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Comparative studies of Acyl-CoA dehydrogenases for monomethyl branched chain substrates in amino acid metabolism

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ABSTRACT

Short/branched chain acyl-CoA dehydrogenase (SBCAD), isovaleryl-CoA dehydrogenase (IVD), and isobutyryl-CoA dehydrogenase (IBD) are involved in metabolism of isoleucine, leucine, and valine, respectively. These three enzymes all belong to acyl-CoA dehydrogenase (ACD) family, and catalyze the dehydrogenation of monomethyl branched-chain fatty acid (mmBCFA) thioester derivatives. In the present work, the catalytic properties of rat SBCAD, IVD, and IBD, including their substrate specificity, isomerase activity, and enzyme inhibition, were comparatively studied. Our results indicated that SBCAD has its catalytic properties relatively similar to those of straight-chain acyl-CoA dehydrogenases in terms of their isomerase activity and enzyme inhibition, while IVD and IBD are different. IVD has relatively broader substrate specificity than those of the other two enzymes in accommodating various substrate analogs. The present study increased our understanding for the metabolism of monomethyl branched-chain fatty acids (mmBCFAs) and branched-chain amino acids (BCAAs), which should also be useful for selective control of a particular reaction through the design of specific inhibitors.

1. Introduction

Three branched-chain amino acids (BCAAs), including isoleucine, leucine, and valine, have been found to play many essential roles in living organisms. For example, BCAAs are used by bacteria as major carbon and energy sources [1,2]. Lowered concentrations of BCAAs result in impaired growth and neurological problems [3]. BCAAs may be useful chemoprevention modality for colon cancer in obese people [4]. BCAAs can suppress insulin-resistance-based hepatocarcinogenesis [5]. BCAAs can modulate the function of proteins engaged in mRNA translation and the selection of specific mRNAs for translation through regulation of a signal transduction pathway, therefore, regulating process related to gene expression [6].

2-Methylbutyryl-CoA, isovaleryl-CoA (3-methylbutyryl-CoA), and isobutyryl-CoA are three short monomethyl branched-chain fatty acids (mmBCFAs) thioester derivatives, and derived from the catabolism of three BCAAs, isoleucine, leucine, and valine, respectively, as shown in Fig. 1. Their oxidations are catalyzed by short/branched chain acyl-CoA dehydrogenase (SBCAD, EC 1.3.99.3), isovaleryl-CoA dehydrogenase (IVD, EC 1.3.99.10), and isobutyryl-CoA dehydrogenase (IBD, EC 1.3.99.12), respectively. SBCAD, IVD, and IBD are mitochondrial homotetrameric flavoproteins containing one molecule of flavin adenine dinucleotide (FAD) per monomer, which all belong to acyl-CoA dehydrogenases (ACDs) family [7]. Deficiencies of these enzymes are important causes of human disease [8–14]. Although these three enzymes share similar sequences, catalytic mechanisms, and structural properties, the positions of their catalytic residues are not conserved in their primary sequences [15,16]. The crystal structure of human IVD has been solved at 2.6 Å resolution [17], and the crystal structure of human IBD with and without substrate has also been determined to 1.76 Å resolution [7]. The coordinates of the human SBCAD structure have been available for some time (pdb code: 2JIF), unfortunately its relevant paper has not been published. The overall fold of these enzymes has been found to be similar, and it is difficult to determine their catalytic differences based on their crystal structures. Besides, three-dimensional structures of the enzymes provide static pictures, while the enzymes are rather flexible in nature. Therefore, it would be interesting to have functional studies of these three enzymes comparatively. In the present paper, we report our comparative studies of these three enzymes through site-direc-

Abbreviations: BCAAs, branched-chain amino acids; BCFAs, branched chain fatty acids; DCPIP, 2,6-dichlorophenolindophenol; IBD, isobutyryl-CoA dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; IVD, isovaleryl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; mmBCFAs, monomethyl branched-chain fatty acids; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMS, phenazine methosulfate; SBCAD, short/branched chain acyl-CoA dehydrogenase; SDS, sodium dodecylsulfate; UV/Vis, ultraviolet-visible spectroscopy.

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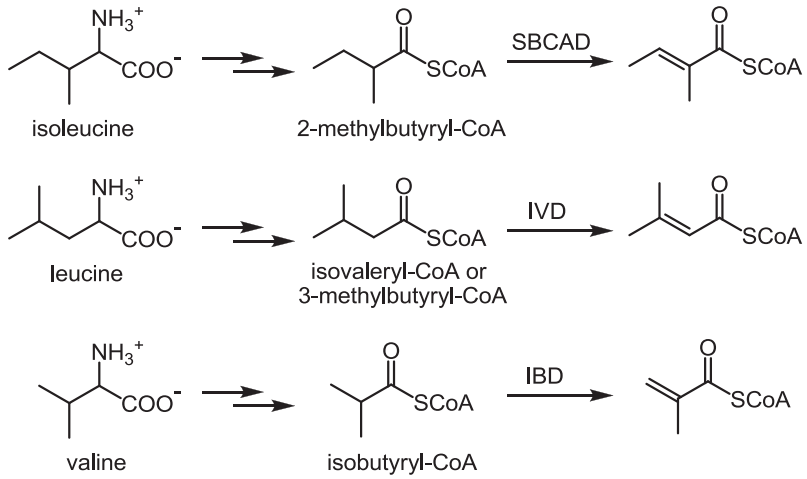

Fig. 1. Metabolism of branched-chain amino acids, isoleucine, leucine, and valine.

ted mutagenesis, kinetics, and incubation with various substrates and substrate analogs.

