Biochemical Studies on Recombinant Human Isobutyryl-CoA Dehydrogenase

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Abstract: Problem statement: Human Isobutyryl-CoA Dehydrogenase (IBD) is member of the Acyl-CoA Dehydrogenases family. It is involved in Val metabolism. In this study we modified the purification method of the IBD and further spectroscopic characterization was conducted. **Approach:** Using DTT in the buffers during the purification, IBD purified to homogeneity by different chromatographic steps. Reactivity of Cys residues were measured using thiol modifying agent DNTB. **Results:** IBD found to be homotetramer with 42.7 KDa for each subunit and the value of its pI was 6.2. **Conclusion:** Thiol modifying reagent showed crucial role (s) of some Cys residues for IBD structure, FAD binding and/or activity.

Key words: Acyl-CoA dehydrogenase, isobutyryl-CoA dehydrogenase, valine degradation, Sudden Infant Death Syndrome (SIDS), cys residues, recombinant human, ferricenium assay, jamaican vomiting, thiol modifying

INTRODUCTION

Acyl-CoA Dehydrogenases (ACADs) form a family of ten members that catalyze the a, ®-desaturation of acyl-CoA thioesters to corresponding trans-2-enoyl-CoA. They are assumed to share the same chemical mechanism, however, they differ in their specificity for different types of fatty acids linked to CoA (Ye *et al.*, 2004). This family can be differentiated into two subclasses. First subclass comprises five members Very long-chain acyl-CoA dehydrogenase1and 2 (VLCAD1, VLCAD2), Long-chain acyl-CoA dehydrogenase (LCAD), Medium-chain acyl-CoA dehydrogenase (MCAD) and Short-chain acyl-CoA dehydrogenase (SCAD) that utilizing ''straight chain'' substrates, that in turn, enable the sequential degradation of the substrates in ß-oxidation cycle (Ye *et al.*, 2004). The second subclass encompasses the enzymes involved in degradation of "branched chain" acyl-CoA thioesters arising from amino acid catabolism. These include Isovaleryl-CoA dehydrogenase (IVD), Short-Branched Chain-CoA Dehydrogenase (SBCAD), Glutaryl-CoA Dehydrogenase (GCAD) and a newer member Isobutyryl-CoA dehydrogenase or IBD. With the exception of VLCAD1 (VLCAD2 not characterized yet) that exists as dimer, the ACADs are found in homotetrameric form with one molecule of Flavin Adenine Dinucleotide (FAD) non-covalently bound to each monomer (Izai *et al.*, 1992). The tenth members ACAD10 is not characterized yet (Ye *et al.*, 2004) (Ye *et al.*, 2004).

Metabolic disorders due to inherited genetic mutations in ACAD genes have two pathological consequences. One is the result of energy deficiency and shortage of substrates for gluconeogensis (generation of accessible glucose supply) due to reduction of the flux through the fatty acid oxidation pathway. The second is the toxic effect of intermediate metabolites that accumulate due to enzyme deficiency. Effects of the latter are the most serious and can cause serious neurological damage. The net result is crisis situations in metabolic decompensation and clinical disease, which may be fatal especially in infants. That in some cases leads to the Sudden Infant Death Syndrome (SIDS) (Eminoglu *et al.*, 2011).

cDNA exhibiting sequence homology with ACAD family was initially isolated from human adult brain and skin fibroblasts as a precursor and was designated as ACAD8 (Telford *et al.*, 1999).

Preliminary activity profiles obtained with heterologously expressed enzyme suggested that the protein was involved in the catabolism of branched chain amino acids which was with the occurrence of specific genetic defects and their physiological consequences (Roe *et al.*, 1998; Sass *et al.*, 2004). It

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was thus deduced that the enzyme was involved in Val catabolism and specifically in the dehydrogenation of isobutyryl-CoA. Accordingly the enzyme was named isobutyryl-CoA dehydrogenase (IBD) (Sass *et al.*, 2004). Recently, different inherited mutations of ACAD8 have been detected and follow-up algorithm diagnosis of IBD deficiency has been reported (Oglesbee *et al.*, 2007). Earlier studies had assigned isobutyryl-CoA degradation activity to SBCAD, as in case of rat SBCAD which can utilize both 2-methyl-butyryl-CoA and isobutyryl-CoA that emerged from Ile and Val degradation, respectively (Willard *et al.*, 1996; Battaile *et al.*, 2004).

