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Identification of isobutyryl-CoA dehydrogenase and its deficiency in humans

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Abstract

The acyl-CoA dehydrogenases (ACDs) are a family of related enzymes that catalyze the α,β-dehydrogenation of acyl-CoA esters. Two homologues active in branched chain amino acid metabolism have previously been identified. We have used expression in *Escherichia coli* to produce a previously uncharacterized ACD-like sequence (*ACAD8*) and define its substrate specificity. Purified recombinant enzyme had a k_{cat}/K_m of 0.8, 0.23, and 0.04 ($\mu M^{-1}\ s^{-1}$) with isobutyryl-CoA, (S) 2-methylbutyryl-CoA, and *n*-propionyl-CoA, respectively, as substrates. Thus, this enzyme is an isobutyryl-CoA dehydrogenase. A single patient has previously been described whose fibroblasts exhibit a specific deficit in the oxidation of valine. Amplified *ACAD8* cDNA made from patient fibroblast mRNA was homozygous for a single nucleotide change (905G > A) in the *ACAD8* coding region compared to the sequence from control cells. This encodes an Arg302Gln substitution in the full-length protein (position 280 in the mature protein), a position predicted by molecular modeling to be important in subunit interactions. The mutant enzyme was stable but inactive when expressed in *E. coli*. It was also stable and appropriately targeted to mitochondria, but inactive when expressed in mammalian cells. These data confirm further the presence of a separated ACD in humans specific to valine catabolism (isobutyryl-CoA dehydrogenase, IBDH), along with the first enzymatic and molecular confirmation of a deficiency of this enzyme in a patient.

Keywords: Isobutyryl-CoA dehydrogenase; Acyl-CoA dehydrogenase: ACAD8, β-Oxidation; Valine metabolism; Inborn error of metabolism; Valine

1. Introduction

The acyl-CoA dehydrogenases (EC 1.3.99.3) are a family of nuclear encoded, mitochondrial flavoenzymes that catalyze the α,β-dehydrogenation of acyl-CoA intermediates in the catabolism of fatty acids and branched chain amino acids [1–7]. Inherited deficiencies of these enzymes are important causes of human disease [8–10]. Early studies of ACDs[1] isolated from rat liver mitochondria suggested the existence of a single enzyme (called 2-methyl-branched chain acyl-CoA dehydrogenase) that could utilize both isobutyryl- and S-2-methylbutyryl-CoAs from the valine and isoleucine pathways, respectively, equally well as substrates [3]. More recently,

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[1] *Abbreviations used*: ACD, acyl-coenzyme A dehydrogenase; ETF, electron transfer flavoprotein; IBDH, isobutyryl-coenzyme A dehydrogenase; SBCADH, short–branched chain acyl-coenzyme A dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; SCADH, short chain acyl-CoA dehydrogenase; MCADH, medium chain acyl-CoA dehydrogenase; VLCAD, very long chain acyl-CoA dehydrogenase; FAD, flavin adenine dinucleotide.

Table 1
Genetic loci and common enzyme names for acyl-CoA dehydrogenases involved in short and branched chain amino acid catabolism

Genetic locus	Enzyme name	Catabolic pathway
IVD	Isovaleryl-CoA dehydrogenase (IVDH)	Leucine
ACADSB	Short–branched chain acyl-CoA dehydrogenase (SBCADH) 2-Methyl-branched chain acyl-CoA dehydrogenase	Isoleucine
ACAD8	Isobutyryl-CoA dehydrogenase (IBDH)	Valine
ACADS	Short chain acyl-CoA dehydrogenase (SCADH)	Mitochondrial fatty acid β-oxidation

the rat and human cDNAs for this enzyme have been cloned and the gene was named short–branched chain acyl-CoA dehydrogenase (*ACADSB*; see Table 1 for a summary of genetic nomenclature and protein designations) [6,7,11]. Recombinant rat SBCADH produced in *Escherichia coli*, like its native counterpart, could efficiently utilize both isobutyryl- and 2-methylbutyryl-CoA as substrate. In contrast, the recombinant human enzyme did not efficiently utilize isobutyryl-CoA as substrate, raising the possibility that another ACD specific to valine metabolism might exist in humans. Two patients with inactivating mutations in the *ACADSB* gene have recently been identified [12,13]. In the first of these patients, valine metabolism was shown to be normal, while this was not examined in the second patient. Finally, a patient has been identified in whom metabolic loading studies in fibroblasts revealed decreased oxidation of labeled valine, with an increase in accumulation of isobutyryl carnitine. Metabolism of labeled isoleucine and leucine was normal and a defect in a valine specific ACD was proposed [14].

A mapping study of human chromosome 11q25 has identified a novel gene that shares strong homology to other members of the human ACD gene family [15]. Initial studies of *ACAD8* cDNA revealed that the protein expressed in an eukaryotic system had high activity towards both 2-methylbutyryl-CoA and isobutyryl-CoA in crude cellular extracts [13]. We now report expression of the cDNA for *ACAD8* in *E. coli*, purification of the recombinant enzyme to homogeneity, and characterization of the kinetic properties and substrate specificity of the purified enzyme. We also report the gene structure of the human *ACD* gene. Mutation analysis of *ACAD8* from the patient with a proposed defect in valine metabolism revealed a mutation in the *ACAD8* coding region leading to loss of enzymatic activity. Our findings identify *ACAD8* as an isobutyryl-CoA dehydrogenase (IBDH) active in valine catabolism, as well as the first patient deficient in this enzyme.