2. Materials and methods

2.1. Materials

A HiTrap chelating metal affinity column was purchased from Amersham Pharmacia Biotech. *Pfu* DNA polymerase, HB101 competent cells, *Escherichia coli* strain BL21(DE3) competent cells and agarose came from Invitrogen Life Technologies. The Plasmid Mini kit and synthesized oligonucleotides were obtained from Tech Dragon Company of Hong Kong. A gel extraction kit, T4 DNA ligase and restriction enzymes came from MBI Fermentas of Germany. Acyl-CoA thiolesters were prepared from the corresponding free acids and coenzyme A by using the mixed anhydride method [18] in our lab, and subsequently purified by using reverse-phase HPLC. All other reagents were of research grade or better and were obtained from commercial sources.

2.2. Cloning of the Gene of Rat SBCAD

A rat liver Quick-Clone cDNA library was purchased from Clontech (Palo Alto, CA). The gene of rat SBCAD was amplified by PCR using following primers. The sequence of the forward primer was 5′ G GAA TTC CAT ATG AAA TCC TC**C** CA**G** CC**G** GAA GCT CT**G** CT**G** AG**C** GTA AC**C** AA**C** AA**C** GC**T** CT**G** TG**C** TTC GCA CCT CTG CAG ACA TTT AC 3′, containing a Nde1 site (CAT ATG), and codons for amino acids 2–26 of rat liver SBCAD. In order to enhance expression, the nucleotide sequence of 12 codons at the 5′-end of the cDNA was altered to accommodate *E. coli* codon usage without altering the amino-acid coding sequence (highlighted with bold and underline). The sequence of the reverse primer was 5′ CTG CAG CTC GAG TCA GTA CTC TGC ATC GAT GTG C 3′, containing a Xho1 site (CTCGAG), a stop anticodon (TCA), and anticodons for the last seven amino acids of rat liver SBCAD. The PCR product was gel purified, double digested, and ligated into a pET28a expression vector resulting in the pET28a::SBCAD plasmid. The constructed pET28a::SBCAD plasmid was transformed into HB101 competent cells according to an electroporation transformation procedure (Bio-Rad) for screening purposes. The identified positive colony was grown in LB medium containing Ampicillin (50 mg/L), and the plasmid pET28a::SBCAD was isolated and transformed to *E. coli* strain BL21(DE3) competent cells for expression purposes. DNA sequencing of the cloned rat liver SBCAD gene was performed, and the inserted gene sequence was identified to be the same as that previously deposited in NCBI without any mutation.

2.3. Cloning of the Gene of Rat IVD

The gene of rat IVD was amplified by PCR using following primers. The sequence of the forward primer was 5′ GG CAA TTC CAT ATG CAC TC**T** ATG TT**T** CC**G** GTG GAT GAT GAT ATC AAC GG**T** CT**G** AAC GA**A** GA**A** CAG AA**A** CAG CT**G** CGT CAT AC**C** AT**C** TCT AAG TTC GTT CAA GAG AAC CTG 3′, containing a Nde1 site (CAT ATG), and codons for amino acids 2–32 of rat liver IVD. In order to enhance expression, the nucleotide sequence of 11 codons at the 5′-end of the cDNA was altered to accommodate *E. coli* codon usage without altering the amino-acid coding sequence (highlighted with bold and underline). The sequence of the reverse primer was 5′ CCG CAG AAG CTT CTA GCG GAA GTC TGC GTT GAA AG 3′, containing a Hind III site (AAGCTT), a stop anticodon (CTA), and anticodons for the last seven amino acids of rat liver IVD. The PCR product was gel purified, double digested, and ligated into a pET28a expression vector resulting in the pET28a::IVD plasmid. The constructed pET28a::IVD plasmid was identified, isolated and transformed to *E. coli* strain BL21(DE3) competent cells for expression purposes. DNA sequencing of the cloned rat liver IVD gene was performed, and the inserted gene sequence was identified to be the same as that previously deposited in NCBI without any mutation.

2.4. Cloning of the Gene of Rat IBD

For the cloning of rat isobutyryl-CoA dehydrogenase gene, the sequence of its forward primer was 5′ GCA CTA CAT ATG CTG GCT CAG ACC GAC CAC AGG 3′, containing a *Nde*I site (CATATG). The sequence of its reverse primer was 5′ CTG CAG CTC GAG CTA GTC CTG AAG CAG GCT CC 3′, containing a *Xho*I site (CTCGAG). The PCR product was gel purified, double digested by using *Nde*I and *Xho*I, and ligated into the pET28a expression vector digested by the same restriction enzymes, resulting in the plasmids pET28a::IBD. The positive colony with the expected gene was identified by single and double restriction digestion of the plasmid, followed with agarose gel analysis, and confirmed further by DNA sequencing.

2.5. Construction of mutants

A QuikChange mutagenesis kit (Stratagene) was applied for constructing the mutant expression plasmids. The plasmid pE-

T28a::SBCAD or pET28a::IVD or pET28a::IBD was used as a template for constructing mutant expression plasmids through PCR reaction. PCR amplification was performed using *Pfu* DNA polymerase and samples were subjected to 13 cycles of 0.5 min of denaturation at 95 °C, 1 min of annealing at 60–63 °C, and 12 min of elongation at 72 °C in a Mastercycler (Eppendorf). The mutant-carrying plasmid was transformed into *E. coli* HB101 competent cells (Novagen) through electroporation (Bio-Rad) for screening purposes. Positive clones were identified and the DNA sequenced to verify the presence of the desired mutations and the absence of any PCR-generated random mutations. Plasmids were then transformed in *E. coli* strain BL21(DE3) cells for expression purposes.

2.6. Expression and purification of soluble proteins

Established methods were again used to prepare the samples [19]. The proteins, all observed to be apparent homogeneity by SDS–PAGE, were stored at −80 °C in 50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol.

