**Molecular Cloning and Characterization of (R)-3-Hydroxybutyrate Dehydrogenase from Human Heart**

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The complete amino acid sequence of human heart (R)-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) has been deduced from the nucleotide sequence of cDNA clones. This mitochondrial enzyme has an absolute and specific requirement of phosphatidylcholine for enzymic activity (allosteric activator) and is an important prototype of lipid-requiring enzymes. Despite extensive studies, the primary sequence has not been available and is now reported. The mature form of the enzyme consists of 297 amino acids (predicted M₉ of 33,117), does not appear to contain any transmembrane helices, and is homologous with the family of short-chain alcohol dehydrogenases (SC-ADH) (Persson, B., Krook, M., and Jornvall, H. (1991) *Eur. J. Biochem.* 200, 537–543) (30%) residue identity with human 17β-hydroxysteroid dehydrogenase). The first two-thirds of the enzyme includes both putative coenzyme-binding and active site conserved residues and exhibits a predicted secondary structure motif (alternating α-helices and β-sheet) characteristic of SC-ADH. Bovine heart peptide sequences (174 residues in nine sequences determined by microsequencing) have extensive homology (89% identical residues) with the deduced human heart sequence. The C-terminal third (Asn-194 to Arg-297) shows little sequence homology with the SC-ADH and likely contains elements that determine the substrate specificity for the enzyme including the phospholipid (phosphatidylcholine) binding site(s). Northern blot analysis identifies a 1.3-kilobase mRNA encoding the enzyme in heart tissue.

(R)-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) is a mitochondrial membrane enzyme with an absolute and specific requirement of phosphatidylcholine (PC) for enzymic activity (1, 2). It has served as a prototype to study the role of lipid and in turn the nature of lipid-protein interactions in membranes (3–6). The enzyme has been purified from bovine heart (7) and rat liver mitochondria (8, 9) and more recently from rat brain (10). The purified enzyme (apodehydrogenase) is devoid of phospholipid and thereby inactive but can be reactivated by reconstitution with phospholipid vesicles containing PC. The apodehydrogenase inserts spontaneously and unidirectionally into preformed phospholipid vesicles or natural membranes, suggesting that it may be amphipathic (11). Optimal activation of the enzyme is obtained only with membranes containing PC (12, 13). However, a phospholipid bilayer is not essential for function since soluble PC (below the critical micellar concentration) will activate the enzyme (12, 14). Target inactivation analysis indicates that the enzyme is a tetramer in the mitochondrial inner membrane and after reconstitution into phospholipid bilayers (15). The activation of 3-hydroxybutyrate dehydrogenase by bilayer PC appears to involve site-site interaction (16) and an allosteric mechanism in which PC enhances binding of nucleotide (coenzyme) by more than an order of magnitude (17).

Chemical derivitization studies have provided insight into structure-function relationships for this novel lipid-requiring enzyme; however, progress has been limited by the lack of the primary sequence. In this study, we report the complete amino acid sequence of human heart (R)-3-hydroxybutyrate dehydrogenase as determined by molecular cloning and partial peptide sequence for the bovine heart form of the enzyme.

**MATERIALS AND METHODS**

_Enzymatic Digestion and Peptide Sequencing—(R)-3-Hydroxybutyrate dehydrogenase_ was purified from bovine heart mitochondria as described previously (7, 9). The protein (1 nmol) was digested with 1 μg of _Achromobacter endoproteinase_ Lys-C (Wako Chemicals, Dallas, TX) in 20 μl of 100 mM ammonium bicarbonate for 5 h at 37 °C. The resulting peptide mixture was then reduced by adding 1 μl of β-mercaptoethanol and incubating for 30 min at 37 °C; it was subsequently S-pyridylethylated with 3 μl of 4-vinylpyridine, also for 30 min at 37 °C, under argon. Peptides were then separated on a Vydac 4.6-mm C₄ column (The Separations Group, Hesperia, CA) as described (18) and sequenced using a modified (19) Applied Biosystems 477 automated sequenator (Applied Biosystems Inc., Foster City, CA). The N-terminal sequence of the enzyme was determined without modification of the protein. An additional peptide, corresponding to...
the C-terminal of the protein, was prepared by cyanogen bromide cleavage, purified by high pressure liquid chromatography, and sequenced using an Applied Biosystems 475A sequenator equipped with a 900A data system controller as described (20, 21).