2. Materials and methods

2.1. Construction of wild type human IBDH expression plasmid

PCR primers were designed to amplify the predicted 1182 bp of the mature coding region of *ACAD8* cDNA. The 5′-primer consisted of 47 nucleotides, including nucleotides 67–95 of the precursor coding sequence followed by *Eco*RI (underlined) and *Nde*I (bold) restriction sites (5′-GAC GAT GAA TTC **CAT ATG** ^{1}CTC GTC CAG ACC GGC CAC CGG AGC TTG AC-3′). The 3′-primer consisted of the last 15 nucleotides of the coding region (stop codon in antisense direction is bolded) followed by a *Hin*dIII restriction site (underlined) (5′-AAT GAG AAG CTT **CTA** CTA CTC CTG AAG CAG-3′). A human liver Marathon-ready cDNA from Clontech (Palo Alto, CA) was used as template for PCR, which was performed with 30 cycles of annealing 60 °C for 30 s, extension 68 °C for 4 min, and denaturing 94 °C for 30 s using the Advantage cDNA PCR Kit with Polymerase Mix (Clontech, Palo Alto, CA). PCR products were purified by electrophoresis on a 1.5% low melting agarose gel and the desired DNA band recovered using the QIAquick Gel Extraction Kit 50 (QIAGEN, Valencia, CA). The recovered fragment was digested with *Eco*RI and *Hin*dIII, and inserted into the prokaryotic expression vector pET-21a (+) (Novagen, Madison, WI). The plasmid containing the mature IBDH insert (*pm*IBDH) was used for expression in *E. coli*. To construct the variant IBDH plasmid, a *Bse*RI and *Nsi*I restriction fragment containing the IBDH patient mutation was substituted into the same sites in the wild type vector. Precursor wild type IBDH was expressed in COS-7 cells using a pcDNA3.1(+) vector as previously described [12,13]. The patient mutation was introduced into the precursor IBDH sequence via the replacement of a *Bsm*I and *Nsi*I fragment with the same fragment containing the patient mutation.

2.2. Amplification of ACAD8 sequences made from fibroblast mRNA

mRNA was isolated from control and patient cultured fibroblasts using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech., Pistcataway, NJ), and first strand cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech.). *ACAD8* cDNA sequences were amplified by 30 cycles of PCR: 62 °C, 4 min, annealing; 72 °C, 7 min, extension; and 94 °C, 30 s denaturing. Amplified products were separated and purified as before, and subjected to automated DNA sequencing by the Molecular Biology Core Facility of the Mayo Clinic.

2.3. Identification and characterization of the human ACAD8 gene structure and sequence analysis of patient and control genomic DNA

tBlastn homology searches of the HTGS and GSS databases in GenBank with the predicted amino acid sequence of the human *ACAD8* were used to identify two BAC clones containing part of the human *ACAD8* gene: AC018780 and AP000859. The AP000859 BAC has been mapped to chromosome 11q25, which is consistent with the previous mapping of the human *ACAD8* gene [15]. Intron sizes were estimated on the basis of migration in agarose gels of PCR products amplified with primers located in separate exons and from the sequence of AC018780 and AP000859. Primers were designed for PCR amplification of the 11 exons and part of the flanking intron sequences of the human *ACAD8* gene (primer sequences are available on request brage@biobase.dk). Genomic DNA was isolated from cultured fibroblasts or blood samples according to standard methods [16]. PCR reactions were performed under standard conditions in an automated Thermal cycler 480 (Perkin–Elmer, Norwalk, CT) and the PCR products were subjected to direct bi-directional cycle sequencing as described above.

2.4. Expression of wild type and mutant IBDH

*pm*IBDH was transformed into *E. coli* host strain BL21(DE3; Novagen, Madison, WI), crude extracts of induced cells were made from 25 ml of cultures grown in 2× YT (31 g/L, BIO 101, Vista, CA) with 80 μg/ml ampicillin. Cultures of *E. coli* were grown to mid-log phase (absorbance 550 nm > 0.5), induced by the addition of IPTG to 0.5 mM final concentration, and incubated with shaking at 37 °C for 4 h or overnight. Cells were harvested by centrifugation and lysed by sonication after treatment with lysozyme as previously described [17–20]. For large scale purification, the wild type and Arg280Gln mutant IBDH plasmids were co-expressed with the bacterial chaperonins GroEL/ES, grown at 37 °C, and harvested after 4 h induction. Expression in CHANG cells, immunostaining, and confocal laser scanning microscopy were performed as described [13,21]. The presence of IBDH protein in prokaryotic and eukaryotic cell extracts was determined through Western blotting with IBDH specific antisera as previously described [12,13].

2.5. Purification of IBDH protein

Wild type IBDH protein was purified from induced *E. coli* cultures by DEAE chromatography, fractionation with ammonium sulfate, and chromatography on 10 μm hydroxyapatite as previously described [17–19]. To prevent loss of FAD from the enzyme molecule, 20 μM FAD was added to the buffer during elution from the hydroxyapatite column. Free FAD was removed from final sample by filtration on Superdex G-200 in 50 mM potassium phosphate, pH 7.5, 0.1 M KCl.

2.6. Enzyme assays

ACD activity was measured with the anaerobic electron transfer flavoprotein (ETF) reduction assay using an LS50B fluorescence spectrophotometer from Perkin–Elmer (Norwalk, CT) with a heated cuvette block set to 32 °C as described [22]. Final substrate concentration in the assay mixtures was 50 μM. For activity units (U) see [22].

2.7. Molecular modeling of IBDH structure

A prediction of the three-dimensional structure of IBDH was obtained with the Insight II 2000 package of modeling software from Accelrys (San Diego, CA) and a Silicon Graphics O2 workstation (Mountain View, CA). Modeling based on the published structures of human isovaleryl-CoA dehydrogenase (IVDH), porcine medium chain acyl-CoA dehydrogenase (MCADH), and butyryl-CoA dehydrogenase from *M. elsdenii* [23–26] was performed using the Homology and Modeler modules included with this software as previously described [22]. The ''Manual Rotomer'' option was used to optimize the position of atoms of the side chains of specific amino acid residues and examine the energy minima of the various possible conformations.

2.8. Computational protein sequence analysis

The protein sequences of 22 selected ACDs were identified from different species via a standard BLAST search of the NCBI databases and compared with the human IBDH sequence using the MacVector software package version 7.0 with the Clustal W algorithm v 1.4 and distance matrix methods. The multiple sequences were aligned and a phylogenetic tree constructed using the following parameters: pairwise alignment mode, slow; open gap penalty, 10.0; extend gap penalty, 0.1; delay divergent, 40%; gap distance, 8; and similarity matrix, blosum. Table 2 shows the GenBank accession numbers of protein sequences analyzed.