2.7. Assay for dehydrogenase activity

The activity of enzyme was assayed spectrophotometrically following the decrease in absorbance at 600 nm using phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) as intermediate and terminal electron acceptor respectively (ε_{600nm} = 21 mM^{-1} cm^{-1}), as described previously [19]. The reaction progress curves were recorded for 1 min on a Hitachi U-2000 UV–visible spectrophotometer. A standard assay mixture contained 33 μM corresponding branched-chain acyl-CoA, 1.5 mM PMS, 48 μM DCPIP, 20 mM phosphate buffer, pH 7.4, 30 μM EDTA, and the final volume was 0.7 mL. The reaction was started by adding 5 μL of appropriate diluted enzyme in the reaction mixture. Unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute. Determination of the K_M and the V_{max} was performed using the same assay buffer with varying substrate concentrations ranging from 1 to 75 μM.

2.8. Assay for isomerase activity

3-Enoyl-CoA thiolester was prepared from the corresponding free acid and coenzyme A by the mixed anhydride method [20] and subsequently purified by reverse-phase HPLC. *cis*-3-Enoyl-CoAs were made from corresponding *cis*-3-enoic acids, which showed different retention time on HPLC profiles from those of corresponding *trans*-3-enoyl-CoAs. A previously described method was used for assaying the activity of enoyl-CoA isomerase [21,22]. The assay mixture contained 50 mM of potassium phosphate (pH 7.5), 100 μM EDTA, 50 μM 3-enoyl-CoA, and appropriate amount of enzyme in a final volume of 1.0 mL. The reaction mixtures were preincubated for 1 min at 25 °C, and were initiated by the addition of the enzyme. The increase in absorbance at 263 nm was monitored, which is characteristic for the conjugated α,β-unsaturated thioester group (ε = 6700 M^{-1} cm^{-1}). One enzyme unit (U) was defined as the activity that converts 1 μmol of 3-enoyl-CoA to 2-enoyl-CoA per minute. Determination of the K_M and

Table 1
Comparative studies on substrate specificity.

SBCAD		IVD		IBD	
(S)-2-Methyl-butyryl-CoA					
K_M (μM)	2.1 ± 0.5	K_M (μM)	1.9 ± 0.3	ND	
k_{cat} (s^{-1})	2.5 ± 0.2	k_{cat} (s^{-1})	0.30 ± 0.01		
k_{cat}/K_M (s^{-1} μM^{-1})	1.2	k_{cat}/K_M (s^{-1} μM^{-1})	0.16		
Isovaleryl-CoA					
ND		K_M (μM)	2.9 ± 0.7	ND	
		k_{cat} (s^{-1})	1.5 ± 0.1		
		k_{cat}/K_M (s^{-1} μM^{-1})	0.52		
Isobutyryl-CoA					
ND		ND		K_M (μM)	11 ± 2
				k_{cat} (s^{-1})	2.1 ± 0.2
				k_{cat}/K_M (s^{-1} μM^{-1})	0.19
Butyryl-CoA					
K_M (μM)	36 ± 8	K_M (μM)	24 ± 3	K_M (μM)	15 ± 1
k_{cat} (s^{-1})	0.90 ± 0.09	k_{cat} (s^{-1})	0.25 ± 0.04	k_{cat} (s^{-1})	1.2 ± 0.1
k_{cat}/K_M (s^{-1} μM^{-1})	0.025	k_{cat}/K_M (s^{-1} μM^{-1})	0.010	k_{cat}/K_M (s^{-1} μM^{-1})	0.080
Hexanoyl-CoA					
K_M (μM)	63 ± 10	K_M (μM)	27 ± 1	K_M (μM)	37 ± 2
k_{cat} (s^{-1})	0.070 ± 0.021	k_{cat} (s^{-1})	0.18 ± 0.04	k_{cat} (s^{-1})	0.31 ± 0.02
k_{cat}/K_M (s^{-1} μM^{-1})	0.0011	k_{cat}/K_M (s^{-1} μM^{-1})	0.0067	k_{cat}/K_M (s^{-1} μM^{-1})	0.0084
Octanoyl-CoA					
K_M (μM)	72 ± 14	K_M (μM)	62 ± 22	K_M (μM)	70 ± 8
k_{cat} (s^{-1})	0.041 ± 0.012	k_{cat} (s^{-1})	0.16 ± 0.06	k_{cat} (s^{-1})	0.11 ± 0.01
k_{cat}/K_M (s^{-1} μM^{-1})	0.00057	k_{cat}/K_M (s^{-1} μM^{-1})	0.0026	k_{cat}/K_M (s^{-1} μM^{-1})	0.0016
Decanoyl-CoA					
ND		K_M (μM)	63 ± 19	ND	
		k_{cat} (s^{-1})	0.14 ± 0.01		
		k_{cat}/K_M (s^{-1} μM^{-1})	0.0022		
Dodecanoyl-CoA					
ND		K_M (μM)	67 ± 10	ND	
		k_{cat} (s^{-1})	0.091 ± 0.005		
		k_{cat}/K_M (s^{-1} μM^{-1})	0.0014		
Myristoyl-CoA					
ND		ND		ND	

ND, no detectable activity.

the V_{max} was performed using the same assay buffer with varying substrate concentrations.

2.9. HPLC analysis of enzymatic incubation mixture

Enzymatic incubations were carried out at 37 °C and the mixtures were analyzed with HPLC. Incubation reactions were terminated by filtering through a Microcon YM-10 filter (Millipore) with centrifugation. The filtrate was applied to a HPLC C_{18} reverse-phase column (3.9 × 150 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 260 nm. Separation was achieved by linearly increasing the methanol/H_2O (85%, v/v) content of 25 mM potassium phosphate elution buffer (pH 5.9) from 20% to 80% in 35 min at a flow rate of 0.8 mL/min.