Cloning—Degenerate synthetic oligonucleotide probes were synthesized based on the amino acid sequence obtained from bovine (R)-3-hydroxybutyrate dehydrogenase peptides as described above. The four synthetic oligonucleotides (each labeled according to the peptide from which the sequence was derived) were synthesized as follows (degenerate nucleotides are listed in parentheses; I denotes inosine): 1) antisense BDH KC9, 5'-(T/A)CC(G/A) TC(G/C) AC CC(G/A) AA-3', based on the peptide sequence Phe-Gly-Val-Glu-Ala, and the first 2 base pairs of Phe; 2) antisense BDH KC10, 5'-ATI CC(G/A) G(C/G) ATC(G/C) AG I(A/H) CCC CA-3', based on the peptide sequence Trp-Gly-Leu-Val-Asn-Ala-Gly-Hle, and the first 2 base pairs of Ser; 3) antisense BDH KC12, 5'-(G/A)TA (G/A)TC CAT IGG (G/A)TG (G/A)TA-3', based on the peptide sequence Tyr-His-Pro-Met-Tyr-Tyr; 4) sense BDH NTERM: 5'-GCI GCI AG(C/T) GT(G/C) GA(C/T) CCI GTI GG-3', based on the peptide sequence Ala-Ala-Ser-Val-Asp-Pro-Val, and the first 2 base pairs of Gly.

A mixture of these degenerate oligonucleotides was labeled at the 5' end with [γ-32P]ATP and hybridized to nitrocellulose filters containing 0.5 X 10^6 clones from a human heart cDNA library in XZAP II (Stratagene, La Jolla, CA). Hybridization was at 37 °C, and final washing was at 42 °C with 0.2 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Washed filters were autoradiographed for 1-3 days. Isolated clones were sequenced using the deoxyxide chain termination method (22). Sequencing analysis was performed using the MacVector software on a MacIntosh I Icx, the Intelligenetics sequence analysis programs on a Sun Systems computer, or the program, Prozyte (Scientific and Educational Software) on a PC-AT computer.

cDNA Probes—The probe used for Northern blot analysis was the 450-base pair EcoRI/PstI fragment from the 5' region of the BDH cDNA clone (see Fig. 1). All probes were uniformly labeled with [α-32P]dCTP to a specific activity of 10^6 cpm/μg.

RNA Preparation and Northern Blot Analysis—RNA was prepared from rabbit tissues using guanidinium-thiocyanate lysis buffer and centrifugation over a cesium chloride cushion (18, 22). RNA was size-separated on formaldehyde-agarose gels, and Northern blot transfer was carried out overnight using 10 X SSC. Hybridization was at 42 °C overnight, and washing was at 55 °C with 0.2 X SSC. Films were autoradiographed with a single intensifying screen at -70 °C. To assure that equivalent amounts of RNA were present in each lane, the BDH cDNA probe was eluted, and the Northern blots were then hybridized with cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from chicken muscle.

RESULTS AND DISCUSSION

cDNA and Amino Acid Sequence

Nine clones were isolated and sequenced (sequencing strategy is shown in Fig. 1). The largest clone was a 1.3-kb cDNA, which contained the full coding sequence of the mature form of human heart enzyme. The nucleotide sequence and deduced amino acid sequence of the cDNA encoding human heart (R)-3-hydroxybutyrate dehydrogenase (BDH cDNA) are shown in Fig. 2. The corresponding amino acid sequence of the peptides from the bovine heart enzyme, as determined by microsequencing, is shown below the deduced amino acid sequence for the human enzyme. Two bovine peptide sequences determined by Prasad and Hatefi (23) are also included (peptides D and G). In the regions for which bovine heart sequence has been determined (total of 174 residues), there is extensive homology (89% identical residues) between the enzymes from the two species.

The deduced amino acid sequence of the human enzyme (343 residues) indicates the existence of a leader peptide sequence of at least 46 residues, which is cleaved to produce the mature form of (R)-3-hydroxybutyrate dehydrogenase (297 residues). The location of the cleavage site is based on alignment with the N-terminal peptide of the bovine enzyme. The putative cleavage site is consistent with but not identical to such cleavage sites in other mitochondrial precursor proteins (24). The calculated size of the preprotein is 38 kDa and of the mature form of the enzyme is 33,117 Da. The size of the leader peptide (~5 kDa) is comparable to the difference in size between precursor and mature forms of rat liver (R)-3-hydroxybutyrate dehydrogenase measured by immunoprecipitation of polypeptides from both in vitro and in vivo translation products (26).

