Primary Structure and Tissue-specific Expression of Human β-Hydroxyisobutyryl-coenzyme A Hydrolase*

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β-Hydroxyisobutyryl-CoA (HIBYL-CoA) hydrolase is responsible for the specific hydrolysis of HIBYL-CoA, a saline catabolite, as well as the hydrolysis of β-hydroxypropionyl-CoA, an intermediate in a minor pathway of propionate metabolism. We have obtained the amino acid sequences of several tryptic peptides derived from purified rat liver HIBYL-CoA hydrolase, and the NH2-terminal peptide sequence was matched to the translated sequence of a human expressed sequence tag present in the data base of the IMAGE Consortium (Lawrence Livermore National Laboratory, Livermore, CA). The complete nucleotide sequence and the deduced amino acid sequence showed no similarity to the sequences of well known thioesterases but showed significant homology to the enoyl-CoA hydratase/isomerase enzyme family. The cDNA fragment corresponding to the mature (processed) protein was expressed in Escherichia coli. The purified recombinant enzyme displayed substrate specificity very similar to that of the rat enzyme and was specifically bound by polyclonal antibodies raised against purified rat liver HIBYL-CoA hydrolase. Northern and Western blot analyses with various human tissues indicated predominant expression in liver, heart, and kidney, with discrepancies occurring in the amounts of HIBYL-CoA hydrolase mRNA compared to stably expressed protein in several tissues.

β-Hydroxyisobutyryl-CoA (HIBYL-CoA)1 hydrolase (3-hydroxy-2-methylpropanoyl-CoA hydrolase, EC 3.1.2.4) catalyzes the hydrolysis of S-HIBYL-CoA, an intermediate in the valine-catabolic pathway, producing free CoA and the branched-chain acid β-hydroxyisobutyrate (1, 2). In most tissues, the β-hydroxyisobutyrate produced by this reaction is oxidized to methylylmalonate semialdehyde, but from certain tissues it enters the blood stream and serves as a substrate for hepatic gluconeogenesis (3). This aspect of the catabolism of valine differs from that of other branched-chain amino acids that are metabolized solely as CoA esters, and the existence of this enzyme was postulated as a mechanism to protect cells from the effects of the highly toxic metabolite, methacrylyl-CoA (2). HIBYL-CoA hydrolase was partially purified and characterized from pig heart in 1957 (1) and more recently purified to homogeneity from rat liver (2). We report here the amino acid sequences of several tryptic peptides derived from the purified rat enzyme and the complete primary structure of human HIBYL-CoA hydrolase derived from a cDNA clone. The enzyme shows no sequence homology to other well known thioesterases but shows significant amino acid sequence homology to the enoyl-CoA hydratase/isomerase family of enzymes (4), raising interesting questions about the enzymatic mechanism of this novel thioesterase.

EXPERIMENTAL PROCEDURES

Materials—Cells harboring the EST (IMAGE Consortium ID 32783, GenBankTM accession number R20241) were obtained from the IMAGE Consortium (Lawrence Livermore National Laboratory, Livermore, CA). The pET28a expression vector and the HMS174(DE3) Escherichia coli cell line were from Novagen, Inc. (Madison, WI). Ni2+–nitritolactone acid resin was from Qiagen, Inc. (Chatsworth, CA). All restriction enzymes were from Life Technologies, Inc. The DNA sequencing kit was from U.S. Biochemical Corp. The pGEM-T vector was purchased from Promega Corp. (Madison, WI). Multiple human tissue Northern and Western blots were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). All other chemicals were from Sigma.

Peptide Sequencing—To obtain an NH2-terminal amino acid sequence, purified rat liver HIBYL-CoA hydrolase (10 μg) was subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. To obtain internal peptides, the purified enzyme was digested with trypsin, and the resulting peptides were purified by C18 reversed phase high performance liquid chromatography using a 130-A microbore separation system (Applied Biosystems). The amino acid sequences of the blotted protein and purified tryptic peptides were determined by Edman degradation using a P1 2090 microsequencing system (Porton Instruments) in the Biochemistry Biotechnology Facility of Indiana University School of Medicine.