3. Results and discussion

3.1. Comparative characterization of SBCAD, IVD, and IBD

Rat liver SBCAD, IVD, and IBD were cloned, and their mutants were constructed using molecular biology techniques. These rat enzymes have high sequence homology with their corresponding human enzymes, especially their corresponding catalytic residues are all the same. Since the early studies of these enzymes have been carried out in rat models, and preclinical trials have to be carried out before clinical trials in medical research, we chose to study rat enzymes for better comparison with those early results. All the wild type and variant enzymes were purified to be more than 95% pure. Determination of their K_M and V_{max} values was performed, and the averages of two assays were used for each point. The result was obtained as shown in Table 1. The straight chain substrate specificity of these three enzymes was also determined as shown in Table 1. When extending acyl chain of substrate, the K_M values increased, while their k_{cat} values decreased. The shorter acyl chain substrates were more suitable for these enzymes. Rat IVD could catalyze the conversion of substrates with up to 12 carbons in the acyl chain, while the other two enzymes could catalyze the conversion of substrates with up to 8 carbons only in the acyl chain. Besides, rat IVD could accommodate two different branched-chain substrates, while the other two enzymes could only accommodate one branched-chain substrate. These results indicated that the active site of IVD should be a little larger than those of the other two enzymes. The k_{cat} values for IVD are relatively low compared with those of the other two enzymes. These may be related with the position of its catalytic residue, which is not aligned with those of the other two enzymes (Fig. 2).

3.2. Comparative inactivation studies

Several mechanism-based inhibitors have been designed to elucidate the mechanisms of reactions catalyzed by ACDs. The mechanism for the inhibition of MCAD by 2-octynoyl-CoA has been extensively studied by Thorpe and coworkers, and it has been shown to be a mechanism-based irreversible inhibitor [23,24]. In our laboratory, oct-2-yn-4-enoyl-CoA has also been previously synthesized and confirmed to be an irreversible inhibitor of rat MCAD [25,26]. Both 2-octynoyl-CoA and oct-2-yn-4-enoyl-CoA were used in the present study to comparatively investigate the reactions catalyzed by rat SBCAD, IVD, and IBD. We found that only

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SBCAD      1 KSSQPEALLSVTNNALCFAPLQTFTDEDIMMQKAVKKFAQEQIAPLVSTMDENS--KMEK 58
IVD        1 -----HSMLPVDD------DINGLNEEQKQLRHTISKFVQENLAPKAQEIDQSNDFKNLR 49
IBD        1 -----LAQTDHRSITFCIDPSLGLNEEQKGFQKVAFDFAAREMAPNMAEWDQKE--LFPV 53
                    :     .          :.:*:  :::.  .*. .::**     *:..

SBCAD     59 SVIQGLFQQGMMGIEVEAKYGGTEASFLCSVLVIEELAKVDASVALLCDIQNTVINKLFR 118
IVD       50 EFWKQLGSLGVLGITAPVQYGGSGLGYLEHVLVMEEISRASAAVGLSYGAHSNLCINQIV 109
IBD       54 DVMRKAAQLGFGGIYVRTDVGGSGLSRLDTSVIFEALATGCTSTTAYISIHN-MCAWMID 112
              .. :   . *. ** . .. **:  . *   :::* ::   ::.    . :. :    :

SBCAD    119 KHGTEEQKATYLPKLVT-EKLGSFCLSEAGAGSDSFALKTRADKSGNYYVINGSKMWISN 177
IVD      110 RNGNEAQKEKYLPKLISGEFIGALAMSEPNAGSDVVSMRLKAEKKGDHYVLNGNKFWITN 169
IBD      113 SFGNEEQRHKFCPPLCTMEKFASYCLTEPGSGSDAASLLTSAKRQGDHYILNGSKAFISG 172
                *.* *: .: * * : * :.: .::*..:***  ::   *.:.*::*::**.* :*:.

SBCAD    178 AEHAELFLVFANVDPPSG--YRGITCFLVDRDTEGFQIGRRENKMGIRASSTCQLTFENV 235
IVD      170 GPDADVLVVYAKTDLTAVPASRGITAFIVEKDMPGFSTSKKLDKLGMRGSNTCELVFEDC 229
IBD      173 GGESDIYVVMCRTGGSGP---KGISCIVVEKGTPGLSFGKKEKKVGWNSQPTRAVIFEDC 229
              . .::: :* .... ..    :**:.::*::.  *:. .:: .*:* ... *  : **:

SBCAD    236 KVPETSVLGKIGHGYKYAIGSLNEGRIGIAAQMLGLAQGCFDYTIPYIKERMQFGKRIFD 295
IVD      230 KVPAANILSQESKGVYVLMSGLDLERLVLAGGPLGIMQAVLDHTIPYLHVREAFGQKIGQ 289
IBD      230 AVPVANRIGTEGQGFLIAMKGLNGGRINVASCSLGAAHASVVLTQEHLKVRKQFGAPLAR 289
              ** :. :.  .:*    : .*:  *: :*.  **  :. .  *  ::: *  **  :

SBCAD    296 FQGLQHQVAHVATQLEAARLLTYNAARLVEAG-RPFIKEASMAKYYASEVAGLTTSKCIE 354
IVD      290 FQLMQGKMADMYTRLMACRQYVYNVARACDEG-HITAKDCAGVILYTAECATQVALDGIQ 348
IBD      290 SQYLQFQLADMATKLVASRLMIRTAAVALQEEREDAVALCSMAKLFVTEECFTICNQALQ 349
              * :* ::*.: *:* *.*    ..*   :   .     .: .  :.:* .     . ::

SBCAD    355 WMGGVGYTKDYPVEKFFRDAKIGTIYEGTSNIQLNTIAKHIDAEY- 399
IVD      349 CLGGNGYINDFPMGRFLRDAKLYEIGGGTSEVRRLVIGRAFNADFR 394
IBD      350 MHGGYGYLKDYAVQQYMRDSRVHQILEGSNEVMRMLISRSLLQD-- 393
              ** ** :*:.: :::**:::  *  *:.::    *.: :  :
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Fig. 2. The sequence alignment of rat SBCAD, IVD, and IBD. The catalytic residue Glu (E) and its corresponding residues of the other enzymes are highlighted. Residues conserved in all sequences are marked with ∗. Residues not conserved in all sequences but conserved in some sequences are marked with: or. based on the degree of conservation. The sequence alignment was made using ClustalX [34,35].

