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Isolation and Characterization of a Complementary DNA Clone Coding for the $E_1\beta$ Subunit of the Bovine Branched-Chain α-Ketoacid Dehydrogenase Complex: Complete Amino Acid Sequence of the Precursor Protein and Its Proteolytic Processing†

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ABSTRACT: A 1.7-kb cDNA clone encoding the entire precursor of the $E_1\beta$ subunit of the branched-chain α-ketoacid dehydrogenase (BCKDH) complex was isolated from a bovine liver cDNA library by screening with a mixture of synthetic oligonucleotide probes corresponding to the C-terminal five-residue sequence of the mature $E_1\beta$ subunit. A partial amino acid sequence was determined by Edman degradation of the intact subunit and the peptides generated by cleavage at the lysyl bonds. Nucleotide sequence analysis revealed that the isolated cDNA clone contained the 5′-untranslated sequence of 186 nucleotides, the translated sequence of 1176 nucleotides, and the 3′-untranslated sequence of 306 nucleotides with a poly(A) tail. A type AATAAA polyadenylation signal was located 17 nucleotides upstream of the start of a poly(A) tail. Comparison of the amino acid sequence predicted from the nucleotide sequence of the cDNA insert of the clone with the partial amino acid sequence of the mature BCKDH $E_1\beta$ subunit showed that the cDNA insert encodes for a 342 amino acid subunit with M_r 37 745 and that the subunit is synthesized as the precursor with a leader sequence of 50 amino acids and processed at the N-terminus. Northern blot analysis using the cDNA insert as a probe showed the presence of a 1.8-1.9-kb mRNA in bovine liver, suggesting that the insert covers nearly a full length of mRNA. Alignment of the deduced amino acid sequence of bovine BCKDH $E_1\beta$ with that of the human pyruvate dehydrogenase (PDH) complex $E_1\beta$ subunit revealed a high degree of sequence homology throughout the two enzymes. The structure and function of mammalian α-ketoacid dehydrogenase complexes are apparently highly conserved.

Mammalian branched-chain α-ketoacid dehydrogenase (BCKDH)[1] (EC 1.2.4.4) is a mitochondrial multienzyme complex that catalyzes the oxidative decarboxylation of branched-chain α-keto acids derived by transamination of branched-chain amino acids such as valine, leucine, and isoleucine:

$$\text{RCOCOOH} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{TPP, Mg}^{2+}} \text{RCOCoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The BCKDH complex consists of three catalytic components: branched-chain α-ketoacid decarboxylase (E_1), dihydrolipoyl transacylase (E_2), and dihydrolipoamide dehydrogensae (E_3) (Pettit et al., 1978; Danner et. al., 1979; Heffelfinger et al., 1983). The enzyme complex also contains two specific regulatory enzymes, a kinase (Lau et al., 1982; Odessey, 1982; Paxton & Harris, 1982) and a phosphatase (Fatania et al., 1983; Damuni et al., 1984), that regulate the catalytic activity. E_1 is composed of two subunits, α and β, with M_r of 46 500 and 37 500, respectively (Heffelfinger et

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[1] Abbreviations: BCKDH, branched-chain α-ketoacid dehydrogenase; PDH, pyruvate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase; bp, base pair(s); kb, kilobase(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MSUD, maple syrup urine disease; SSC, 0.15 M NaCl containing 0.015 M trisodium citrate; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin derivative; *S*-CAM, *S*-carbamoylmethyl; TFA, trifluoroacetic acid; TPP, thiamin pyrophosphate.

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al., 1983). $E_1\alpha$ is probably the catalytic subunit, phosphorylated at two serine residues, responsible for regulation of the catalytic activity by covalent modification (Cook et al., 1984). The function of $E_1\beta$ is unknown. E_2 catalyzes transfer of the acyl group from the lipoyl moiety to coenzyme A and forms the structural core of the enzyme complex. E_1, E_3, kinase, and phosphatase are bound to this core through noncovalent interactions (Pettit et al., 1978; Fatania et al., 1983; Cook et al., 1985). E_3 is a dimer consisting of identical subunits (M_r 55 000) and is common among α-ketoacid dehydrogenase complexes (Otulakowski & Robinson, 1987; Pons et al., 1988).