3. Results

The clinical history of the patient studied has previously been reported [14]. Briefly, she presented at 2-years of age to first cousin parents of Hispanic origins. She was well for the first one and one-half months of life on breast feeding, then developed feeding intolerance on formula. At 11 months of age she was found to have

Table 2
Species of origin and accession numbers of ACD-like protein sequences

Species name and enzyme	Abbreviation in Fig. 6	Accession Nos.
Arabidopsis thaliana (plant) IVDH	IVDH A.t.	CAA73227
Bacillus halodurans IBDH[a]	ACDH B.h.1	BAB07517
Bacillus halodurans SBCADH[a]	ACDH B.h.2	BAB07518
C. elegans IVDH	IVDH C.e.	T16568
C. elegans SBCADH[a]	SBCADH C.e.	T15088
Drosophila melanogaster IVDH	IVDH D.m.	AAF50398
Drosophila melanogaster SBCADH	SBCADH D.m.	AAF49216
Drosophila melanogaster SCADH	SCADH D.m.	AAF55709
Human IBDH	IBDH human	AAF12736
Human IVDH	IVDH human	P26440
Human SBCADH	SBCADH human	AAA74424
Human SCADH	SCADH human	P16219
Mouse IVDH	IVDH mouse	AAF35888
Mouse SCADH	SCADH mouse	AAA16714
Mycobacterium tuberculosis IBDH[a]	IBDH M.t.	C07825
Pig SCADH	SCADH pig	BAA13964
Pisum sativum (pea) IVDH	IVDH pea	CAB55554
Potato IVDH	IVDH potato	CAC08233
Pseudomonas aeruginosa IBDH[a]	IBDH P.a.	AAG04135
Pseudomonas aeruginosa IVDH	IVDH P.a.	AAG05403
Rat IVDH	IVDH rat	P12007
Rat SBCADH	SBCADH rat	AAB17136
Rat SCADH	SCADH rat	B30605

[a] Most likely predicted enzyme.

failure to thrive, a severe carnitine deficiency, and dilated cardiomyopathy. She responded well to carnitine therapy and has been well without episodes of decompensation since. She is now 6 years old with normal growth and development. Metabolic flux studies originally revealed a defect in valine catabolism, and the existence of a valine specific acyl-CoA dehydrogenase was suggested. We hypothesized that the recently identified *ACAD8* might be such an enzyme and that it might be deficient in this patient. To examine this, we amplified *ACAD8* sequences from control and patient fibroblasts. Amplification of *ACAD8* from cDNA made from control fibroblast mRNA yielded a fragment of 1250 bp in size, in good agreement with the size of the predicted precursor form of *ACAD8* [15]. Direct DNA sequencing of the amplified product confirmed that the sequence of the PCR product matched that published for *ACAD8* (not shown). In contrast, *ACAD8* sequences amplified from cDNA made from patient fibroblasts revealed a homozygous substitution of a guanine by an adenine residue at position 905 (905G > A) of the precursor coding region (Fig. 1). This leads to an Arg302Gln alteration in the precursor, full-length protein, which corresponds to amino acid 280 in the predicted mature protein.

To characterize this mutation in genomic DNA, we defined the genomic structure of the human *ACAD8* gene (Table 3; Accession Nos. AF260679–AF260689). The human *ACAD8* gene structure was confirmed by PCR and direct sequencing of all exons from genomic DNA from several control samples. Exon 1 is located in AC018780 (*Homo sapiens* chromosome 11 clone RP11-153cl4). Exons 2–11 are located in AP000859 (mapped to 11q25). PCR amplification and sequence analysis of all 11 exons of the *ACAD8* gene from the index patient showed that the 905G > A mutation observed in patient *ACAD8* cDNA was also present in homozygous form in *ACAD8* exon 8 in genomic DNA (not shown). Both parents of the index patient were heterozygous for the 905G > A mutation by sequence analysis of exon 8 amplified from genomic DNA. Sequence analysis of exon 8 amplified from genomic DNA from 59 control individuals (118 alleles) showed that the 905 G > A alteration was not present, though the samples were not matched for ethnic origin.

Extracts from CHANG cells expressing *ACAD8* were previously reported to have nearly equal activity using isobutyryl-CoA and 2-methylbutyryl-CoA as substrates at high concentrations [12,13]. To characterize better the substrate specificity of ACAD8 and the effect of the amino acid substitution on its activity, the predicted mature coding region of *ACAD8* (beginning with amino acid residue Leu23 of the precursor as predicted by consensus processing signals for mitochondrial precursor proteins) was amplified via PCR and cloned into a prokaryotic expression vector. Expression of the insert was induced with IPTG following transformation into *E. coli*, crude cellular extracts were prepared and the ACD activity of the extracts was measured in triplicate with a variety of acyl-CoA substrates using the sensitive and highly specific anaerobic ETF fluorescence reduction assay. Extracts from cells containing the wild type

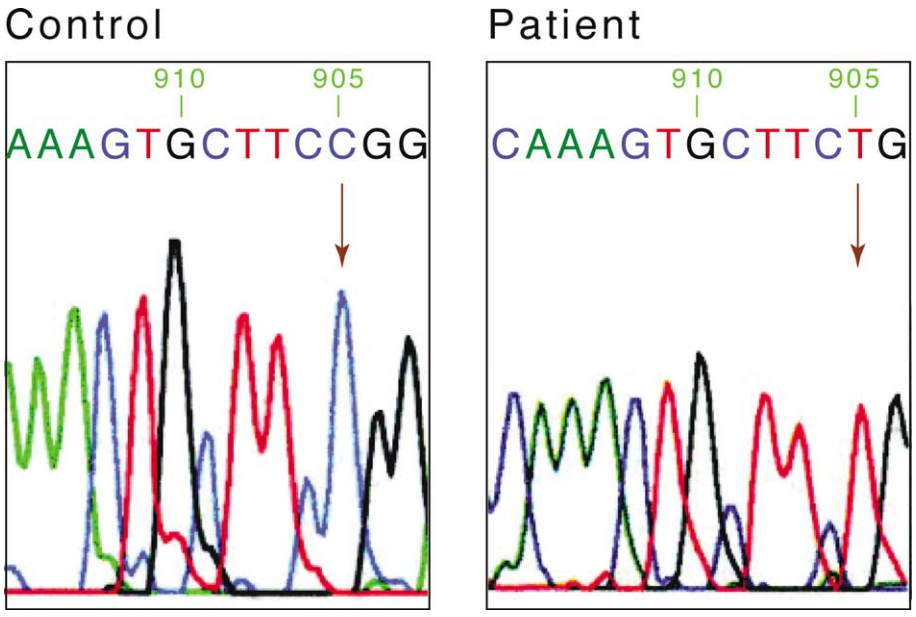

Fig. 1. DNA sequencing of control (A) and patient (B) IBDH cDNA. A homozygous nucleotide substitution (905 G > A) was identified in the patient cDNA by direct sequencing of sequences amplified from patient fibroblasts. The chromatogram shows a sequence obtained in the reverse direction. This predicts an Arg280Gln alteration in the mature IBDH subunit (position 302 of the precursor protein). Sequencing of genomic DNA from the patient confirmed that the patient was homozygous for the mutation (not shown).