SBCAD was inactivated by both 2-octynoyl-CoA and oct-2-yn-4-enoyl-CoA, while the activity of the other two enzymes was not affected by either of these two compounds (Fig. 3).

The K_I and k_{inact} of 2-octynoyl-CoA for rat SBCAD were determined to be 26.1 μM and 0.029 min^{-1}, respectively. While the K_I value was similar to that obtained previously for inactivation of MCAD by the same inhibitor [23], the k_{inact} value for this inhibition is much lower. The K_I and k_{inact} of oct-2-yn-4-enoyl-CoA for rat SBCAD were determined to be 19.8 μM and 0.012 min^{-1}, respectively. It should be noted that oct-2-yn-4-enoyl-CoA has been identified as an irreversible inhibitor of MCAD in our earlier study, and the kinetic parameters K_I and k_{inact} for MCAD have been determined to be 11 μM and 0.025 min^{-1} [26]. This result indicated that the active site of SBCAD is relatively similar to those of straight-chain acyl-CoA dehydrogenases such as MCAD, while the active sites of IVD and IBD are relatively different.

3.3. Comparative studies of isomerase activity

In ACD family, the bacterial short-chain acyl-CoA dehydrogenase has been found to have intrinsic isomerase activity besides its normal dehydrogenation activity, which can remove a proton from C2 of 3-butenoyl pantetheine and re-protonate at C4 to form the thermodynamically favored crotonyl pantetheine [27]. The same isomerase reaction has also been observed for MCAD [28,29]. In the present study, we found that rat SBCAD also displayed intrinsic enoyl-CoA isomerase activity, while rat IVD and IBD did not show such intrinsic isomerase activity. This result also

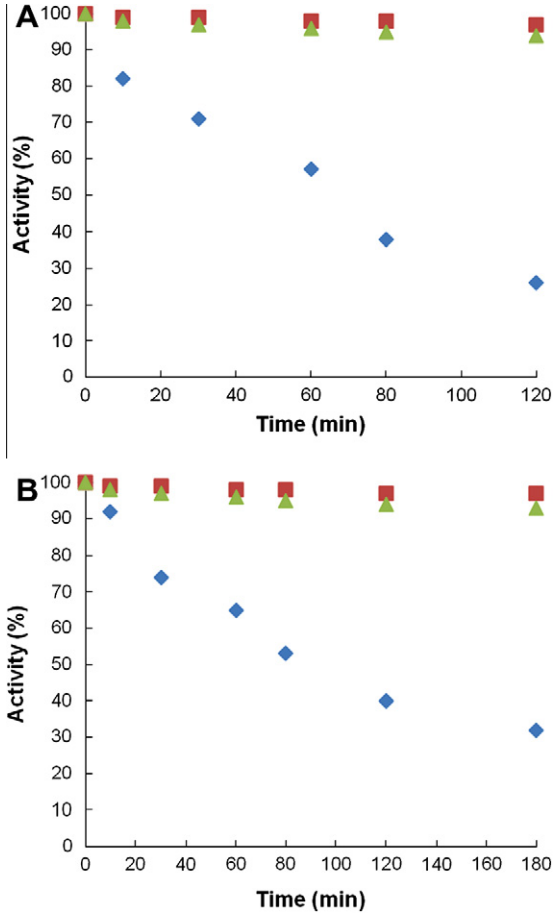

Fig. 3. Inhibition of SBCAD by 2-octynoyl-CoA and 2-octyn-4-enoyl-CoA. (A) Rat SBCAD (♦), rat IVD (▲), and rat IBD (■) were incubated with 5 equivalents of 2-octynoyl-CoA. (B) Rat SBCAD (♦), rat IVD (▲), and rat IBD (■) were incubated with 5 equivalents of 2-octyn-4-enoyl-CoA.

indicated that the active site of SBCAD is relatively similar to those of straight-chain acyl-CoA dehydrogenases.

Both short branched chain and straight chain enoyl-CoAs were chosen as substrates for the steady-state kinetic characterization of the isomerase activity of rat SBCAD, and the result is shown in Table 2. It was found that the k_{cat} value of its isomerase activity for 3-butenoyl-CoA is 23 ± 2 s^{-1}, which is significantly higher than that of its dehydrogenase activity. However, its k_{cat} value dropped a lot to 0.55 ± 0.04 s^{-1} for *trans*-3-hexenoyl-CoA, which might be due to unfavourable binding of *trans*-3-hexenoyl-CoA to the enzyme. The isomerase activity of rat SBCAD for *trans*-3-octenoyl-CoA and longer chain *trans*-3-enoyl-CoAs was not detected. For branched chain substrates, the position of methyl group did not influence substrate binding affinity, but the catalytic efficiency of the enzyme for 2-methyl-3-butenoyl-CoA was 3.6-fold higher than that for 3-methyl-3-butenoyl-CoA, indicating that the methyl group at C2 makes the conformation of substrate much more favorable for the catalysis. The straight chain 3-butenoyl-CoA was found to be better substrate than monomethyl branched chain 3-enoyl-CoA substrates. The introduction of a double bond could make the conformation of 3-butenoyl-CoA more suitable for the catalysis without affecting its binding affinity. The isomerase activity of the enzyme was confirmed through HPLC analysis of incubation mixtures, which was shown in Fig. 4. The further validation of *trans*-2-butenoyl-CoA resulting from isomerization was shown in Fig. 5, because enoyl-CoA hydratase can only add water into double bond on C2. In another control experiment, SBCAD was treated with a modified acid-ammonium sulfate procedure to remove its FAD cofactor [30], and the apoprotein was found to lose its isomerase activity. Its isomerase activity could be recovered after the addition of FAD into SBCAD apoprotein, indicating that the isomerase activity of SBCAD required the participation of FAD cofactor, and was not due to contamination of *E. coli* isomerase.