IBD crystallographic structure data indicated that Glu-376 is the catalytic base and that the substrate-binding cavity is shorter and wider relative to SCAD, which allows the optimal binding of the isobutyryl-CoA substrate. Another difference between IBD structure and other ACAD is that the conserved Tyr or Phe that defined side of the binding cavity is replaced by Leu residue (Leu-375). In comparison with IVD, the lateral expansion of the binding cavity is not observed in IBD (Battaile *et al.*, 2004). Here, we describe modified purification method for recombinant human IBD and studies on its biochemical characters.

MATERIALS AND METHODS

Q-Sepharose FF and Superdex 200 prep grade HiLoad 26/60 (Amersham Biosciences, GE). Hydroxyapatite CHT10-I Ceramic (Bio-Rad). Hydroxyapatite (Fluka). Ferricenium hexafluorophosphate (Aldrich). Cleland´s reagent (DTT), Ellman's reagent and all other chemicals are from Sigma.

Expression and purification of recombinant human IBD: Recombinant IBD was expressed and purified as previously mentioned (Willard *et al.*, 1996) with some modifications. The modification includes using of 5 ⌈M DTT in all buffers, using ammonium sulfate precipitations and two hydroxyapatite columns following the Q-Sepharose one.

Molecular weight determination: IBD molecular weight was determined by MALDI-TOF (Bruker Biflex time-of- flight mass spectrometer, Bruker Daltonik, Bremen, Germany) equipped with a 26-sample SCOUT source, a nitrogen UV laser (⌊$_{max}$ = 337 nm) and a delayed extraction system. A concentrated sample of IBD, 1 µl ($\approx$ 7 mg mL^{-1}), was mixed with 1 µL matrix solution (super saturated a-cyano-4-hydroxycinnamic acid in acetonitrile: 0.1% trifluoroacetic acid in water, 2: 1) on the MALDI-TOF target and allowed to dry at room temperature (Mann and Talbo, 1996). IBD native molecular weight was estimated by gel filtration (Superdex 200, 25 mM KPi, pH 7.8, 150 mM KCl, 5% Glycerol, 5 ⌈M DTT).

Isoelectric focusing: ProteoGel IPG strips (7 cm, Sigma) with pH range 5-8 and IPGphor IEF system (Amersham Biosciences, GE) were used. A lyophilized sample, from 150-250 µL of 4 µM IBD was re-dissolved in rehydration buffer (2D-Manual, Amersham Biosciences, GE) and used for rehydration of the strip for 15 h. After IEF, the strip was equilibrated in the equilibration buffer containing DTT for 1 h. and then applied to an SDS-PAGE gel. IBD was detected by chemiluminescence using antibody specific for His-tagged IBD kindly provided by Dr. P. Bross (Aarhus, Denmark).

Spectroscopic methods: Visible and ultraviolet spectra were recorded with a Kontron Uvikon 930 spectrophotometer. Anaerobic reactions were followed in Thunberg type cuvettes equipped with two side arms. Oxygen depletion was achieved by at least four flushing and evacuation cycles using O$_2$-free argon in the presence of an oxygen scavenging system (0.1 µM glucose oxidase, 0.1 M glucose and 0.1 µM catalase enzyme) in a sidearm of the cuvette. Rapid reaction studies were conducted with a stopped-flow spectrophotometer with 1.0 cm path length cell and a diode array detector (J and M Aalen), using BioKine. The fastest acquisition time of 1 spectrum/0.7 ms in the range of 300-650 nm. Unless otherwise stated all spectrophotometer measurements were carried out at 25°C in 40 mM potassium phosphate buffer, pH 7.8, containing 5% glycerol. Acyl-CoA solutions were standardized by spectrophotometer in10 mM KPi, pH 6.0 using $\sum_{260}$ = 15.4 mM^{-1} cm^{-1}. Absorption coefficients for IBD were determined using the SDS method and $\sum_{450}$ = 11.3 mM^{-1}.cm^{-1} for free FAD (Mayhew and Massey, 1969; Whitby, 1953).