Subcloning of the cDNA for Sequencing and Expression—The published sequence of EST 32783 was first confirmed by dyeoxy DNA sequencing. To subclone the cDNA fragment corresponding to the mature peptide, as required for prokaryotic expression, a polymerase chain reaction primer (5′-AAAGCGGCGGCGAGAAGGTGCTATTGGAA-3′) was synthesized corresponding to the NH2 terminus of the mature protein sequence, and also including a NotI restriction site. M13 forward primer (5′-TAAACGACGGCCAGT-3′) was used as the downstream primer and amplifies a NotI site present in the plasmid carrying the EST. These primers were used to amplify the sequence using the purified EST DNA as template. A single product of 1.6 kb was obtained and subcloned into pGEM-T vector (Promega). This subclone, pGEM-T-HHYD, was used for complete sequencing of the cDNA. For expression of the cDNA in E. coli, the 1.6-kb cDNA was isolated from the pGEM-T-HHYD plasmid by digestion with NotI and ligated to NotI-digested pET28a, placing the cDNA in the frame with the 6-histidine tag present on this vector. The reading frame and fidelity of this...
expression vector, pET28-HHYD, was confirmed by restriction analysis and dideoxynucleotide DNA sequencing.

DNA Sequencing—Double stranded plasmids were purified and sequenced on both strands, after alkaline denaturation, by the dideoxynucleotide chain termination method with Sequenase version 2.0 (U.S. Biochemical Corp.). The complete nucleotide sequence reported in this paper has been submitted to the GenBankTM/EMBL Data Bank.

Expression and Purification of Recombinant Enzyme—HMS174(DE3) cells were transformed with the pET28-HHYD plasmid and grown at 37°C in TY medium containing kanamycin (70 μg/ml). When the cell culture obtained an absorbance at 600 nm of 0.8, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM, and growth of the cultures was continued for 18–20 h at 37°C.

The recombinant enzyme was purified by nickel chelation chromatography following previously published procedures (6). Approximately 5 mg of purified recombinant enzyme were obtained per liter of E. coli culture.

Circular Dichroism Spectropolarimetry—Circular dichroism spectra were recorded at 21°C with a Jasco J-720 spectropolarimeter. Spectra were recorded with a cell path length of 0.1 cm and a wavelength range of 300–190 nm. Each spectrum was averaged from four separate recordings.

Enzyme Assay—CoA thioester hydrolase activities were measured spectrophotometrically as described previously (2), using a Cary 1E spectrophotometer.

Northern Blot Analysis—Multiple human tissue Northern blots, obtained from Clontech, were probed with random primed 32P-labeled probe derived from rat HIBYL-CoA hydrolase cDNA and hybridization conditions were as follows: 5 × saline/sodium/phosphate/EDTA (1 × saline/sodium/phosphate/EDTA = 180 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA, pH 7.5), 10 × Denhardt’s solution, 0.5% (w/v) SDS, 50% (v/v) deionized formamide, 0.1 mg/ml denatured salmon sperm DNA, and radiolabeled probe (2 × 106 cpm/ml) at 42°C for 12 h. Blots were washed four times in 2 × standard saline-citrate, 0.5% (v/v) SDS at room temperature, and twice in 0.1 × standard saline-citrate, 0.5% (v/v) SDS at 55°C for 15 min and then subjected to autoradiography.