3.4. Sequence alignment and construction of variant enzyme plasmids

In the above catalytic studies, it was found that catalytic properties of IBD and IVD are similar, in terms of their isomerase activity and enzyme inhibition, while SBCAD is different from these two enzymes. In order to further compare and understand the catalytic properties of rat SBCAD, IVD, and IBD, the sequence alignment of these three enzymes was carried out, and the result is shown in Fig. 2. Glu381 and Glu376 have been confirmed as the catalytic residues of SBCAD [16] and IBD [7] respectively, which are replaced by a glycine (Gly375) at the equivalent position of rat IVD. Interestingly, Glu254 has been found to be the catalytic residue of IVD [17], which is replaced by Gly260 in rat SBCAD and Gly254 in rat IBD at the equivalent position. Therefore, in terms of the position of catalytic residue, SBCAD and IBD are similar, while IVD is different from these two enzymes. These results indicated that SBCAD and IVD are different in both the positions of catalytic residues and their catalytic properties. It is then interesting to know if we can change their catalytic properties through mutating their catalytic residues. Therefore, several mutant expression plasmids, pSBCAD(E381G), pSBCAD(E381D), pSBCAD(G260E), pSBCAD(G260D), pSBCAD(E381G/G260E), pIVD(E254G), pIVD(G375E), and pIVD(E254G/G375E), were constructed through site-directed mutagenesis, and transformed into *E. coli* strain BL21(DE3) for protein expression and purification. All these variant enzymes were purified to apparent homogeneity as shown in supporting information.

3.5. Comparative studies of rat SBCAD and IVD wild-type and variant enzymes

Kinetic studies of the purified rat SBCAD and IVD wild-type and variant enzymes were carried out with *(S)*-2-methylbutyryl-CoA as

Table 2
Kinetic characterization for the isomerase activity of SBCAD wild-type and variant enzymes toward 3-enoyl-CoAs.

	wild-type	G260E	G260D	E381G	E381D	E381/ G260E
2-Methyl-3-butenoyl-CoA						
K_M (μM)	20 ± 2	ND	ND	ND	34 ± 3	ND
k_{cat} (s^{-1})	2.1 ± 0.1	ND	ND	ND	0.090 ± 0.011	ND
k_{cat}/K_M (s^{-1} μM^{-1})	0.11	ND	ND	ND	0.0026	ND
3-Methyl-3-butenoyl-CoA						
K_M (μM)	26 ± 1	ND	ND	ND	28 ± 3	ND
k_{cat} (s^{-1})	0.84 ± 0.01	ND	ND	ND	0.081 ± 0.002	ND
k_{cat}/K_M (s^{-1} μM^{-1})	0.030	ND	ND	ND	0.0029	ND
3-Butenoyl-CoA						
K_M (μM)	23 ± 2	ND	ND	22 ± 1	22 ± 1	ND
k_{cat} (s^{-1})	23 ± 2	ND	ND	0.15 ± 0.01	0.12 ± 0.03	ND
k_{cat}/K_M (s^{-1} μM^{-1})	1.0	ND	ND	0.0068	0.0055	ND
trans-3-Hexenoyl-CoA						
K_M (μM)	45 ± 8	ND	ND	ND	ND	ND
k_{cat} (s^{-1})	0.55 ± 0.04	ND	ND	ND	ND	ND
k_{cat}/K_M (s^{-1} μM^{-1})	0.012	ND	ND	ND	ND	ND

ND, not detectable.