Enzyme assays and pH-dependence of activities: Activities were measured using the ferricenium assay (Lehman *et al.*, 1990). Measurements of pH-dependences were carried out in 40 mM buffer containing 5% glycerol and 150 mM KCl at the indicated pH values and at 25°C. IBD concentration was $\approx$ 20 nM and the buffers used were: pH 5.0: acetate/KOH; pH 5.5: MES/KOH; pH 6.0-6.5: HEPES/KOH; pH 7.0-7.5: MOPS/KOH; pH 8.0: Tris/HCL; pH 8.5-9.0: Tricine/KOH; pH 9.5-10.0: Glycine/KOH. For anaerobic measurements, oxygen was removed from enzyme samples in Thunberg type cuvetts by 5-6 cycles of flushing with oxygen-free argon/vacuum.

Reactivity of cys thiol groups: IBD, 1 ⌈M in 40 mM KPi, pH 7.8, containing 5% glycerol was used. Concentrations of DTNB were 4 ⌈M and 1.3 mM DTNB, as indicated on results section. Both absorbance at $\sum_{412}$ and activities were measured at time intervals.

The amount of reacted sulfhydryl groups was calculated using $\llcorner_{412} = 14150$ M^{-1}cm^{-1}. Ellman (1959); Wright and Viola (1998) and Riddles *et al*. (1983). The activity was measured using the ferricenium assay.

RESULT AND DISCUSSION

Purification: IBD stability during purification was substantially increased by addition of 5 µM DTT to all buffers used. This improves dramatically the yield of active IBD with ammonium sulfate cut-off and Q-Sepharose chromatography steps. The reason for this was traced to an unexpectedly high reactivity of solvent accessible thiol groups of Cys residues. The purity of the isolated IBD was > 95 %.

Biochemical characterization: The subunit molecular weight obtained by MALDI-TOF spectroscopy is 42.689 KDa ± 100 Da, which is comparable to calculated one (MW = 42.692 KDa) according to amino acid composition derived from the cDNA sequence. The oligomeric state was estimated by gel filtration and IBD found to be homotetramer with a MW ≈170,000 KDa (not shown). The extinction coefficient of the flavin absorption band in the visible spectrum (Fig. 1) was estimated to be ≈ 14.1 mM^{-1}cm^{-1} using the SDS method (Whitby, 1953). This compares to 11.3 mM^{-1}cm^{-1} for free FAD (Lehman *et al*., 1990) and to values 12.3-14.8 for various ACADs; it is thus within the usual range for this family of flavoproteins.

The visible and ultraviolet absorbance spectrum of purified IBD is depicted in Fig. 1. Interestingly, pure IBD was obtained in a yellow form, although an associated green color can be noted during purification, which is gradually lost. This contrasts with some other ACADs that are obtained in ''green forms'' due to tightly bound CoA-persulfide (Engel and Massey, 1971). IBD spectrum is typical for other ACAD flavoproteins in having the two main bands centered on 450 and 370 nm. Maxima absorbance ratio (268/444 nm) for IBD found to be ≈ 5.4 (Fig. 1), comparing to 5.7 for human MCAD (Kuechler *et al*., 1999).

