistic studies in the past years (1). The three-dimensional structures of MCADH from pig liver (2) and from pig kidney[2] are currently being determined and should shortly result in a map of the enzyme active site at a resolution of 2.5 Å. The cDNA sequences of human liver and placental MCADH (4), rat liver MCADH (5), and pig liver MCADH[3] have been determined, and amino acid sequence identity among them is 86–89%.

Inherited defects resulting in deficiency of MCADH are a known cause of hypoglycemic coma, liver dysfunction, and sudden death in infants (7). This is now recognized as a relatively common inherited metabolic defect (8). Preliminary studies have shown that many affected patients have a normal sized immunodetectable protein (9, 10). Therefore, these naturally occurring mutant MCADH proteins likely contain a crucial point mutation which results in a catalytically inactive enzyme.

Powell and Thorpe (11) employed a mechanism-based inhibitor to identify a glutamic acid residue which becomes covalently modified during inactivation. They also determined the amino acid sequence of a peptide containing this glutamic acid. This residue most probably corresponds functionally to that identified by Fendrich and Abeles (12) at the active center of a bacterial short chain acyl-CoA dehydrogenase.

In this report, we describe a plasmid which directs synthesis of mature active human MCADH in *Escherichia coli*. Further, we also analyzed expression of a plasmid containing a mutation at this crucial glutamic acid which did not produce active enzyme. We describe the purification and selected properties of both these enzymes.

MATERIALS AND METHODS

Recombinant DNA procedures (13, 14), immunoblotting (15), and growth and maintenance of bacteria were performed adapting standard procedures. The pMON2665 plasmid construct was a gift from the Monsanto Co. This construct is a derivation of pBR327 (16) and contains the recA promoter (17) followed by the T7 bacteriophage gene 10-leader sequence which was inserted via *Bgl*II and *Nde*I restriction sites. The protocol for enzyme purification was an adaptation of that published by Thorpe *et al.* (18). Extracts were prepared by disruption of cells with a French press followed by ammonium sulfate precipitation (80%) and Sephadex G-25 chromatography. The activity of the extracts and the purified enzymes were determined using 2,6-dichlorophenolindophenol as acceptor, phenazine methosulfate as mediator, and octanoyl-CoA as substrate (19). Methylenecyclopropyl acetyl-CoA (MCPA-CoA), an irreversible inhibitor of acyl-CoA dehydrogenase, has been described (19). Amino acid sequencing of the NH_2 terminus was performed using an Applied Biosystems 477A protein sequenator.

RESULTS AND DISCUSSION

Cloning of the Mature Form of Human MCADH—Plasmid p9 contains the cDNA for human MCADH (4) in pGEM-3Z

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[1] The abbreviations used are: MCADH, medium chain acyl-CoA dehydrogenase; MCPA-CoA, methylenecyclopropyl acetyl-CoA; bp, base pair(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[2] E. Pai, personal communication.

[3] A. W. Strauss, D. P. Kelly, J.-J. P. Kim, and R. A. Alpers, unpublished data.

(Promega Biotec) assembled from two overlapping cDNA clones from a human placenta library (m-2) and a human liver library (m-3). Plasmid p9 was constructed by joining the two cDNA clones via an internal *Eco*RI site at bp 706 of the MCADH cDNA. p9 contains the entire 1263-bp coding region of the MCADH cDNA and 18 and 626 bp of the 5′- and 3′-nontranslated regions, respectively (Fig. 1, *left panel*). Plasmid p9 was used as template for the polymerase chain reaction. Two primers were employed, a 21-mer complementary to the sense strand at a position just 3′ to the stop codon (TAA) and a mutagenic sense strand primer (5′-TGGAGCTCA-CAGCATATGAAAGCCAAT-3′) which introduces a *Nde*I site containing a start codon (ATG) directly 5′ of the putative first codon (AAA) of the mature protein. Thus, the product of the polymerase chain reaction would contain the entire coding sequence of mature MCADH without the transit peptide. After polymerase chain reaction, the newly synthesized DNA was treated with *NdeI and Eco*RI (*Eco*RI cuts at nucleotide position 706, within the coding region), and the 617-bp fragment containing the 5′ portion of the coding sequence was purified. The fragment was introduced into the expression vector pMON2665 and will be referred to as plasmid pWTMCAD-1. It encodes a truncated version of mature MCADH with the NH_2-terminal 210 amino acids.