Secondary Structure Predictions

Apparent Lack of Transmembrane Helices—The hydrophilicity profile (28) of the mature form of (R)-3-hydroxybutyrate dehydrogenase (Fig. 3A, leader peptide deleted) does not indicate hydrophobic stretches sufficient to span a phospholipid bilayer. Similar hydrophilicity profile patterns were obtained using algorithms for antigenicity (29) or exposed residues (30) (not shown). Three additional methods (31-33) also failed to detect transmembrane helices. With the Eisenberg et al. (31) method, the maximal average hydrophilicity value for a 21-residue segment was 0.42 for positions 21-41 in the mature human enzyme, whereas a value of 0.68 is considered minimal for a transmembrane helix.

Secondary Structure Motif—The predicted secondary structure (α-helix, β-sheet, turns, and random coil) of the sequence is illustrated in Fig. 3B. The first 200 amino acids are characterized by alternating segments of α-helix and β-sheet, consistent with the secondary structure motif for this region of the family of short-chain alcohol dehydrogenases (27) to which this enzyme belongs (see below). In the C-terminal region, both the Chou-Fasman (34) and the Garnier et al. (35) methods predict two helices (Fig. 3B, residues 207-225 and 228-241) connected by a predicted turn at Tyr-226 and Gly-227. Recent studies indicate that the C-terminal region of the bovine enzyme is important for binding of (R)-3-hydroxybutyrate dehydrogenase to membranes or phospholipid bilayers (36).

Comparison of Amino Acid Sequence with Known Sequences

Sequence analysis by alignment to either of three data banks (Genbank, v. 69; Swiss-Prot 20; and EMBL 29) reveals substantial homology of human (R)-3-hydroxybutyrate dehydrogenase with the family of short-chain alcohol dehydrogenases (SC-ADH) (27) (see Fig. 2). The proteins in this family have subunits of about 250 amino acids (27). Sequence alignment shows that the 6 strictly conserved residues in all 20 related SC-ADH (residues Gly-14, Gly-19, Asp-64, Gly-69; Swiss-Prot 20, and EMBL 29) are

2 P. Adami, T. M. Duncan, C. E. Carter, C. Fu, M. Melin, N. Latruffe, J. O. McIntyre, and S. Fleischer, manuscript in preparation.
FIG. 2. Nucleotide sequence and deduced amino acid sequence of human heart BDH and alignment with bovine heart BDH peptides. The probable cleavage site to remove the leader peptide from precursor human BDH is indicated by the enlarged arrowhead. Amino acids are numbered from the first residue of mature human BDH. Residues that correspond with positions of conserved amino acids of the short-chain alcohol dehydrogenases are in boldface (77 residues identical in 10 or more of 20 dehydrogenases using the alignment of Persson et al. (27)). Of these residues, 37 are identical in human BDH (indicated by an asterisk), including 5 of the 6 residues (denoted by △) that are identical in all 20 short-chain alcohol dehydrogenases. Underlined residues (Glu-120 and Cys-242) in the human BDH sequence correspond with the first residue of mature human BDH. The N-terminal sequence (AMN) differs from a cyanogen bromide cleavage of bovine heart BDH. In this peptide, no additional residues were detected after His-297, consistent with its location at the C-terminus of bovine BDH.
The other highly conserved residues of the SC-ADH. Of the and Doolittle (28) values of 5 adjacent residues and is plotted at the middle (3rd) residue. Hydrophobic regions deflect downward (positive values) with the longest continuous hydrophobic segments (10 residues each) at positions 33-42 and 182-191 (average hydrophobicity of 1.7 and 1.0, respectively). The graphic representation of the secondary structure of mature human 3-hydroxybutyrate dehydrogenase shown in B is based on the secondary structure prediction method of Chou and Fasman (54) using an average of 4 adjacent residues.

(residues Gly-16, Gly-22, Asn-67, Gly-142, Tyr-162, Lys-166). In addition, the sequence exhibits significant homology with the other highly conserved residues of the SC-ADH. Of the 77 residues that are conserved (i.e., identical) in 10 or more of the 20 dehydrogenases (alignment given in Ref. 27), 37 are identical in human 3-hydroxybutyrate dehydrogenase (denoted by an asterisk in Fig. 2). Alignment of the bovine peptides with the sequence of the human enzyme conforms to this homology; 27 of 59 are conserved in the aligned bovine peptides.

The homology of (R)-3-hydroxybutyrate dehydrogenase (from Lys-10 to Gly-193) with the SC-ADH extends over about the first two-thirds of the complete sequence and encompasses both the putative coenzyme-binding and active site domains of this class of dehydrogenases (27). The coenzyme-binding site was assigned to the N-terminal halves of the short-chain dehydrogenases and includes Gly-14, Gly-17, and Gly-19, which are conserved in human and bovine (R)-3-hydroxybutyrate dehydrogenase (Fig. 2, residues 16, 20 and 22). Putative active site residues, identified as Ser-139, Tyr-154, and Lys-156 in SC-ADH, are also conserved in human (R)-3-hydroxybutyrate dehydrogenase (Fig. 2; residues Ser-149, Tyr-162, and Lys-166).