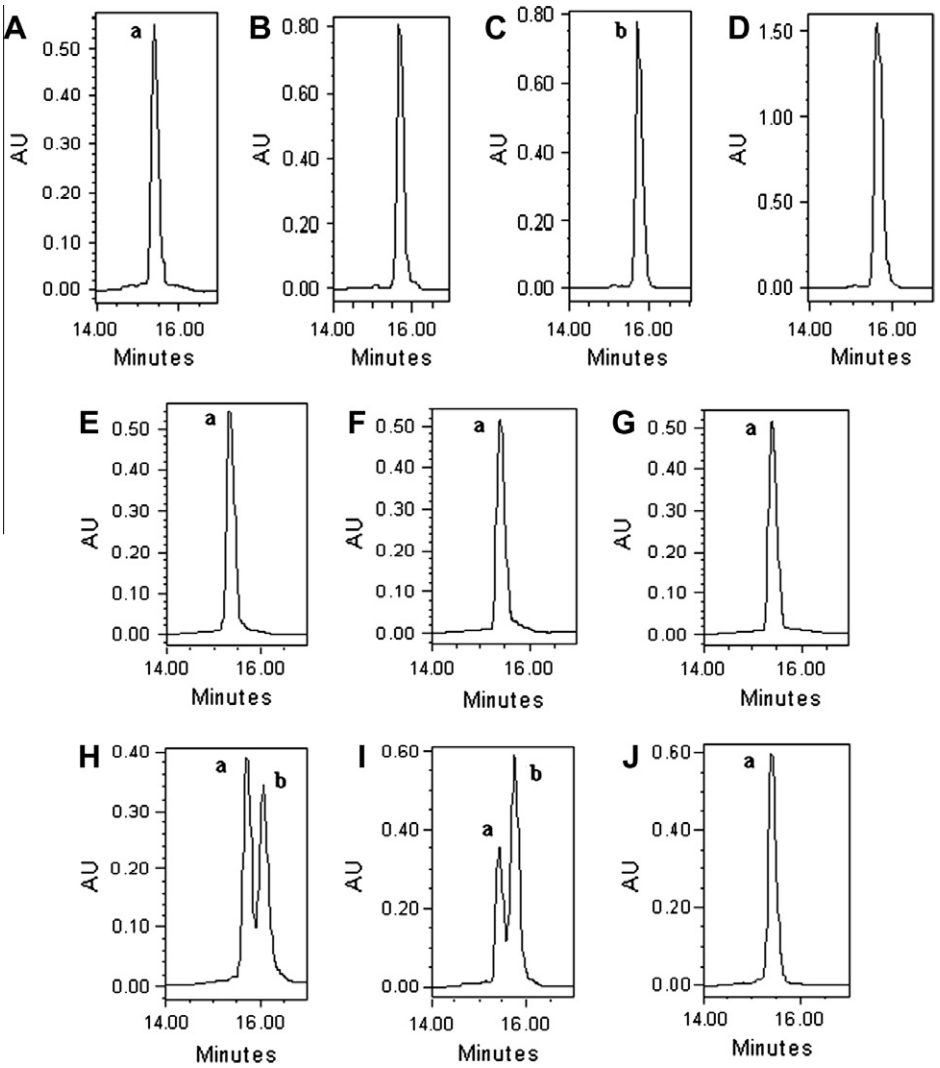

Fig. 4. HPLC analysis for incubation mixture of 3-butenoyl-CoA and SBCAD. A, 3-butenoyl-CoA; B, *trans*-2-butenoyl-CoA; C, incubation of 3-butenoyl-CoA with SBCAD; D, co-injection of B and C; E, incubation of 3-butenoyl-CoA with SBCAD G260E mutant; F, incubation of 3-butenoyl-CoA with SBCAD G260D mutant; G, incubation of 3-butenoyl-CoA with SBCAD E381G/G260E double mutant; H, incubation of 3-butenoyl-CoA with SBCAD E381D mutant; I, incubation of 3-butenoyl-CoA with SBCAD E381G mutant; J, incubation of 3-butenoyl-CoA with IVD. Possible identity of substrate or product peaks: a, 3-butenoyl-CoA; b, *trans*-2-butenoyl-CoA.

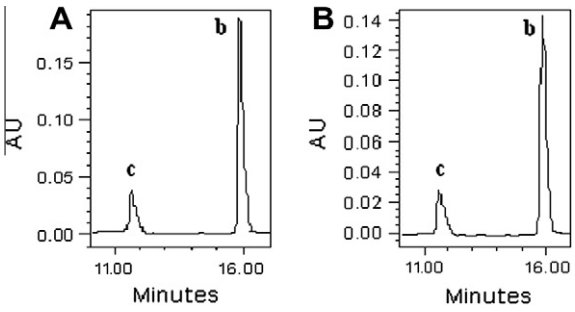

Fig. 5. HPLC analysis of enoyl-CoA hydratase catalyzed conversion for the mixture of SBCAD and 3-butenoyl-CoA. A, Incubation of 3-butenoyl-CoA with SBCAD and enoyl-CoA hydratase (add enoyl-CoA hydratase to mixture C in Fig. 4); B, incubation of *trans*-2-butenoyl-CoA with enoyl-CoA hydratase. Possible identity of substrate or product peaks: b, *trans*-2-butenoyl-CoA; c, 3-hydroxybutanoyl-CoA.

substrate (Table 3). Rat SBCAD wild-type was most active when *(S)*-2-methylbutyryl-CoA was used as the substrate. SBCAD E381G completely lost enzyme activity while E381D remained partially active, which confirmed that the catalytic residue of rat SBCAD was Glu381. The activity of SBCAD G260 variant enzymes was reduced, probably because their side chains affect the substrate binding. SBCAD double mutant E381G/G260E could not convert *(S)*-2-methylbutyryl-CoA, which was different from the result of human SBCAD [16]. It has been reported that human SBCAD double mutant E381G/G260E could restore partial activity. Rat SBCAD E381G and SBCAD E381G/G260E had complete loss of SBCAD activity, which were further confirmed through HPLC analysis of substrate and product peaks in HPLC profiles after enzyme substrate incubation. It has been found that 10 amino acids located in or near the substrate binding pocket, are different between rat and human SBCADs [31], which may explain the above different experimental results.

Rat IVD wild-type enzyme could convert *(S)*-2-methylbutyryl-CoA with low k_{cat} value of only about one tenth of that for rat SBCAD wild-type. IVD G375E had the same k_{cat} value as IVD wild-type enzyme, but lower catalytic efficiency (k_{cat}/K_M) due to its slightly increased K_M value. IVD E254G and IVD double mutant E254G/G375E could not catalyze the conversion of *(S)*-2-methylbutyryl-CoA, indicating that E254 should take part in the dehydrogenation of *(S)*-2-methylbutyryl-CoA. It has been reported that rat IVD wild-type enzyme could not transform *(S)*-2-methylbutyryl-CoA [32,33], which is possibly because their enzyme has been purified from rat liver directly and many steps of purification process may have decreased its enzymatic activity.

Kinetic studies of the purified rat SBCAD and IVD wild-type and variant enzymes were also carried out with isovaleryl-CoA as substrate (Table 4). Rat IVD wild-type enzyme was most active when isovaleryl-CoA was used as the substrate. The IVD E254G lost enzyme activity completely, which confirmed that Glu254 is the catalytic residue of rat IVD, same as that previously suggested for human IVD [15,17]. The activity of IVD G375E was reduced to 11% of that of the wild-type enzyme, suggesting that this mutation might affect the orientation of the substrate in the active site due to the bulky side chain of glutamate. It was found that the catalytic residue E254 of IVD was aligned with G260 of SBCAD, while the catalytic residue E381 of SBCAD was aligned with G375 of IVD. Therefore, IVD E254G/G375E double mutant was constructed to restore the enzyme activity, and its catalytic efficiency was found to be higher than that of IVD G375E single mutant. The double mutant might rearrange the substrate binding pocket to bring α-hydrogen of the substrate close to Glu375 compared with IVD G375E single mutant.