Western Blot Analysis—For Western blot analysis of rat tissues extracts, 1 g of freeze-clamped rat tissues was homogenized in 5 volumes of a buffer consisting of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3 mM dithiothreitol, 1 mM N-p-tosyl-L-lysine chloromethyl ketone, 0.1 mg/ml hen egg white trypsin inhibitor, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 0.5% Triton X-100. The homogenates were centrifuged at 60,000 × g at 4°C for 30 min. Equal amounts of protein (20 μg) from each extract were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated at 37°C for 30 min in TBST buffer containing 3% bovine serum albumin to block nonspecific binding of antibodies. The membranes were then incubated at 4°C for 18 h in the same solution with the addition of polyclonal rabbit antiiserum specific for HIBYL-CoA hydrolase. This polyclonal antiserum specifically detected 10 ng of purified rat liver HIBYL-CoA hydrolase at a working dilution of 1:10,000. The membrane was washed five times for 5 min in TBST buffer at room temperature. The membrane was then incubated with 125I-labeled protein A (4.5 μCi, 20 μCl/μg) in TBST buffer containing 3% bovine serum albumin at room temperature for 1 h. Finally, the membranes were washed as above and subjected to autoradiography. For Western blot analysis of various human tissues, a human multiple tissue Northern blot, obtained from Clontech, was probed with random primed 32P-labeled probe derived from rat HIBYL-CoA hydrolase NH2-terminal and trypsin peptides were undetected.

RESULTS AND DISCUSSION

Sequencing of Rat HIBYL-CoA Hydrolase Tryptic Peptides—The purified rat HIBYL-CoA hydrolase displayed an unblocked amino terminus as is characteristic of mitochondrial proteins. The 24-residue NH2-terminal sequence was obtained as follows: ETAEVLLERG?AGVITLNRPKLL. Four tryptic peptides obtained, three were of sufficient purity for determining the amino acid sequence by Edman degradation. The sequences of these peptides are as follows: PETGIGLFPDVGGGYFLP, AGIATHFVDSEKL?VL, EGHDFHEV.

Sequencing of HIBYL-CoA Hydrolase cDNA—The NH2-terminal amino acid sequence determined for rat HIBYL-CoA hydrolase matched with 75% identity to a translated human EST (GenBank accession number R20241) present in the data base of the IMAGE Consortium. This clone was obtained and found to contain an open reading frame of 1146 base pairs encoding a protein of 352 amino acid residues with a calculated molecular weight of 39,398 (Fig. 1). The calculated molecular weight is close in size to the previously reported molecular weight of rat HIBYL-CoA hydrolase (36,000) estimated by SDS-PAGE. Furthermore, sequences matching each of the peptides derived from rat HIBYL-CoA hydrolase were present with high levels of homology (83, 88, and 75% identities) to the human cDNA (Fig. 1). The human cDNA encodes a 28-amino acid mitochondrial leader sequence (residues −28 to −1) which contains numerous basic residues (Arg and Lys) which align on a single face when applied to a helical wheel, as is characteristic of mitochondrial leader sequences (6).

Expression of Recombinant HIBYL-CoA Hydrolase in E. coli—Further evidence that this cDNA encodes HIBYL-CoA hydrolase was obtained by prokaryotic expression studies. Expression from the pET28-HHYD plasmid and purification of recombinant, 6-histidine-tagged protein by nickel chelation chromatography resulted in the isolation of homogenous protein with a molecular weight of ~40,000 as determined by SDS-PAGE (data not shown). This purified enzyme hydrolyzed HIBYL-CoA with a specific activity slightly over 400 units/mg of protein, similar to the specific activity of native rat HIBYL-CoA hydrolase (Table I). Also similar to the native rat enzyme, the purified recombinant enzyme hydrolyzed 3-hydroxypropionyl-CoA at approximately one-half the rate of hydrolysis of HIBYL-CoA (Table I). Such high levels of acyl-CoA thioesterase activity were observed with no other substrates tested, although slower rates of hydrolysis could be measured with several structurally related substrates in the order: 1,3-hydroxybutyryl-CoA > Dl-3-hydroxybutyryl-CoA > isobutyryl-CoA > 3-hydroxyisovaleryl-CoA (Table I). Thus, the enzyme is highly specific with regard to the acyl-CoA substrate.