Addition of the substrate isobutyryl-CoA under anaerobic conditions leads to formation of the reduced enzyme-2-methyl-acrylyl-CoA complex.
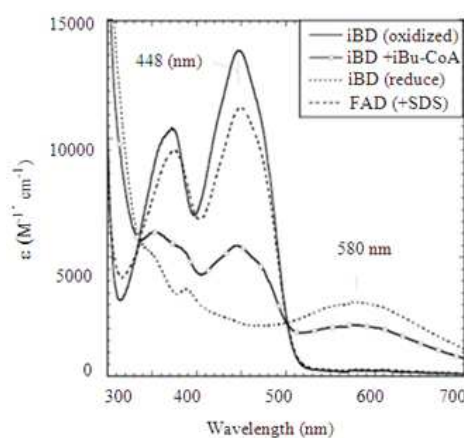

Fig. 1: (Ibrahim and Mohsen)

This is characterized by the decreasing of the absorption at 450 nm and formation of new maxima at 580-425 nm and a shoulder at ≈ 350 nm. The latter two belong to the reduced flavin chromophore, while the long wavelength absorption is due to the charge-transfer interaction between the reduced flavin as donor and enoyl-CoA product as acceptor (Mueller, 1991). This band is characteristic for most ACADs, it varies depending on the ACAD and the bound product in its position and intensity (Finocchiaro *et al*., 1987) and can be used to identify the species (Fig. 1-3). IBD pKa value with isobutyryl-CoA was found to be 8.2 while the pI was estimated as ≈ 6.2, value compares to 4.8 for MCAD (Coates and Tanaka, 1992).

Interaction with substrate analogs and inhibition: There are several substrate analogs that have gained prominence as inhibitors or inactivators for ACADs. Some of these were found to be useful for mechanistic studies (Schmidt *et al*., 1981). Methylenecyclopropyl-acetyl-CoA (cf. Structure in Fig. 4) which is naturally metabolite of hypoglycin and the causative agent of Jamaican vomiting sickness found to inactivate MCAD and SCAD via irreversible formation of covalent adducts (Wenz *et al*., 1981; Tanaka and Ikeda, 1990). None of these analogs listed in Fig. 4 found to inactivate IBD.

Reactivity of Cys thiol groups: Based on the observation that DTT substantially increases the stability of IBD, the role of 13 Cys residues in IBD tested using Ellman's reagent. Time dependence of the reaction of IBD with 4 folds and 100 folds molar excess of DTNB versus IBD activity is shown in Fig. 4. The data showed that, apparently, all 13 Cys residues present in IBD (extrapolated value for T_∞ ≈ 12.7) react at similar rates in monophasic processes.
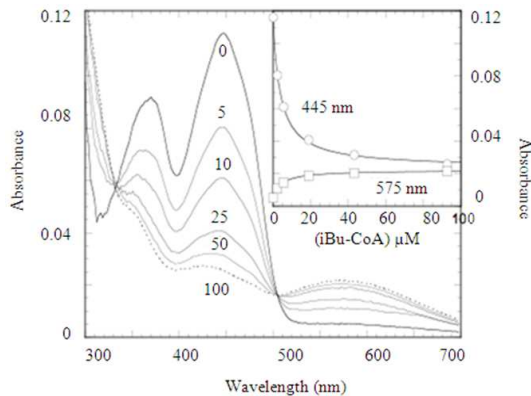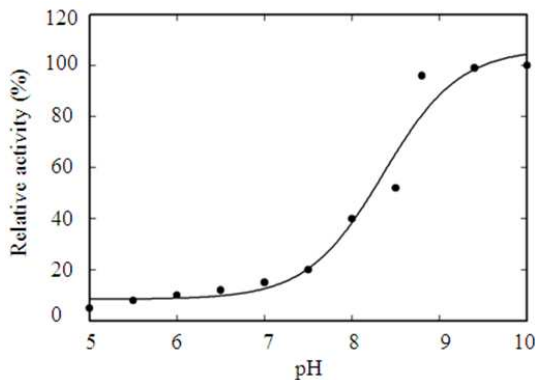
Fig. 2: (Ibrahim and Mohsen)
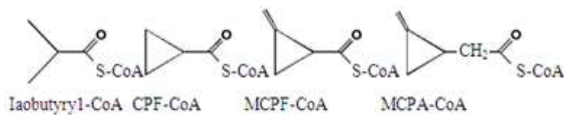