Subsequently, a cDNA encoding full-length mature MCADH was created in the expression vector in the following way (Fig. 1, *right panel*). Plasmid pWTMCAD-1 was digested with *Eco*RI, and the kanamycin cartridge of pUC4k (20) was introduced as an *Eco*RI fragment. A clone with the resistance gene in the same orientation as the MCADH segment was selected and digested with *Bcl*I (which cuts at position 432 of MCADH cDNA) and *Hin*dIII (which cuts in the middle of the kanamycin cartridge), and the small fragment was replaced with the *Bcl*I/*Hin*dIII fragment of p9 which encodes the carboxyl-terminal portion of MCADH. This plasmid, designated pWTMCAD-2, carries DNA encoding the mature form of MCADH preceded by an initiator codon (ATG) under control of the inducible recA promoter.

Construction of Active Site Mutants—The cDNA insert of the full-length MCADH-containing plasmid, p9, was introduced as a *Sal*I/*Hin*dIII fragment into M13mp18. This construct was used to prepare single-stranded DNA for oligonucleotide-directed mutagenesis. Two oligonucleotides were employed for mutagenesis (Fig. 2). The first created an A to G transition at position 1185, generating a *Bgl*II site without causing a change in the amino acid coding sequence. The second oligonucleotide created both a G to C transversion at position 1201, causing a change of the codon for glutamic acid to one encoding glutamine, and a silent T to C transition at position 1209, introducing a *Kpn*I site. Both the single and the double mutants were constructed. After successful mutagenesis, the segment containing the mutations was spliced as a *Bcl*I/*Hin*dIII fragment into pWTMCAD-2 (Fig. 2). The plasmid with the *Kpn*I/Gln mutations was designated pGLN-1; that with the *Bgl*II mutation, pWTMCAD-Bgl; and that with both mutations, pGLN-2. Plasmid pGLN-2 will be employed for cassette mutagenesis of the active site, because the small fragment coding for amino acids 372–376 can be excised as a *Bgl*II/*Kpn*I fragment and exchanged by suitable synthetic complementary mutant oligonucleotides.

MCADH Expression in Bacteria—The plasmids were transformed into *E. coli* strain TG1, the bacteria were grown to a cell density of approximately 1×10^9 cells/ml, and plasmid expression was induced with nalidixic acid. Samples were taken without induction and 16 h after induction. Aliquots were subjected to SDS-PAGE followed by immunoblotting using anti-porcine kidney MCADH antibodies. A representative blot is shown in Fig. 3*A*. Analysis of total protein (*lane 2, i*) from cells containing pWTMCAD-1, encoding truncated MCADH, reveals an immunoreactive band with an estimated molecular weight of 23,000, demonstrating synthesis of the appropriately sized protein product. In *lanes 2–4, i*, proteins present after induced expression of pWTMCAD-2, pWTMCAD-Bgl, and pGln-2 were analyzed. MCADH immunoreactive bands with exactly the same electrophoretic mobility as purified pig kidney MCADH (*lane 1*) are present (Fig. 3*A*). In cells not induced with nalidixic acid (*lanes 2–4, u*), no immunoreactive MCADH antigen is detected. Therefore, as expected, the wild-type (pWTMCAD-2) and MCADH active site mutant plasmids express stable immunodetectable MCADH of the same subunit size as the native enzyme purified from tissue.

Activity Measurements in Cell Extracts—Transformed cells were grown and, following induction with nalidixic acid, harvested, disrupted, and processed as described under "Materials

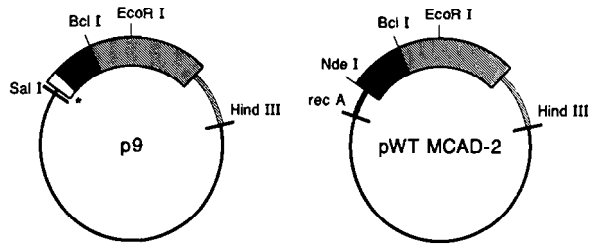

FIG. 1. **Creation of the wild-type MCADH expression vector.** The *left panel* shows p9 plasmid, which contains the entire MCADH cDNA coding sequence and portions of the 3′- and 5′-untranslated region as described in the text. The MCADH sequence begins after the *Sal*I restriction site and ends at the *Hin*dIII site. The *large boxed region* corresponds to the coding region. The *white portion* of the coding region indicates the transit peptide. The *asterisk* denotes the original start codon. The *right panel* demonstrates the pWTMCAD-2 construct which contains the mature MCADH wild-type cDNA coding region (*large box*) and 626 bp of the 3′ untranslated region ending at the *Hin*dIII site. The *black portion* of the coding region is retained in all subsequent constructs, and the *stippled portion* (*Bcl*I-*Hin*dIII fragment) is replaced in the mutant constructs shown in Fig. 2. The MCADH coding sequence is downstream from the inducible rec A promoter as indicated.