ACAD8 sequence after 4 h of induction showed the highest activity with isobutyryl-CoA (149 ± 37 mU/mg protein). Activity of the same quantity of crude extract measured with (R/S) 2-methylbutyryl-CoA and (S) 2-methylbutyryl-CoA as substrates was 63 ± 2 and 69 ± 15 mU/mg protein, respectively. No activity was detectable in the cellular extract when *n*-butyryl-CoA, *n*-valeryl-CoA, or isovaleryl-CoA were used as substrates. The recombinant enzyme was purified to >95% homogeneity (Fig. 2A) and kinetic parameters determined using the ETF fluorescence reduction assay. Results (Table 4) confirm that expressed ACAD8 protein has maximal activity towards isobutyryl-CoA and thus we name this enzyme isobutyryl-CoA dehydrogenase (IBDH).

To characterize the effect of the Arg280Gln amino acid substitution identified in the patient on IBDH activity, the mutation was introduced into the wild type

Table 3
Organization of the human *ACAD8* gene

Intron 3′ splice site	Exon[a]	5′ Exon sequence	Exon size	3′ Exon sequence	Intron 5′ splice site	Intron size
	Exon 1	([b])		*TGCATCGACC*	gtaaggatct	ND[c]
ctgcccacag	Exon 2	CTTCCATGGG	111	GGACCAGAAG	gtaggcgttt	439
ttgtacatag	Exon 3	GAGCTGTTCC	170	GCATCCACAA	gtgagtgccc	ND[c]
tacctatcag	Exon 4	CATGTGTGCC	120	ACTGAACCAG	gtgaatttgc	383
ccctcctcag	Exon 5	GAAGTGGGAG	77	TGGCTCCAAG	gtactagcgt	51
acccccacag	Exon 6	GCCTTCATCA	138	GGAGAAAAAG	gtgagtggct	1238
tttttgccag	Exon 7	GTGGGGTGGA	136	ATCAATATTG	gtgagatacg	95
tcccctctag	Exon 8	CTTCCTGCTC	98	CAGTAACCAG	gtaacctctg	351
tgtgctgcag	Exon 9	TACTTGCAAT	153	ATGCTTTGCC	gtaagtgatt	629
ctccctgcag	Exon 10	ATCTGCAACC	103	ATTCTAGAAG	gtaaaaattg	2225
cctcttacag	Exon 11	GTAGCAATGA	943	Poly(A) addition site		
Consensus Splice signal						
YYYYYYYYYNAG		G		AG	GTRAGT	

[a] The start position of each exon is indicated relative to the corresponding position in the cDNA sequence. Intron and exon sizes are indicated, and 10 bp of the exon and intron sequences at each junction are presented. Exon 1 is located in AC018780 (*Homo sapiens* chromosome 11 clone RP11-153c14). Exons 2–11 are located in AP000859, mapped to 11q25. The sequence data have been submitted to the GenBank database (Accession Nos. AF260668 –AF260678).

[b] Transcription initiation site not determined.

[c] Not determined.

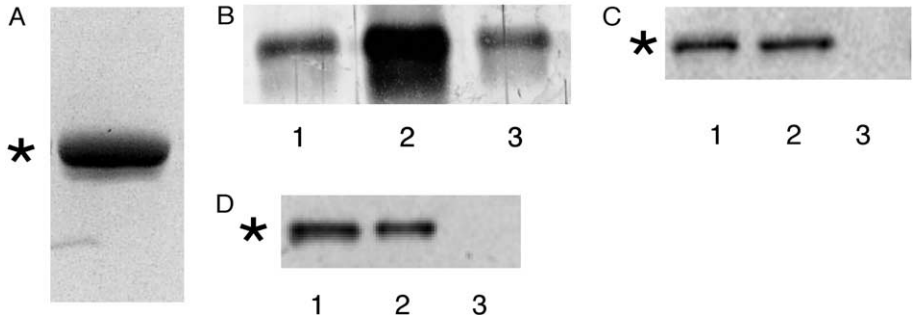

Fig. 2. Western blot of wild type and Arg280Gln mutant IBDH. (A) Commasie A blue staining of a 10% polyacrylamide SDS gel with 15 ng of purified hIBDH wild type IBDH. (B) Western blot of crude *E. coli* cell extracts following expression of wild type (lane 2) and Arg280Gln IBDH (lane 3). Samples were separated on a 10% polyacrylamide SDS gel. Equal amounts of 300 ng cellular protein were loaded on the gel. Lane 1 shows purified IBDH. (C) Western blot of crude cell extracts from CHANG cells following expression of wild type (lane 1) and Arg280Gln (lane 2) IBDH. Samples were separated on a 12% polyacrylamide SDS gel. Five micrograms of cellular protein were loaded in each lane. Lane 3 shows extract from cells trasfected with vector containing no insert. (D) Western blot of crude extracts from normal (lane 2) and patient (lane 3) fibroblasts after separation on a 10% polyacrylamide SDS gel. 120 μg of cellular protein were loaded for each sample. Lane 1 shows purified IBDH.

Table 4
Comparison of the kinetic parameters of purified wild type IBDH measured with different substrates and the ETF fluorescence reduction assay

Substrate	K_m (μM)	k_{cat} (s^{-1})	Tetramer catalytic efficiency (μM^{-1} s^{-1})
Isobutyryl-CoA	2.6 ± 0.7[a] ($N = 24$)[b]	2.0 ± 0.14	0.8 ± 0.3
(S)-2-Methylbutyryl-CoA	18 ± 3 ($N = 29$)	4.1 ± 0.3	0.23 ± 0.06
n-Propionyl-CoA	24 ± 7 ($N = 20$)	0.83 ± 0.07	0.04 ± 0.01

[a] All values represent the standard deviation and 95% confidence intervals calculated as described in the text.
[b] *N*, number of determinations.