Fig. 3: (Ibrahim and Mohsen)
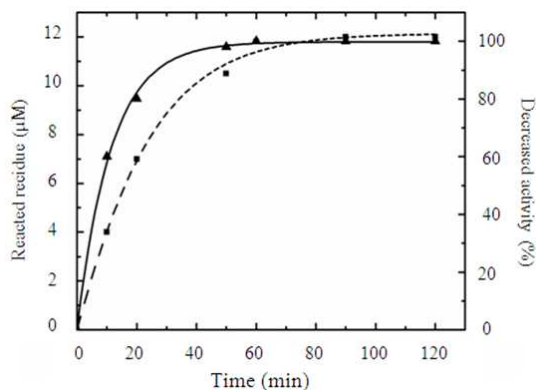

Fig. 4: (Ibrahim and Mohsen)

Fig. 5: (Ibrahim and Mohsen)

With 4 folds excess DTNB, 5 Cys residues reacted with DNTB decreasing the IBD activity by about 40% after the reaction with fourth residue (data not shown).

However, with 100 folds excess, almost all Cys residues reacted with DNTB and IBD activity decreased by ≈ 60% after the reaction with first four Cys residues and diminished completely diminished after reaction with 8-10 Cys residues (Fig. 5).

CONCLUSION

Upon the discovery of MCAD and SCAD by Boyer's group in the mid 60' the other members of the ACAD family were discovered in rather rapid succession (Boyer *et al.*, 1964). However, only few years ago IBD was identified by Telford *et al.* (1999) and later, genetic defect in 2-years old child with reversible cardiomyopathy and deficiency in IBD activity in fibroblasts was identified (Sass *et al.*, 2004).

IBD has relatively high pI (6.2) in comparison to values of other ACADs. However, it has pKa ≈ 8.4, similar to that of MCAD (pK ≈ 8.2) (Finocchiaro *et al.*, 1987). Different types of ACADs inhibitors were examined for IBD inhibition. Acetyl-CoA and acetoacetyl-CoA are known to inhibit ACADs enzymes. With IBD, no inhibition found with either acetyl-CoA or acetoacetyl-CoA. Propionyl-CoA also known to inhibit both human and bovine SCAD (Coates and Tanaka, 1992; Ikeda ad Tanaka, 1983). On contrary, human IBD found to utilize propionyl-CoA as substrate. Interestingly, IBD not inhibited by cyclopropyl-formyl-CoA or its related compounds, that might be due to spatial configuration of these compounds to favore substrate, isobutyryl-CoA.

IBD has the highest number of Cys residues, 13 residues, in all known ACADs, while there are 5 in SCAD, 7 in MCAD and 9 residues in IVD.

First trial of IBD purification without using DTT in purification buffers produced apoenzyme and IBD could not retained its activity upon FAD addition. It is well known that sulfhydril reagents inhibit ACADs enzymes (Coates and Tanaka, 1992; Ikeda andTanaka, 1983). We started to study the reasons of losing IBD activity and its FAD. We measured the reactivity and accessibility Cys residues by using –SH modification reagent DNTB. With 4 folds DTNB to IBD, the activity decreased by 40-50% by modification of 3-4 Cys residues.

The maximum number of Cys residues that can be modified at this DTNB conc. was about 5 residues. With higher DTNB conc., more than 100 folds, about 60-80% of the activity diminished upon modification of 4-7 Cys residues and completely abolished upon

modification of the 10th residue (Fig. 5). These results suggest that Cys residue (s) are important for the proper structure, FAD and/or activity of human IBD in vivo. Our results consistent with previous reported results that showed presence of essential Cys residues located in the vicinity of the FAD- and substrate-binding sites within the active center of rat SCAD and MCAD enzymes (Okamura-Ikeda *et al*., 1985).

ACKNOWLEDGEMENT

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