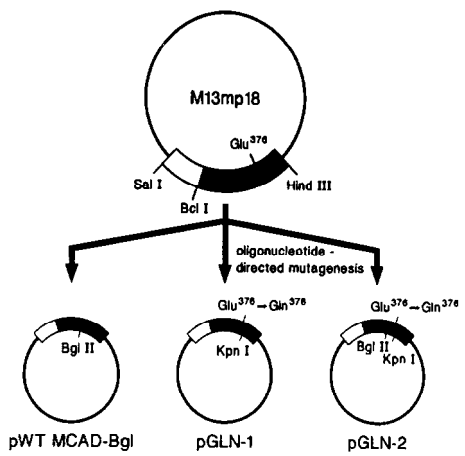

FIG. 2. **Strategy used for construction of MCADH expression vectors containing the $Glu^{376} \rightarrow Gln^{376}$ mutation.** The three constructs shown in the lower portion of this figure are the products of the oligonucleotide-directed mutagenesis experiments performed in the bacteriophage vector M13. After the mutants were created, they were placed into pWTMCAD-2 (see Fig. 1) as *Bcl*I/*Hin*dIII fragments (the *black boxes*). The newly created restriction sites and $Glu^{376} \rightarrow Gln^{376}$ mutation are labeled.

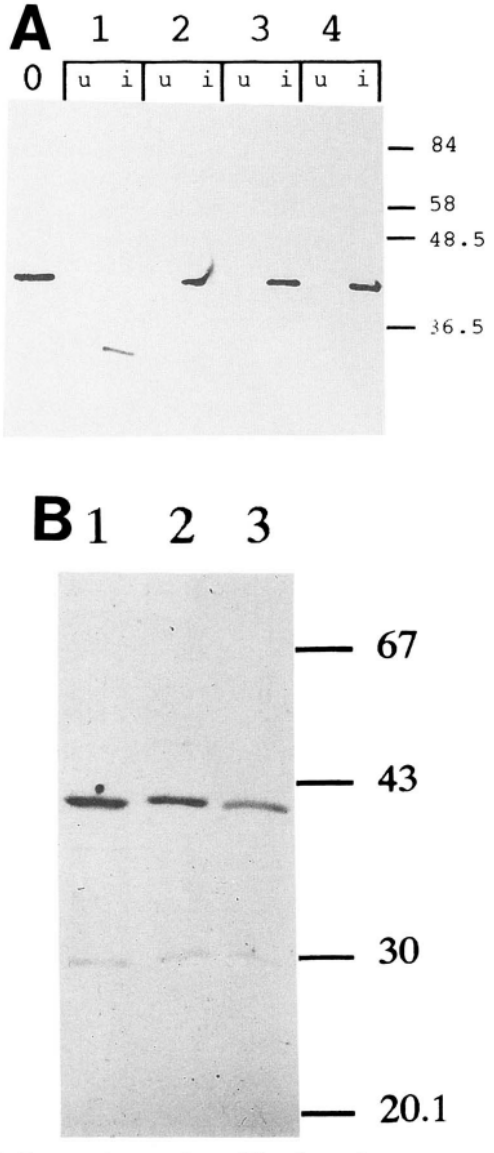

FIG. 3. **Expression and purification of mutant and wild-type MCADH in *E. coli*.** *Panel A* is an immunoblot of proteins derived from whole cells transformed with several plasmids. Aliquots (about 5×10^6 cells/*lane*) of the cultures before (*u*) and 16 h after induction (*i*) with nalidixic acid were loaded on SDS-PAGE. For detection after transfer, anti-pig kidney MCADH antibodies and phosphatase-conjugated goat anti-rabbit secondary antibodies were employed. The *lanes* contain the following: *0*, purified pig kidney MCADH; *1*, TG1/pWTMCAD-1; *2*, TG1/pWTMCAD-2; *3*, TG1/pWTMCAD-Bgl; *4*, TG1/pGLN-2. The position and molecular mass (in kDa) of coelectrophoresed marker proteins are indicated on the *right margin*. *Panel B* is the analysis of purified MCADHs on SDS-PAGE. MCADH was purified (18) from *E. coli* cells transformed with plasmid pWTMCAD-2 (*lane 2*) and pGLN-2 (*lane 3*) and subjected to SDS-PAGE and stained with Coomassie Blue. The major band comigrates with purified pig kidney enzyme (*lane 1*).