The BCKDH complex is similar to PDH and KGDH complexes in both structure and function (Yeaman, 1989). The isolation and characterization of cDNAs encoding all or a part of the mammalian BCKDH $E_1\alpha$ subunit (Zhang et al., 1987, 1988; Hu et al., 1988; Fisher et al., 1989) and the E_2 component (Hummel et al., 1988; Lau et al., 1988; Griffin et al., 1988; Danner et al., 1989; Nobukuni et al., 1989) have been reported. Thus, BCKDH $E_1\alpha$ shares a high degree of sequence homology with the $E_1\alpha$ subunit of the PDH complex (Zhang et al., 1988). Similarly, BCKDH E_2 was shown to be homologous with PDH E_2 (Lau et al., 1988; Griffin et al., 1988). The amino acid sequence of BCKDH $E_1\beta$ has not been reported, and the function of this subunit is poorly understood. We therefore attempted to determine the primary structure of BCKDH $E_1\beta$ for a better understanding of structure–function relationships, gene organization, biosynthetic regulatory mechanisms, etc.

A lack of BCKDH activity results in maple syrup urine disease (MSUD), an autosomal recessive inherited disorder (Danner & Elsas, 1989). Several different phenotypes of MSUD have been characterized, i.e., classical, intermittent, intermediate, thiamin responsive, and E_3 deficiency (Danner & Elsas, 1989). Etiology of MSUD is variable as the BCKDH complex is encoded by at least six different structural genes. Mutations in various regions of any of the BCKDH constituent proteins could lead to a decreased function of the entire complex.

Recently, it was shown that a defect in BCKDH $E_1\beta$ is one cause of MSUD (Indo et al., 1987, 1988; Fisher et al., 1989). In an attempt to elucidate the molecular basis of this disease, we carried out cDNA cloning of BCKDH $E_1\beta$. We describe herein the isolation and characterization of a 1.7-kb cDNA clone that encodes the entire $E_1\beta$ precursor of bovine liver BCKDH.

Materials and Methods

Purification of BCKDH and Its $E_1\beta$ Subunit. The BCKDH (E_1–E_2) complex was purified from bovine kidney as previously described (Indo et al., 1987). The specific activity of the purified BCKDH was 3–7 units/mg of protein. The $E_1\beta$ subunit was isolated from the complex by preparative SDS–PAGE (Laemmli, 1970). Protein bands were visualized with 4 M sodium acetate (Higgins & Dahmus, 1979). The gel portion containing the $E_1\beta$ subunit was cut out and the protein recovered by electrophoresis as previously described (Stephens, 1975).

Amino Acid and Sequence Analyses. The purified protein was reduced with dithiothreitol and S-carbamoylaminomethylated with monoiodoacetamide by a slight modification of the method of Titani et al. (1984). Lysyl bonds of the *S*-CAM-modified protein were cleaved by digestion with *Achromobacter* protease I (a gift from Dr. T. Masaki, Ibaraki University, Ibaraki, Japan) at 37 °C for 15 h in 50 mM Tris-HCl, pH 9.0 (Masaki et al., 1981), in the presence of 2 M urea. The *S*-CAM-modified protein was dissolved in a small volume of 8 M urea and diluted with 3 volumes of 50 mM Tris buffer, pH 9.0.

Peptides were separated by reversed-phase HPLC on a SynChropak RP-8 column (4.1 × 250 mm) (SynChrom, Inc.) with gradients of acetonitrile into diluted aqueous TFA (Mahoney & Hermodson, 1980).

Amino acid compositions of the intact *S*-CAM-modified protein and peptides were determined in a Hitachi L-8500 amino acid anaylzer or with a Waters Pecotag system (Bidlingmeyer et al., 1984). Sequence analysis was carried out in an Applied Biosystems 470A protein sequencer connected to a 120A PTH analyzer.

