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Purification and Partial Characterization of Human Isobutyryl-CoA Dehydrogenase: A Novel Member of the Acyl-CoA Dehydrogenases Family.

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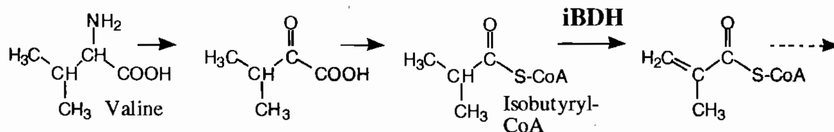
Introduction

Acyl-CoA dehydrogenases (ACDs) are a family of mitochondrial enzymes that catalyze the first dehydrogenation step in the β-oxidation of fatty acyl-CoA derivatives (1). Seven human ACDs have already been described, including four involved in the initial step of mitochondrial β-oxidation of straight chain fatty acids (SCAD, MCAD, LCAD and VLCAD) and three involved in the degradation of amino acids (IVD, SBCAD and GCD). All ACDs catalyze the same initial dehydrogenation of the substrate at the □,β-position and require electron transfer flavoprotein (ETF) as electron acceptor (2). With the exception of VLCAD, the ACDs are found in homotetrameric form with one molecule of flavin adenine dinucleotide (FAD) non-covalently bound to each monomer (3). A cDNA encoding the precursor for the new eighth member of this family, ACAD-8, was originally isolated by RT-PCR on RNA from human adult brain and skin fibroblasts and was characterized (4). Based on sequence homologies it was proposed that ACAD-8 codes for a new enzyme of the ACD family (4). Based on the occurrence of specific genetic defects (6), and preliminary activity profiles, it was deduced that the protein is an isobutyryl-CoA dehydrogenase (hiBDH). Earlier reports (5) assigned this activity to "2methyl-branched chain butyryl-CoA dehydrogenase". However, latter studies indicated that the enzyme is involved in valine metabolism only (6,7). The proposed role of hiBDH is shown in Scheme 1.In this report we describe the purification and a partial characterization of this novel enzyme.



Scheme 1. Proposed role of hiBDH in valine metabolism.

Material and Methods

Enzyme expression and purification.

The wild type hiBDH gene coding for the mature form of the enzyme was obtained by PCR of cDNA obtained from human fibroblast cells using two primers that included *Nde* I and *Hin*d III restriction site sequences at the 5′and 3′ ends, respectively. The PCR fragment was inserted into pET21(a) vector multi cloning site using *Nde* I and *Hin*d III sites. The gene was sequenced to confirm absence of PCR errors. Following overnight growth of BL21(DE3) (Novagen) cells containing the expression plasmid, cells were induced for 4 hours with 0.5 mM IPTG. During first purification attempts it was observed that hiBDH activity decreases dramatically after each step resulting in rather poor yields of hiBDH. To improve expression in *E. coli*, the expression plasmid was introduce into RosettaBlue(DE3) (Novagen), which harbor rare tRNAs. This lead to an improvement in expression, but not to levels sufficient for an efficient purification. The co-expression with chaperonins GroEL/GroES lead to a substantially higher production of active hiBDH suggesting that folding problems are very important. Upon protein production the cells were collected by centrifugation, and the enzyme was extracted in buffer A (25 mM potassium phosphate (KPi), 1 mM EDTA, pH 7.8, 5% Glycerol) and cleared by centrifugation at 18,000 rpm for 30 min and at 55,000 rpm for 60 min. The protein was precipitated using polyethyleneimine (8), redissolved in buffer A and loaded on a DEAE column (Pharmacia, 35 ml void volume) equilibrated with the same buffer. The column was washed with 150 ml of buffer A, and the enzyme was eluted with a 550 ml gradient from 0-40% of buffer B (buffer A + 1 M KCl). The fractions with highest hiBDH activity, eluting at approx. 20-25% of buffer B, were pooled and concentrated by Ultrafiltration (Amicon 30 KD). Salt was removed using Sephadex G15 filtration and the protein precipitated with ammonium sulfate (45 and 65% cut-off). The 65% pellet was dissolved in 10 mM KPi, pH 7.5, desalted by Sephadex G15 filtration and loaded on a ceramic hydroxylapatite CHI10 column. After washing-off unbound proteins with 150 ml buffer A (10mM KPi; pH 7.5, 5% Glycerol), 550 ml of a gradient (0-16.5%) of buffer B (0.5M KPi; pH 7.5, 5% Glycerol) was applied. Upon application of buffer C (buffer A + 20 µM FAD) with 16.5% buffer B the enzyme elutes in a volume of approximately 150 ml. Gel filtration was used to purify the protein further as needed.