and Methods." MCADH activity of the crude extracts was measured (Table I) using the standard coupled assay (18), which requires phenazine methosulfate and 2,6-dichlorophenolindophenol as electron acceptors. The extracts of cells transformed with pWTMCAD-2 (encoding mature wild-type MCADH) and pWTMCAD-Bgl (with the silent mutation creating a *Bgl*II site) display MCADH activity. To prove that the measured activity was due to MCADH, extracts were incubated with MCPA-CoA, a specific and irreversible inhibitor of this enzyme (19). Upon this treatment, all activity was

TABLE I

MCADH specific activity in extracts from cells transformed with the wild-type and mutant plasmids

Extracts were prepared and assayed as described under "Materials and Methods." Activity was determined in the untreated extracts after addition of MCPA-CoA (final concentration 1.74×10^{-5} M) or after treatment with octanoyl-CoA (final concentration 1×10^{-3} M) prior to addition of MCPA-CoA. The values are given in units per mg of protein. ND, not detectable.

Treatment	pWTMCAD-2	pWTMCAD-Bgl	pGLN-2	pWTMCAD-1
None	0.023	0.012	ND	ND
+MCPA-CoA	ND	ND		
+Octanoyl-CoA + MCPA-CoA	0.021	0.011		

suppressed (Table I). Also shown in Table I, the inhibitory effect of MCPA-CoA could be strongly suppressed by adding excess octanoyl-CoA prior to incubation with MCPA-CoA. Thus, expression of mature MCADH in *E. coli* results in active enzyme.

In contrast, extracts of cells containing the plasmid with an insert which encodes a truncated protein (pWTMCAD-1) and the plasmid with the $Glu^{376} \rightarrow Gln$ mutation (pGLN-2) had no detectable MCADH activity (Table I), in spite of the fact (see Fig. 3 above) that similar amounts of immunoreactive antigen were present in these extracts. The lack of activity of the truncated MCADH protein is consistent with an important role of the COOH-terminal region in catalysis. Similarly, the absence of activity of the $Glu^{376} \rightarrow Gln$ mutant protein strongly suggests that this glutamic acid residue is crucial for proper catalytic activity, as suggested previously (11). However, because indirect effects of either mutation on conformation (with alteration of the active site secondarily) are also possible, we elected to express sufficient amounts of the mutant enzyme for further kinetic and structural studies.

Purification and Selected Properties of the Enzymes—The active mature MCADH and the Gln^{376} mutant protein were isolated from large scale cultures (40 liters) of bacteria transformed with plasmids and induced with nalidixic acid, using an adaptation of the procedure published for the purification of the pig kidney enzyme (18). SDS-PAGE of the purified proteins (Fig. 3*B*) demonstrates purity (>95%) and subunit molecular weights indistinguishable from pig kidney MCADH. In a typical experiment, we obtained 0.9 mg of pure enzyme per liter of cell culture. The specific activity of the purified active MCADH is 1.36 units/mg enzyme, which is 53% of that observed with highly purified pig kidney MCADH in our laboratory. This is clearly within the range of pure MCADH from different sources (21, 22).

The UV-visible absorption spectrum of the active wild-type enzyme (Fig. 4) is similar, although not identical, in extinction coefficient, maxima, and general shape to that of the pig kidney MCADH, suggesting a similar environment of the flavin chromophore.

Interestingly, the purified Gln^{376} mutant is a green protein. This color has been reported with flavin enzymes, and the mechanisms has been a subject of debate (23). To demonstrate that the green enzyme was the expected mutant, NH_2-terminal protein sequence analysis was performed. The chemically derived sequence of the NH_2-terminal 25 residues is identical to that predicted by the plasmid cDNA (4). The green color is due to a long wavelength band extending into the infrared, which is probably of charge transfer origin. The maxima at 675 and 437 nm, general shape, and extinction coefficients of this spectrum (Fig. 4) are similar to the green enzyme described by Williamson *et al.* (24), who demonstrated that the effect was due to tightly bound CoA persulfide. We performed