Oligonucleotide Probes. A mixture of 24 oligonucleotide probes (17-mer) corresponding to the C-terminal sequence of the $E_1\beta$ subunit (Figure 3) was synthesized and radiolabeled at the 5′ ends, with [γ-^{32}P]ATP (sp act. 3000 Ci/mmol) and T4 polynucleotide kinase (Maniatis et al., 1982).

Screening of cDNA Library. A bovine liver cDNA library, constructed by inserting cDNA copies of poly(A^+) RNA from bovine liver into the *Eco*RI site of bacteriophage vector λgt11, was purchased from CLONTECH Laboratories, Inc. Approximately 6×10^5 recombinant phage plaques were screened from the cDNA library by hybridization with the radiolabeled nucleotide probe. Prehybridization, hybridization, and washing of nitrocellulose filters were carried out at 32 °C. The filters were prehybridized for 4 h in 6× SSC, 10× Denhardt's [1× Denhardt's = 0.02% poly(vinylpyrrolidone), 0.02% bovine serum albumin, and 0.02% Ficoll], and 0.05 M sodium phosphate, pH 7.0. Hybridization took place for 40 h in prehybridization buffer containing (0.5–1.0) × 10^6 cpm/mL of the probe. The filters were washed for 1 h in 6× SSC with two to three changes. Hybridization plaques detected by autoradiography were plaque purified.

Restriction Endonuclease Map and Nucleotide Sequence Analysis. Recombinant phage DNA was prepared as described (Maniatis et al., 1982). *Eco*RI-excised cDNA inserts were subcloned into plasmid vector pUC18 and characterized by restriction endonuclease mapping. Restriction fragments were subcloned into pUC18 for sequencing. In addition, ordered serial deletions from the 5′ → 3′ end of both strands of the pUC18 insert were produced with exonuclease III/mung bean nuclease for sequencing (Henikoff, 1984). DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) using an alkali-denatured plasmid as the template (Hattori & Sakaki, 1986).

Northern Blot Analysis. Total RNA was prepared from bovine liver as previously described (Chomczynski & Sacchi, 1987). Poly(A^+) RNAs were further purified on an oligo(dT)–cellulose column (Maniatis et al., 1982), separated on a 1.0% formaldehyde–agarose gel, and transferred to nitrocellulose filters. The cDNA insert was radiolabeled (Feinberg & Vogelstein, 1984) with a random primer labeling kit (Takara Shuzo Co., Kyoto, Japan) and [α-^{32}P]dCTP (sp act. 3000 Ci/mmol). The filters were hybridized with a radiolabeled cDNA insert as described (Maniatis et al., 1982).

Protein Data Base Search, Secondary Structure Prediction, and Hydropathy Profile. Homologous amino acid sequences were sought in the GenBank (Release 59.0)/EMBL (Release 18.0) protein data base on a VAX computer using the WORDSEARCH program (Wilbur & Lipman, 1983) (Version 6.0, April 1989). The alignment procedure used the SEGMENTS program (Smith & Waterman, 1981). The secondary structure was predicted by the method of Chou and Fasman (1978). The hydropathy index was calculated by the method of Kyte and Doolittle (1982).