prokaryotic expression vector, the cells were induced, and crude lysate was prepared as above. No activity could be detected in the extract with isobutyryl-CoA as substrate using a maximum amount of 127 μg of extract protein (up to 30-fold higher than used for assay of the extract from cells expressing the wild type vector). Western blot experiments with antiserum produced to the purified recombinant IBDH confirmed that the mutant enzyme was expressed and was in the soluble cell supernatant from mutant cells, though at lower levels than for the wild type expression vector (Fig. 2B). The precursor forms of the mutant and wild type IBDH were also expressed in CHANG cells. Extracts from cells expressing the wild type IBDH sequence had a specific activity of 3.6 ± 0.4 mU/mg cellular protein. No activity was detectable in extracts from cells transfected with the mutant vector, even when 2.5-fold more cellular protein compared to wild type extract was used in the assay (75 vs. 30 μg, respectively). Western blotting with IBDH antiserum confirmed the presence of immunoreactive protein in extracts from both transfected cell lines, though the level of mutant IBDH was less than wild type (Fig. 2C). Immunostaining and confocal laser scanning microscopy of transfected CHANG cells revealed localization of both wild type and mutant IBDH proteins to the mitochondria, the predicted location for the normal enzyme (Fig. 3). Finally, fibroblasts from the patient showed no immunoreactive IBDH, while enzyme was present in control cells (Fig. 2D).

Molecular modeling was used to generate a structural model of IBDH as well as predict the effects of the patient mutation on IBDH structure. Modeling of other ACDs has previously proven to be a robust method for this purpose due to the high level of conservation of the primary carbon backbone configuration in this gene family [20,22]. The model generated for IBDH, shown in Fig. 4, reflects the conserved nature of the input structures, including the location of the predicted catalytic base (Glu376 in the mature protein). Arg302Gln (position 280 in the mature protein), mutated in the patient, is predicted to lie at the interface of interacting dimers within the mature homotetramer.

The relatively high specific activity of rat SBCADH for both 2-methylbutyryl-CoA and isobutyryl-CoA compared to the human enzyme led us to hypothesize that IBDH might be specific to humans, and thus represent a relatively recent gene duplication event. To examine this, Southern blotting of rat and human genomic DNAs was performed using the human IBDH and SBCADH cDNAs as probes. Southern mapping studies confirmed the presence of a single copy each of the IBDH and SBCAD genes (data not shown), indicating a more ancient evolutionary event. This is substantiated by the presence of a mouse sequence reported

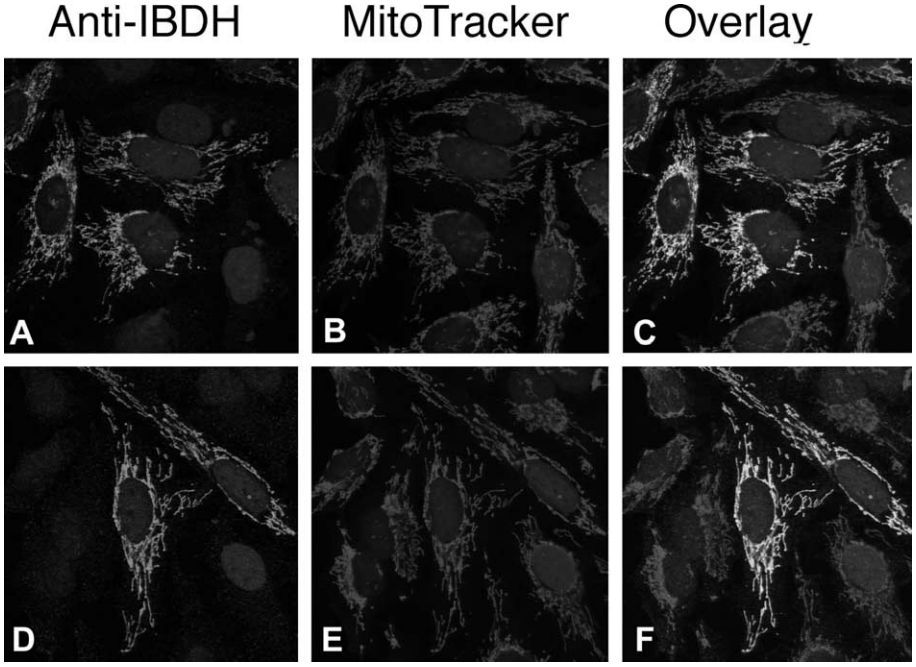

Fig. 3. Localization of wild type and mutant IBDH to mitochondria after expression in transfected CHANG cells. Forty-eight hours after transfection with wild type IBDH (A)–(C) and Arg280Gln (D)–(F) expression vectors, cells were incubated with the rhodamine (red) labeled MitoTracker (B) and (E) for 30 min at 37 °C. Following fixation and permeabilization, cells were immunostained using anti-IBDH polyclonal primary antibody, detected by an Alexa 488-conjugated (green) secondary antibody (A) and (D) and analyzed by CLSM. (C) and (F) Overlay of the different optical sections shown in (A), (B) and (D), (E), respectively. Nuclear DNA is counterstained with Hoechst 33258 (blue). Original magnification 1000×.

in the genetic databases with up to 90% homology to human IBDH (Stratagene mouse macrophage Mus musculus cDNA clone #937306).

To examine this question further, we searched GenBank for sequences related to the ACDs (Table 2) and classified them on the basis of homology to each of the individual gene family members. Substrate specificity was then predicted on the basis of overall sequence homology, as well as conservation of key residues previously identified to be important in determining this feature. A phylogenetic tree constructed from 23 ACDs from various species predicted to have branched chain activity is shown in Fig. 5. Full length coding sequences likely to be IVDHs were found in at least nine species, including four already shown by us to be IVDHs (human, rat, *Caenorhabditis elegans*, and pea). Sequences predicted to be IBDHs and SBCADHs were also clearly identified in a similarly wide range of species. These analyses identify human IBDH as belonging to a distinct branch of the ACD gene family with 20% amino acid identity and 32% similarity to other family members. It is more homologous to SCADH and SBCADH than IVDH. Interestingly, bacterial proteins from *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* share the highest overall homology of the identified ACDs to IBDH (64%), and thus might be potential candidates to be IBDH homologues. Highly conserved key residues in the various ACDs are shown in Fig. 6.