Results

The purified enzyme was assayed by SDS-PAGE gel and the best fractions were found to be > 95% pure (Fig. 1). The enzyme appears to be rather unstable and significant activity is lost during the purification and ammonium sulfate precipitation steps. One property hampering purification is the propensity of hiBDH to release the FAD cofactor during purification. Thus, chromatography steps were carried out in the presence of FAD at concentrations around μM. This was found to have a positive effect on stability and yields. Further steps aiming at obtaining homogeneous protein are currently being pursued.

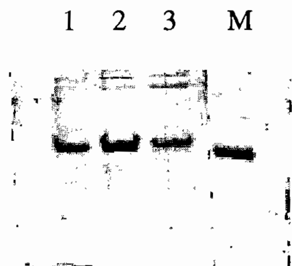


Figure 1. SDS PAGE gel of hiBDH. Three main fractions (1,2,3) from the last purification step (hydroxy apatite column) are shown. M: MCAD (MW 45000 D) purified protein as a marker.

The pI for hiBDH is ≈ 6.4 as determined by 2D gel electrophoresis. After the 2D gel run, the protein was blotted to a membrane and detected using ant-His_6-hiBDH antibodies kindly provided by Dr P. Bross, Univ. of Århus, DK. There is partial cross-reactivity of ant-His_6-hiBDH with MCADH. This is not surprising in view of the rather high sequence coincidence of hiBDH and MCADH that amounts to 34% (4). A study of the relative activity of hiBDH with substrates having different acyl residues indicates that it is comparatively very specific for isobutyryl-CoA (Table 1).

Table 1. Dependence of the activity of human isobutyryl-CoA dehydrogenase from the chemical constitution of the acyl-CoA substrate. The activity is related to that of isobutyryl-CoA, the best substrate.

R (in R-CoA)	Relative activity
isobutyryl	100
propionyl	40
2-methylbutyryl	10
n-butyryl	0
isovaleryl	0

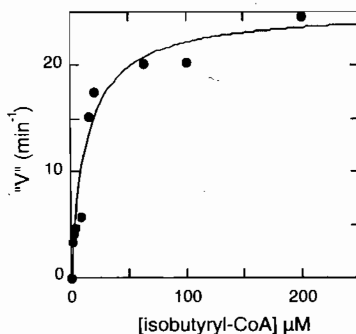


Figure. 2. Dependence of the hiBDH Activity from the Isobutyryl-CoA concentration.

Table 2. Dependence of human isobutyryl-CoA dehydrogenase activity from the temperature and incubation time.

Temp. (°C)	% activity after 3 h	% activity after 16 h
0	99	85
28	98	85
37	95	77

Conclusions

We have purified the hiBDH enzyme and partially characterized some of its properties. One salient feature is the high specificity for isobutyryl-CoA, and a very low, or absent activity with acyl-CoA substrates of similar molecular mass but different stereochemistry such as n-butyryl-CoA. In comparison to other members of ACD family the enzyme appears to be much less stable. This is due, at least in part, to its tendency to loose its cofactor FAD. This has lead to some difficulties during its purification and characterization. Work is underway to complete the characterization of the enzyme and compare its properties to those of other ACDs enzymes in more detail and also to investigate the reasons for its lability.

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