SBCAD wild-type enzyme, two Glu381 mutants, and E381G/G260E double mutant cannot use isovaleryl-CoA as the substrate. The SBCAD E381G/G260E double mutant did not show the activity toward isovaleryl-CoA, and it is possible that the glutamate at 260 position is distant from the α-hydrogen of the substrate. The k_{cat} of SBCAD G260D was higher than that of SBCAD G260E, which suggested that the aspartate was more suitable as catalytic base of variant enzyme. The optimal substrate of IVD has a methyl group at the C3, while the optimal substrate of SBCAD has a methyl group at the C2. IVD can convert the substrate of SBCAD, however SBCAD cannot convert the substrate of IVD. Therefore, IVD has relatively broader substrate specificity than SBCAD.

As mentioned earlier, SBCAD wild-type enzyme had intrinsic isomerase activity, while IVD and IBD did not show the isomerase activity. SBCAD E381D and E381G mutants had about 0.6% of the isomerase activity of the wild-type enzyme when *trans*-3-butenoyl-CoA was used as substrate, as shown in Table 2. Such low activity was confirmed through HPLC analysis, which showed that over 40% of 3-octenoyl-CoA was converted to 2-octenoyl-CoA after incubation of SBCAD E381 mutants with the substrate, as shown in Fig. 4. It is interesting that E381G showed a little isomerase activity, but had no dehydrogenase activity. It is possible that another acidic residue around could function as catalytic residue for its isomerase activity, but could not act as catalytic residue for its dehydrogenase activity. For example, E259 was found to be close to the catalytic residue in the 3D structure of the enzyme. In comparison, SBCAD G260 mutants did not show isomerase activity, which was also confirmed through HPLC analysis, as shown in Fig. 4. One possible reason for the loss of their isomerase activity is that G260 is close to the catalytic residue in the 3D structure, and its mutation affected the function of the catalytic residue. Another possible reason is that the mutation influenced the substrate binding.

Table 3
Kinetic characterization of SBCAD and IVD wild-type and variant enzymes toward *(S)*-2-methylbutyryl-CoA.

	K_M (μM)	V_{max} (μmol/mg/min)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})
SBCAD wild-type	2.1 ± 0.5	3.5 ± 0.2	2.5 ± 0.2	1.2
SBCAD E381G	ND			
SBCAD E381D	3.1 ± 0.5	0.30 ± 0.02	0.22 ± 0.01	7.1×10^{-2}
SBCAD G260E	3.7 ± 1.3	0.38 ± 0.06	0.27 ± 0.04	7.3×10^{-2}
SBCAD G260D	3.0 ± 1.2	0.93 ± 0.16	0.67 ± 0.11	0.22
SBCAD E381G/G260E	ND			
IVD wild-type	1.9 ± 0.3	0.42 ± 0.01	0.30 ± 0.01	0.16
IVD E254G	ND			
IVD G375E	3.5 ± 1.8	0.42 ± 0.09	0.30 ± 0.06	8.6×10^{-2}
IVD E254G/G375E	ND			

ND, no detectable activity.

Table 4
Kinetic characterization of SBCAD and IVD wild-type and variant enzymes toward isovaleryl-CoA.

	K_M (μM)	V_{max} (μmol/mg/min)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})
SBCAD wild-type	ND			
SBCAD E381G	ND			
SBCAD E381D	ND			
SBCAD G260E	5.2 ± 2.6	0.20 ± 0.05	0.14 ± 0.04	2.7×10^{-2}
SBCAD G260D	2.4 ± 0.3	0.98 ± 0.05	0.70 ± 0.04	0.29
SBCAD E381G/G260E	ND			
IVD wild-type	2.9 ± 0.7	2.1 ± 0.1	1.5 ± 0.1	0.53
IVD E254G	ND			
IVD G375E	3.8 ± 1.8	0.24 ± 0.06	0.17 ± 0.04	4.5×10^{-2}
IVD E254G/G375E	3.1 ± 0.4	0.45 ± 0.02	0.32 ± 0.01	0.10

ND, no detectable activity.

In summary, the catalytic properties of rat SBCAD, IVD, and IBD, including their substrate specificity, isomerase activity, and enzyme inhibition, were comparatively studied. Our results indicated that SBCAD has its catalytic properties relatively similar to those of straight-chain acyl-CoA dehydrogenases in terms of their isomerase activity and enzyme inhibition, while IVD and IBD are different. IVD has relatively broader substrate specificity than those of the other two enzymes in accommodating various substrate analogs, which is consistent with those reported previously based on protein crystal structures [7]. The comparison of protein crystal structures of these enzymes have been previously carried out, however, since these enzymes have very similar fold, it is difficult to determine their catalytic differences. It should be noted that three-dimensional structures of the enzymes provide static pictures only, while the enzymes are rather flexible in nature. The present studies of the catalytic properties of these three enzymes compensate the deficiency of previous crystal structural studies, which increased our understanding of the metabolism of monomethyl branched-chain fatty acids (mmBCFAs) and branched-chain amino acids (BCAAs). It is interesting that nature designs three instead of one or two enzymes to convert the short fatty acids from amino acid degradation, which is probably due to the importance of these metabolic pathways. The nature optimizes the efficiency of every pathway, and the deficiency of one enzyme due to genetic error can be partially compensated by another similar enzyme. This study should also be useful for selective control of particular oxidation reaction through the design of specific inhibitors. For example, since IVD has a relatively broader active site, a larger inhibitor should be more specific for IVD. Since only SBCAD has isomerase activity, those inhibitors targeting isomerase activity could also inactivate SBCAD, without affecting the other two enzymes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2012.12.001.

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