4. Discussion

Dehydrogenation of 2-methylbutyryl-CoA and isobutyryl-CoA in the catabolism of isoleucine and valine was originally postulated to be mediated by a single enzyme, termed 2-methyl-branched chain acyl-CoA dehydrogenase [3]. The gene for this enzyme was subsequently termed *ACADSB* (denoting short–branched chain acyl-CoA dehydrogenase) to reflect the broad substrate specificity of the enzyme purified after expression in *E. coli* [6,7]. More recently, we have suggested that separate enzymes might exist to catalyze each reaction in the isoleucine and valine pathways [6,7,12–14], and preliminary studies of the substrate specificity of human *ACAD8* overexpressed in CHANG cells indicated that it had significant enzyme activity with isobutyryl-CoA [13]. The current report confirms the existence of an isobutyryl-CoA dehydrogenase (IBDH) specific to valine metabolism, unequivocally demonstrates identification of an ACD with highest relative activity towards isobutyryl-CoA as substrate, and characterizes a mutation in the gene for this enzyme in a patient with cellular based evidence of a specific defect in valine metabolism [14]. The identification of IBDH completes the complement of ACDs for reactions known to be catalyzed by this family of enzymes. While it is, of course, possible that tissue specific forms of one or more of the ACDs may exist, as well as ACDs for

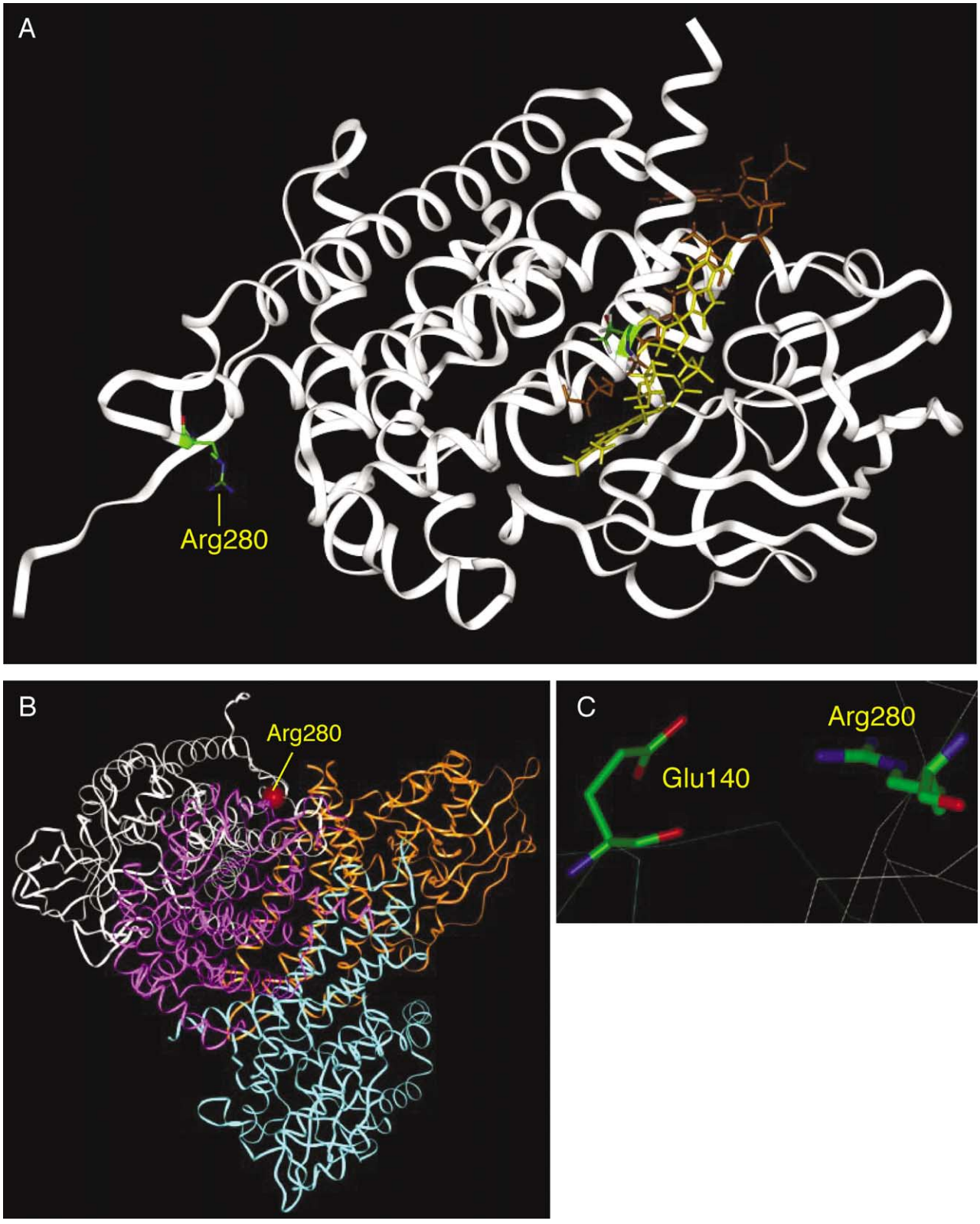

Fig. 4. The predicted three-dimensional structure of IBDH and localization of the patient's mutation in the cleaved, mature protein. (A) The structure of human IBDH was predicted by molecular modeling as described in the text. A monomer of IBDH with substrate and FAD is depicted. The location of Arg280 in the mature protein is indicated. The rendered atoms of Arg280 have been colored green for carbon, red for oxygen, and blue for nitrogen. (B) The position of Arg280 (rendered as a red sphere) in the predicted IBDH tetramer structure is shown. Arg280 lies on the interface of interacting dimers within the mature homotetramer. (C) Measurement of the molecular distance between the Arg280 from one IBDH subunit and Glu140 from a neighboring subunit. Atom colors are as in (A).