For the complete sequence of human 3-hydroxybutyrate dehydrogenase (mature form), the highest homology (30% identity) is with human 17β-hydroxysteroid dehydrogenase, a SC-ADH which has 327 amino acids. In the region corresponding to the conserved domains of the SC-ADH (Lys-10 to Gly-193), the homology was 57% whereas the C-terminal domain (Asn-194 to Arg-297) shows only 20% identity. Since the first 193 amino acids of (R)-3-hydroxybutyrate dehydrogenase exhibit homology with a variety of dehydrogenases using different substrates, but which are not known to exhibit a phospholipid requirement, it would seem probable that the C-terminal region (residues 194-297) includes regions that form the substrate- and phosphatidylycholine-binding sites in this lipid-requiring dehydrogenase.

Functional Groups of (R)-3-Hydroxybutyrate Dehydrogenase

Chemical derivatization studies suggest that (R)-3-hydroxybutyrate dehydrogenase has essential arginyl, carboxyl, histidyl, and thiol residues (6, 37-39). The enzyme can also be derivatized with photoactivatable phospholipid analogues (40, 41). In the bovine enzyme, there are two di-thiol bridges per monomer and two sulphydryls (42), the more reactive of which (SH1), although not involved in catalysis (43), is essential for optimal function (37, 44). The reactive sulphydryl appears to be localized in the nucleotide-binding domain of the enzyme since the same residue was derivatized by arylazido-NAD (23, 45). There are also 6 cystine residues in the sequence of mature human (R)-3-hydroxybutyrate dehydrogenase (residues 17, 40, 81, 163, 175, and 242). The sequence of the bovine peptide containing the reactive sulphydryl was determined by Hatefi and colleagues (23, 45) (Fig. 2, peptide G) and is homologous with residues 238-254 of the human enzyme sequence (reactive sulphydryl located at Cys-242). The sequence of the N,N'-dicyclohexylcarbodiimide-reactive peptide (23) (Fig. 2, peptide D) is homologous with residues 117-128 of the human enzyme sequence (essential carboxyl at Glu-120). Thus, the reactive sulphydryl and the N,N'-dicyclohexylcarbodiimide-reactive glutamic acid, both of which appear to be in the vicinity of the active center of the enzyme (23), are located in separate regions of the sequence. It is to be noted that the sulphydryl that reacts with arylazido-NAD is in the C-terminal domain whereas the three conserved glycines (residues 16, 20, and 22), suggested to be in proximity to the nucleotide-binding site (27), are in the N-terminal domain. This may indicate that, in the ternary or quaternary structure of (R)-3-hydroxybutyrate dehydrogenase, the C-terminal domain (that appears to be important for binding to membranes) is folded over the N-terminal domain (which is homologous with the family of the SC-ADH). The proximity of the two domains in the ternary or quaternary structure of (R)-3-hydroxybutyrate dehydrogenase may provide for the catalytic specificity of this novel lipid-requiring dehydrogenase.

Tissue Expression

(R)-3-Hydroxybutyrate dehydrogenase is known to be widely distributed in tissues of higher animals (25). Northern blot analysis demonstrates that the 1.3-kb mRNA, encoding (R)-3-hydroxybutyrate dehydrogenase (BDH mRNA), is ex-
pressed in all forms of muscle (smooth (not shown), fast and slow twitch, and cardiac muscle (Fig. 4)). To control for the amount of mRNA loaded in each lane, the Northern blots were subsequently probed with cDNA encoding GAPDH. BDH mRNA was expressed in rabbit heart at stable levels from the fetal stage (5 days prenatal) through the adult stage of development (Fig. 4, lanes 1–3). Expression of the BDH mRNA was equivalent in atrium and ventricle (Fig. 4, lanes 4 and 5, respectively). There was higher expression of BDH mRNA in slow twitch versus fast twitch skeletal muscle (normalized for GAPDH expression) (Fig. 4, lanes 7 and 6, respectively), consistent with the higher content of mitochondria in slow twitch as compared with fast twitch skeletal muscle.

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Note Added in Proof—The primary structure for the rat liver d-β-hydroxybutyrate dehydrogenase has recently been reported (Churchill, P., Hempel, J., Romovacek, H., Zhang, W-W., Brennan, M., and Churchill, P. (1992) Biochemistry 31, 3793–3799).

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