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BIOCHEMICAL CHARACTERIZATION OF A MUTANT HUMAN MEDIUM-CHAIN ACYL-COA DEHYDROGENASE PRESENT IN PATIENTS HAVING DEFICIENT ACTIVITY.

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Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a relatively common inherited metabolic defect causing liver dysfunction and hypoglycaemic coma and accounting for some of the cases of sudden death in infants [1,2]. The mutation responsible for MCAD deficiency in five families is a point mutation causing a lysine to glutamic acid change at position 304 of the mature protein. According to the three-dimensional structure, Lys-304 is not part of the active site but is part of the helix which forms the interface between the subunits [3]. In this report we demonstrate expression of mutant MCAD-glu-304 in *E.coli* and biochemical characterization of the defective enzyme in comparison with wild-type MCAD.

Results

Construction of plasmids: We have described the construction of a plasmid (pWTMCAD-2), which directs expression of mature human MCAD in *E.coli* [4]. A derivative of pWTMCAD-2, pBMCK2-, which expresses mature active human MCAD under control of the lac promoter, was constructed (fig.1). The EcoRI/BamHI fragment of pBMCK2- carrying the information for the C-terminal half of MCAD was replaced with an EcoRI/BamHI fragment originating from a PCR clone with the glu-304 mutation (fig.1). The plasmid encompassing the sequence of MCAD-glu-304 was called

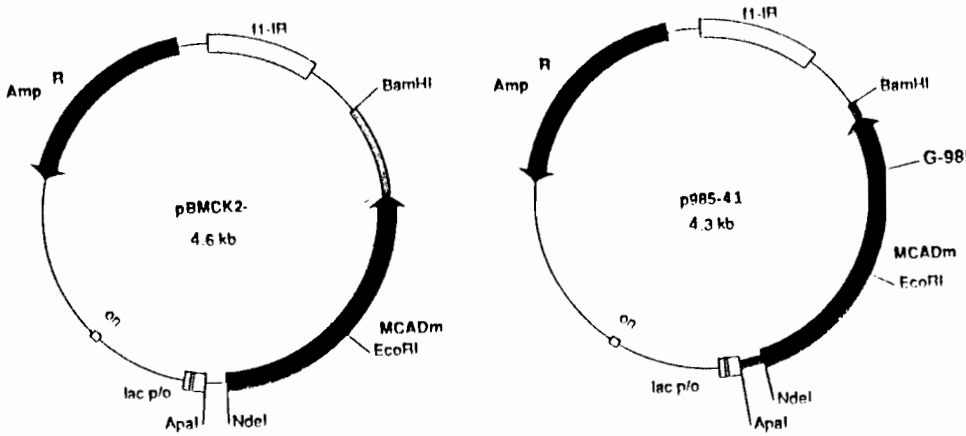

Figure 1: Maps of MCAD wild-type and mutant expression plasmids. Relevant restriction sites, location and orientation of the genes and the position of the A to G transition causing a lys to glu codon change at amino acid position 304 of the mature protein are indicated.

p985-41. In order to ensure that no PCR errors are contained, the whole EcoRI/BamHI fragment was sequenced and found to be identical to the wild-type sequence with the exception of the lys to glu codon change.

Expression of wild-type and glu-304 mutant MCAD in E.coli: Both plasmids, pBMCK2- (wild-type) and p985-41 (glu-304 mutant), express correctly-sized, immunoreactive MCAD protein (fig.2). In comparison between wild-type and lys-304, cells disrupted by boiling in electrophoresis sample buffer display additional and more pronounced degradation bands for the mutant. In extracts prepared by lysozyme disruption of the cells, the band for the mutant protein is much weaker than that of the wild-type. This indicates that after disruption the mutant protein is more susceptible to proteolytic degradation than the wild-type.

Activity in extracts: Extracts of cells expressing wild-type or glu-304 MCAD respectively were assayed for enzyme activity. Extracts were prepared by lysozyme disruption followed by NH_4-sulfate precipitation (20-70%) and activity was measured as described by Thorpe *et al* (5) In this assay, extracts from cells expressing wild-type MCAD display activities in the range of 20-25 mu/mg soluble protein. No activity could be detected in extracts from cells expressing MCAD-glu-304. Due to the high background in the extract the assay would be sensitive enough to detect >5% of the activity measured for the wild-type extract.

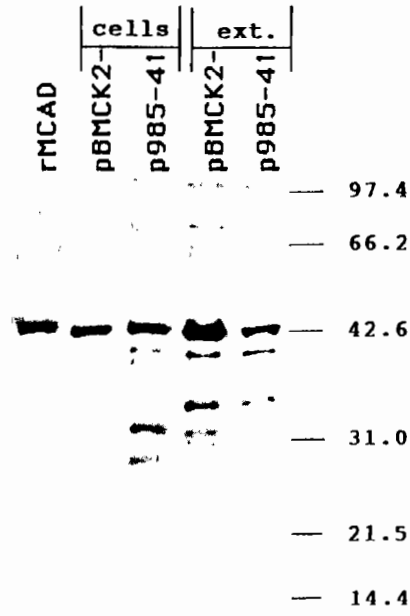

Figure 2: Western blot of E.coli JM109 transformed with pBMCK2⁻ or p985-41 as indicated. Cells harvested and disrupted by boiling with sample buffer (cells) or extracts prepared by sonification followed by ammonium sulfate precipitation of the supernatant (ext.) were loaded. rMCAD: recombinant human MCAD protein purified from induced cells transformed with pWTMCAD-2. The position and molecular mass of co-electrophoresed marker proteins is indicated on the right margin.

Purification and characterization of glu-304 mutant MCAD: Wild-type and glu-304 MCAD have been partly purified from extracts of induced cells transformed with pBMCK2⁻ or p985-41 respectively. Soluble proteins were precipitated with NH_4-sulfate (20-70% saturation). The pellet was dialysed and chromatographed on a hydroxylapatite column

(Merck; linear gradient 10-400mM K-phosphate). Fractions containing MCAD protein were identified by immunoblotting. Part of MCAD-glu-304 eluted at the same position in the gradient as purified recombinant human MCAD. However, the yield was dramatically lower compared to wild-type MCAD purified from induced cells. From the intensity of the band in SDS-PAGE we roughly estimated that MCAD-glu-304 constitutes only about 5% of the protein contained in the peak fraction from the hydroxylapatite column. This means that the quantity of MCAD-glu-304 purified from 0.5 l culture (ca 100mg soluble protein) is 1-5µg (<0.01% of soluble protein). The yield for wild-type MCAD in the same purification scheme is approximately 50-150µg (about 0.5-1%). Furthermore, there are two peaks eluting at lower salt concentration, which also contain correctly sized immunoreactive MCAD-glu-304. These fractions also comprise very low amounts of enzyme.

MCAD activity in the fractions containing MCAD-glu-304 detected by western blotting was measured as described above. No MCAD activity was found. From the estimation of the amount of MCAD-glu-304 present in the assay, it should be sufficient to warrant detection of activity. This indicates that the mutant protein is inactive or at least has decreased activity.

Conclusions

Our results strongly suggest that the lys to glu mutation in MCAD found in patients with MCAD deficiency renders the protein highly susceptible toward proteolytic degradation. This is probably the reason for lack of activity. Whether the mutant protein has some intrinsic activity cannot be conclusively excluded since the amount of purified enzyme is very low and estimation of the concentration is not sufficiently reliable with our assay system. The fact that MCAD-glu-304 elutes from the hydroxylapatite column at various salt concentrations may imply that different forms of the protein are present. Whether this reflects different tertiary and/or quarternary structure remains to be determined.

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