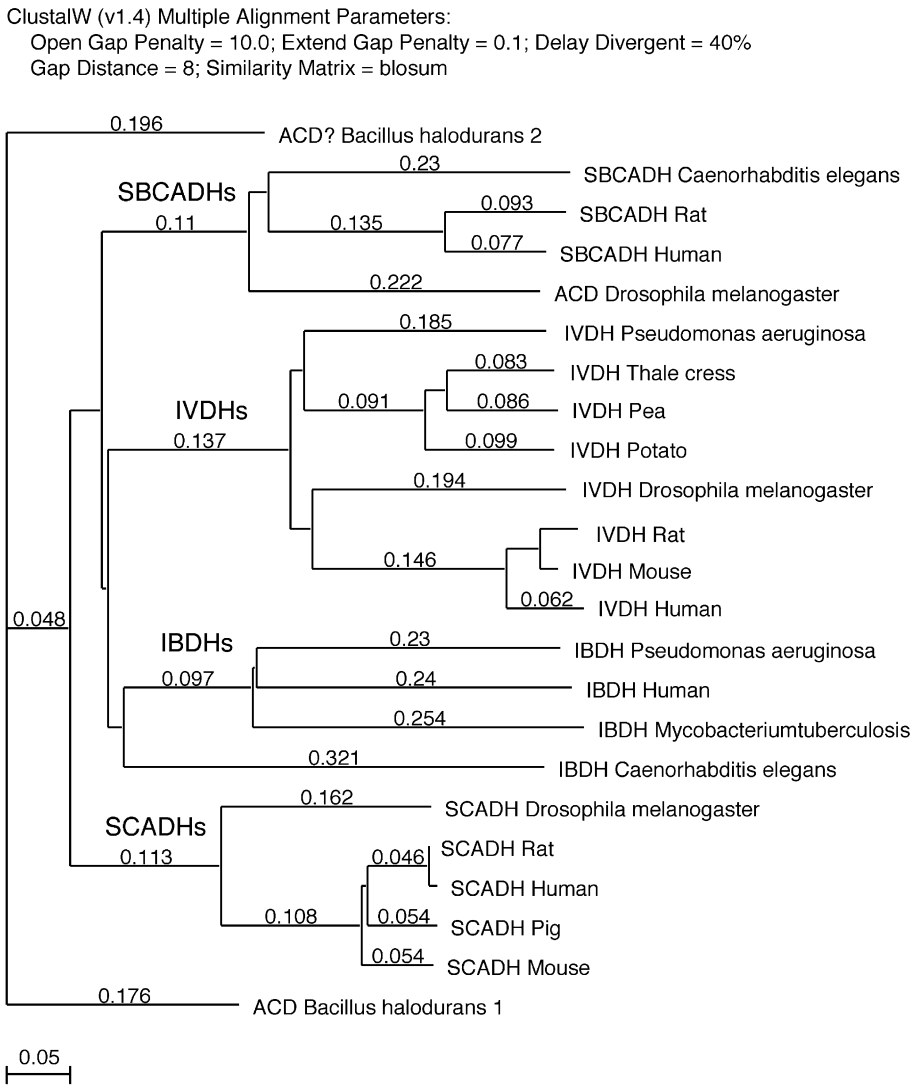

Fig. 5. Phylogenetic tree of predicted IBDH, IVDH, SBCADH, and SCADH amino acid sequences constructed using MacVector sequence analysis software version 7.0. Details regarding the species of origin and the database accession numbers of the various sequences are presented in Table 3.

reactions not previously associated with this family of enzymes, an extensive search of the available genetic databases including the human genome draft sequence has failed to identify any such candidate genes (J. Vockley, unpublished). In the context of cellular metabolism, it is likely that metabolism of isobutyryl-CoA and 2-methylbutyryl-CoA are mediated primarily by IBDH and SBCADH, respectively. There are lines of evidence in support of this. First, while both enzymes can utilize both substrates, their K_m's towards their optimum substrate are much higher for the non-optimum than optimum substrates (7- and 50-fold higher, respectively for IBDH and SBCADH for their non-optimum substrates). Second, patients with IBDH and SBCADH deficiency show accumulation of only isobutyryl-CoA and 2-methylbutyryl-CoA derivatives, respectively, in blood and urine. The rather non-specific presentation of patients with both disorders makes consideration of each necessary in any child with unexplained developmental delay, failure to thrive, or apparent secondary carnitine deficiency. Furthermore, we have now identified additional individuals with deficiencies of both enzymes through expanded state newborn screening programs in North Carolina and Minnesota using tandem mass spectrometry (unpublished), all of whom were asymptomatic at diagnosis, and remain so on therapy. Long term follow-up of especially the patients diagnosed through newborn screening will be necessary to define the natural history of these diseases.

The Arg302Gln substitution identified in the IBDH deficient patient leads to a loss of enzyme activity when the mutant enzyme is expressed in prokaryotic and eukaryotic systems. The mutant enzyme is appropriately targeted to mitochondria when expressed in CHANG cells, but shows reduced stability in both expression