Fig. 1. Nucleotide and deduced amino acid sequences of human HIBYL-CoA hydrolase. Numbering begins at the 5′ end for the nucleotide sequence and at position −28 for the deduced amino acid sequence. Positions −28 through 1 of the deduced amino acid sequence represent the mitochondrial leader sequence. Amino acid sequences of the purified rat HIBYL-CoA hydrolase NH2-terminal and trypsin peptides are underlined.

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nant enzyme was also specifically bound by polyclonal antibodies raised against the purified native rat liver HIBYL-CoA hydrolase. Western blot analysis of the purified recombinant HIBYL-CoA hydrolase revealed a single, cross-reacting species of with a molecular weight of ~40,000 (data not shown).

**CD Spectropolarimetry**—Circular dichroism spectropolarimetry was also used to compare the structural similarity of the recombinant human and native rat liver enzymes. The CD spectra of the purified enzymes were found to be almost identical (data not shown). For comparison of the spectra, the ratio of molar ellipticities at 208 and 222 nm was calculated and found to be 0.94 ± 0.05 for both rat and human HIBYL-CoA hydrolases. This ratio is considered useful as a comparative index of the α-helical content of related proteins (7) and therefore suggests a high level of secondary structural similarity between the human and rat HIBYL-CoA hydrolases.

**Tissue-specific Expression**—Because of its essential role in the catabolism of valine, the tissue distribution of stably expressed HIBYL-CoA hydrolase is of considerable interest. Previous studies of HIBYL-CoA hydrolase activities in various rat tissue extracts demonstrated a tissue-specific distribution of activity with the highest activities in liver, heart, and kidney (2). Northern blot analysis of mRNA from various human tissues with a human HIBYL-CoA hydrolase cDNA probe showed predominant message of ~2 kb. Densitometric analysis indicated levels of expression in skeletal muscle and demonstrated that the RNA present on the blot was

| CoA ester                   | Units/mg | %  |
|-----------------------------|----------|----|
| S-HIBYL-CoA                 | 462      | 100|
| 3-Hydroxypropionyl-CoA      | 249      | 53.9|
| L-3-Hydroxybutyryl-CoA       | 2        | 0.43|
| M-3-Hydroxybutyryl-CoA       | 1.6      | 0.35|
| Isobutryl-CoA                | 0.6      | 0.13|
| 3-Hydroxyisovaleryl-CoA      | 0.3      | 0.06|

* The enzyme activity was assayed under standard conditions. For HIBYL-CoA and 3-hydroxypropionyl-CoA, 0.01 µg of enzyme protein was used; for other substrates, 1.0 µg of enzyme protein was used.

b Generated from methacrylyl-CoA during the assay by crotonase.

c Generated from acrylyl-CoA during the assay by crotonase.

d Generated from crotonyl-CoA during the assay by crotonase.

e Generated from 3-methylcrotonyl-CoA during the assay by crotonase.
Compared with mRNA, Western blot analysis also demonstrated tissue-specific expression of stably expressed HIBYL-CoA hydrolase protein, however, with a quantitatively different tissue distribution as compared to mRNA levels. Analysis of various human tissues showed a Mr 39,000 protein which is highly reactive with the rat HIBYL-CoA hydrolase antiserum. Densitometric analysis indicates levels of expression liver > kidney > heart > muscle = brain (Fig. 3A). The same Mr 39,000 protein is also highly expressed in rat liver and heart but less predominantly in kidney as compared with human kidney and is also present at low levels in muscle and brain (Fig. 3B). Thus, the tissue distribution of stably expressed HIBYL-CoA hydrolase protein appears to be similar in rats and humans but different from the distribution of specific mRNA in the same human tissues. The largest discrepancy between mRNA and protein levels occurs in soleus muscle. Although the cause of this discrepancy is not currently known, it is possible that translation of the HIBYL-CoA hydrolase mRNA is highly regulated in muscle or that the enzyme is less stable in this tissue. Furthermore, another species with a molecular weight of ~42,000 appears to cross-react with antibodies present in the HIBYL-CoA hydrolase antiserum. This species appears to be most highly expressed in human and rat heart as well as brain and soleus muscles of both rat and human origin (Fig. 3). Another high molecular weight cross-reacting species is also specifically expressed in the soleus muscles of both rat and human origin but not detected in any other tissue. The identities of the higher molecular weight cross-reacting species are currently not known. It is possible that these species represent proteins that share some epitopes present on HIBYL-CoA hydrolase. It is also possible that these proteins represent posttranslationally modified forms of HIBYL-CoA hydrolase, other isoforms of HIBYL-CoA hydrolase, or structurally similar enzymes with different functions. Indeed, the peptide sequence data and CD spectra of rat and human HIBYL-CoA hydrolase suggest a high degree of structural similarity, and the structures of these enzymes might include highly antigenic elements present on other related, but unidentified, enzymes. Further studies will be required to elucidate the nature of the presently unidentified proteins that cross-react with HIBYL-CoA hydrolase antibodies and whether these proteins are structurally or functionally related to HIBYL-CoA hydrolase.