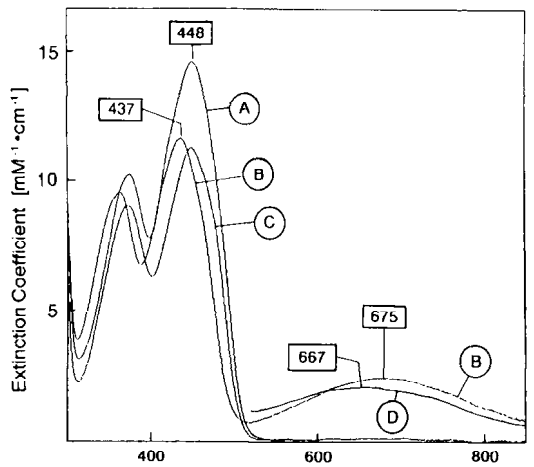

FIG. 4. **Absorption spectra of human MCADH and of the Gln^{376} mutant expressed in *E. coli*.** *Curve A* is the analysis of active human MCADH, approximately 1.25×10^{-5} M in 20 mM sodium phosphate buffer, pH 8.0. *Curve B* is the spectrum of purified Gln^{376} mutant MCADH protein in the same buffer. *Curve D* is the spectrum of the Gln^{376} mutant protein 10 min after addition of a 33-fold excess of octanoyl-CoA (spectrum corrected for dilution). The shape of the spectrum below 500 nm (not shown) is similar to that of *curve B*, but the extinction at 437 nm is approximately 8% lower. *Curve C* is the analysis of purified Gln^{376} mutant protein recorded 40 min after addition of 0.6% SDS (final concentration). This spectrum is identical to that of normal FAD under the same conditions.

a similar analysis of the green Gln^{376} mutant. Addition of dithionite to a solution of the purified mutant enzyme and subsequent dialysis caused loss of the green color and yielded a yellow protein with a spectrum very similar to that of wild type MCADH (*cf. curve A*, Fig. 4). The green color was restored quantitatively to this degreened mutant MCADH by addition of excess CoA-SH and Na_2S in the presence of oxygen, a treatment reported to regenerate CoA persulfide *in situ* (24). These results are consistent with the presence of tightly bound CoA persulfide in our purified mutant MCADH. Comparison of the absorption coefficients (Fig. 4) to the values reported by Williamson *et al.* (24) shows that the content of CoA persulfide in the green mutant MCADH is greater than 90%, implying a very tight binding. In agreement with this finding, the degreened Gln^{376} mutant protein (after oxidation) binds acetoacetyl-CoA approximately an order of magnitude more tightly (K_d about 10^{-6} M) than purified pig kidney MCADH ($K_d = 1.2 \times 10^{-5}$ M) (3). These results and the observation that CoA persulfide ligand is retained during purification of the mutant enzyme imply that residue 376 specifically participates in substrate binding.

Further support for this interpretation was obtained by studies of substrate (octanoyl-CoA) binding to the mutant enzyme. After binding of octanoyl-CoA to the degreened and oxidized mutant MCADH, a blue shift of the 448-nm band is observed, similar to the shift which occurs after substrate binding of native pig kidney MCADH (22). This is typical for formation of the enzyme-substrate Michaelis complex. In the presence of a 5-fold molar excess of octanoyl-CoA at 25 °C under anaerobic conditions, a slow multiphasic bleaching (reduction) of the oxidized mutant enzyme ensues. The first phase comprises about 40% of the total and has a half-time of 5 min. Complete reduction is attained after 24 h of incubation. The rate of the first rapid phase of bleaching, which is assumed to reflect the step of substrate α-proton abstraction, is less than 1/5000th that observed with pig kidney MCADH under anaerobic conditions (22).

These results prove that amino acid 376 plays an important role in the initial step of dehydrogenation catalysis. This residue may be the protein-abstracting base itself, although Glu^{376} is not conserved in all acyl-CoA dehydrogenases (6). Alternatively, this residue may be involved in the charge relay system or the activation of the CoA thioesters via an interaction with the —C=O–S carbonyl.

The work described here is an important initial step in the study of the structure and function relationships of a variety of enzymes including the acyl-CoA dehydrogenase family and all proteins which interact with a fatty acyl substrate. It confirms the important role of Glu^{376} in the catalytic function of MCADH. The expression system will allow enzymological and crystallographic analysis of MCADHs containing a variety of mutations, including those which occur naturally in patients with inherited MCADH deficiency. In addition to the study of the active site, this approach will be useful in the study of the important determinants of flavin binding, CoA binding, and interaction with electron transfer proteins.

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