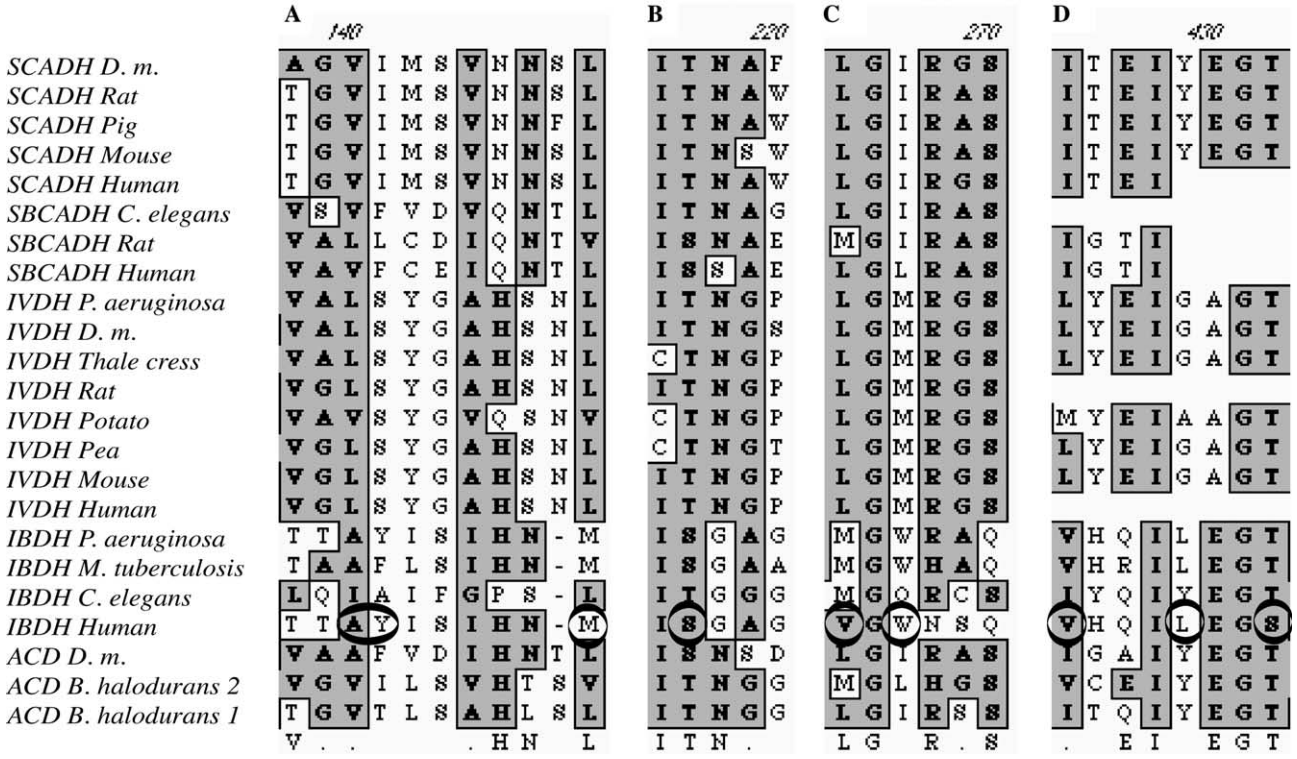

Fig. 6. Alignment of key amino acid residues from predicted branched chain specific ACDs. Residues listed in Table 5 are circled. (A) Ala99, Tyr100, and Met106; (B) Ser171; (C) Val215 and Trp217; (D) Val371, Leu375, and Ser378.

systems and patient fibroblasts. Molecular modeling offers some insight into this phenomenon. The mutated amino acid residue at position 280 in the mature protein is conserved in IVDH and SBCADH, homologous to Arg280 of IVDH, and Arg286 of SBCADH. In the IBDH model, the distance from the catalytic site makes a direct effect of the patient mutation on substrate conversion unlikely. Rather, this residue lies in a position to interact with Glu140 of the opposite mature subunit (Fig. 4), and likely plays a role in the interaction of the enzyme monomers/dimers, thus affecting their stability. Though the predicted distance between these residues in our model is greater than is optimal for such an interaction (4.6 vs <3 Å), it is important to note that the only positions of the amino acid residues within the individual subunits, and not their relative position to one another, have been optimized in the model. Thus it is possible that the two residues are actually in closer approximation than seen in our model. Consistent with this, it is has been suggested, based on the crystal structure of porcine MCADH, that the homologous arginine in this mature enzyme (Arg281) is important for FAD binding and monomer dimerization, forming a hydrogen bond with the pyrophosphate moiety of FAD of the neighbouring subunit of the MCAD dimer [24]. Interestingly, mutation of the homologous residue has also been observed in patients with VLCADH (Arg326 in the mature protein) and IVDH (Arg282 in the mature protein) deficiency, underscoring the importance of this residue for correct ACD function [20,27,28].

Examination of ACDs in the phylogenetic tree predicted to have branched chains specificity from evolutionarily distant species provides an opportunity to evaluate the importance of various amino acid residues in determining substrate utilization in these enzymes. Selected portions of a multiple sequence alignment of 22 such ACDs are shown in Fig. 6. Glycine residues at precursor positions 116, 186, 211, 243, 266, 297, 318, 406, 431 (75, 144, 165, 204, 215, 245, 264, 352, 355 in the mature IBDH sequence) are highly conserved in all of the branched chain ACDs. The position of the presumed catalytic base of the enzymes is also highly conserved (numbered as residue in mature protein): Glu381 for human SBCADH [11], Glu368 for human SCADH [17], and Glu254 for human IVDH [18,23]. The divergence of the position of the catalytic base in IVDH suggests that it is evolutionarily more distant from the other family members. It has previously, been suggested that the gene for *IVD* evolved earlier than that for *ACADSB*, and that the gene for *ACADS* diverged recently relative to *ACADSB* [29,30]. The more extensive sequence data now available confirm that *ACADS* belongs to an evolutionary branch of this gene family, which diverged earlier than the *ACADSB* and *IVD* genes. *ACADSB*, *IVD*, and *IBDH*, however, appear to be more closely related to each other than with *ACADS*. Overall, the divergence

Table 5
Amino acid residues predicted to be important in determining utilization of branched chain substrates among IBDH, IVD, and SBCAD

Amino acid residue			Predicted effect on substrate utilization
IVD	IDBH	SBCAD	
Leu95	Ala99	Val104	Allows deeper pocket for substrate binding in IBDH and SBCADH
Ser96	Tyr100	Phe105	Similar structure in IBDH and SBCAD. Allows binding of other branched chain substrates
Leu103	Met106	Leu112	Allows accommodation of longer branched side chains in substrates in SBCAD
Thr168	Ser171	Ser176	Blocks binding of other branched chain substrates in IVDH
Leu214	Val215	Leu220	Next to Leu95 in IVD. Affects depth of substrate binding pocket
Met216	Trp217	Leu222	Affects depth of binding pocket and accommodation of long chain substrates
Leu370	Val371	Ile376	Next to IVD Leu95 in binding pocket. Determines depth of pocket
Gly374	Leu375	Tyr380	Blocks isovaleryl-CoA binding in IBDH substrate binding pocket
Thr377	Ser378	Ala383	Alters trajectory of α-helix. Differentiates branched chain ACDs from others

in the branched chain specific ACDs appears to be an evolutionarily ancient event as evidenced by the presence of apparent IBDH sequences in *M. tuberculosis* and *P. aeruginosa*.

Comparison of the known structure of human IVDH with our model of IBDH, and one of human SBCAD that we have previously generated, allows prediction of a number of amino acid residues which are likely to be important in determining the ability to use branched chain substrates, along with optimum substrate specificity [22]. These are listed in Table 5 and shown in Fig. 6. In vitro mutagenesis experiments designed to alter these residues in a systematic fashion will allow a better understanding of the factors important in determining substrate specificity.

5. Conclusion

Our data confirmed further the exist of a new ACD (ACD8 or IBDH) that utilizes isobutyryl-CoA as its optimal substrate. Additionally, we have characterized a deficiency of this enzyme in a patient previously shown to have a defect in cellular metabolism of valine, indicating that IBDH is specific to valine metabolism. Database searches reveal that the divergence of IBDH from other ACDs active toward branched chain substrates is likely an evolutionarily ancient event. Further study of this group of enzymes will be useful in elucidating the molecular mechanisms for their utilization of branched chain substrates.

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