Amino Acid Sequence Homology with Enoyl-CoA Hydratases/Isomerases—Surprisingly, the deduced amino acid sequence of human HIBYL-CoA hydrolase (HCHYD) was aligned with the sequences of human mitochondrial enoyl-CoA hydratase (MECH), human mitochondrial 3,2-trans-enoyl-CoA isomerase (MECI), dihydroxynaphthoate synthase (DHNAS) of Bacillus subtilis, and 4-chlorobenzoyl-CoA dehalogenase (CDBC) of Pseudomonas sp. All sequences, except for HIBYL-CoA hydrolase, were obtained from NCBI data bases, and alignments were performed using the NCBI BLAST network service. Residues identical in more than 50% of the sequences are shown in black with reversed text. Partially conserved residues are shown in gray with reversed text.

**FIG. 4.** Amino acid sequence homology of human HIBYL-CoA hydrolase with enoyl-CoA hydratases/isomerases. The deduced amino acid sequence of human HIBYL-CoA hydrolase (HCHYD) was aligned with the sequences of human mitochondrial enoyl-CoA hydratase (MECH), human mitochondrial 3,2-trans-enoyl-CoA isomerase (MECI), dihydroxynaphthoate synthase (DHNAS) of Bacillus subtilis, and 4-chlorobenzoyl-CoA dehalogenase (CDBC) of Pseudomonas sp. All sequences, except for HIBYL-CoA hydrolase, were obtained from NCBI data bases, and alignments were performed using the NCBI BLAST network service. Residues identical in more than 50% of the sequences are shown in black with reversed text.
with methacrylyl-CoA and acrylyl-CoA, respectively. Thus, the pathway for valine catabolism appears to utilize two subsequent reactions catalyzed by members of the enoyl-CoA hydratase/isomerase enzyme family, which most likely share a common evolutionary origin. The existence of a highly specific CoA-thioesterase in this catabolic pathway was proposed as a possible mechanism to protect cells from the effects of the highly toxic metabolite, methacrylyl-CoA (2). It is possible that the evolution of a thioesterase structurally related to enoyl-CoA hydratase provides this metabolic pathway with a highly active thioesterase which is also sufficiently specific to prevent hydrolysis of other short chain thioesters of metabolic importance such as acetyl-CoA and propionyl-CoA. While much data are available concerning the enzymatic mechanisms of more common thioesterases, considerably less is known about the enzymatic mechanism of enoyl-CoA hydratases. However, site-directed mutagenesis studies of mitochondrial enoyl-CoA hydratase and mitochondrial 3,2-trans-enoyl-CoA isomerase suggest that a conserved glutamate residue may function as a critical active site residue in both of these enzymes (4). Although the reaction catalyzed by HIBYL-CoA hydrolase is quite different from those of the mitochondrial hydratase and isomerase, it is interesting that the conserved glutamate residue proposed as an active site residue in the former enzymes is also conserved in HIBYL-CoA hydrolase as glutamate 136. However, it is not presently known if this residue is important in the catalytic mechanism of this enzyme.

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