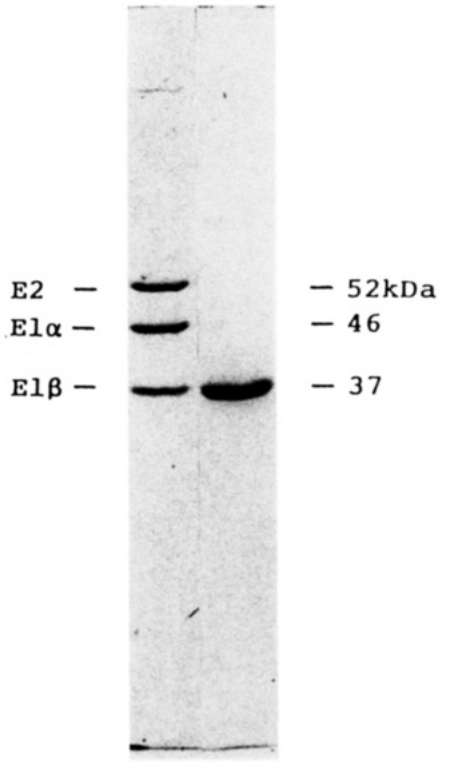

FIGURE 1: SDS-PAGE pattern of purified bovine kidney BCKDH complex and its $E_1\beta$ subunit. Coomassie brilliant blue stain of an SDS-10% polyacrylamide gel (Laemmli, 1970). Lane 1, BCKDH complex (15 μg); lane 2, $E_1\beta$ subunit (15 μg).

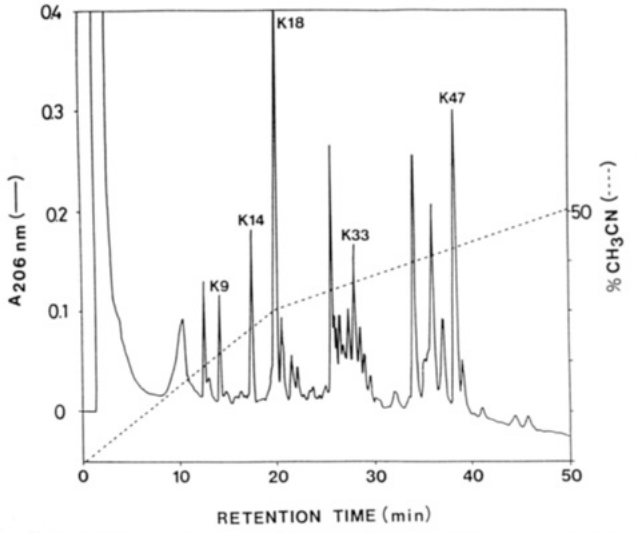

FIGURE 2: Reversed-phase HPLC profile of peptides generated by digestion of 9 nmol of *S*-CAM-BCKDH $E_1\beta$ with *Achromobacter* protease I. Peptides were separated on a SynChropak RP-P column (4.1 × 250 mm) with a gradient of acetonitrile (containing 0.08% TFA) into 0.1% aqueous TFA as indicated by the broken line. Flow rate, 2.0 mL/min. Peptides were monitored by UV absorbance at 206 nm.

Results

Protein Purification and Peptide Sequence Analysis. The purified preparation of mature $E_1\beta$ was homogeneous, with a molecular weight (estimated by SDS-PAGE) of approximately 37 000 (Figure 1). The N-terminal sequence of 16 residues was determined by analysis of the intact *S*-CAM-modified protein (50 pmol). The *S*-CAM-modified protein (9 nmol) was digested with *Achromobacter* protease I, and the digest was separated on reversed-phase HPLC (Figure 2). Among the peptides, denoted by the prefix K, the amino acid sequences of four (K18, K14, K33, and K9) were determined through the C-terminus. The partial sequence of K47 was also determined. The sequence of K18 was identical with that of the N-terminal 16 residues of mature $E_1\beta$, indicating that K18 was derived from the N-terminal portion of the mature protein. Peptide K9 (four residues) contained no lysine residue, suggesting that K9 represents the C-terminus of mature $E_1\beta$.

The amino acid compositions of mature $E_1\beta$ and peptides K18, K14, K33, K47, and K9 were determined (data not shown), to provide support for the predicted sequence of the mature protein and also to verify the obtained sequence.

Synthesis of Oligonucleotide Probe and Isolation of cDNA Clones. Sequence analysis of peptide K9 indicated that the C-terminal sequence of BCKDH $E_1\beta$ is probably -Lys-Met-Ile-Asn-Tyr-end. On the basis of this finding, we synthesized a mixture of 24 combinations of 17-mer oligonucleotides (Figure 3). With the synthetic oligonucleotide mixture as a probe, approximately 6×10^5 plaque-forming units were screened from the bovine liver cDNA library. Five positive clones were plaque purified to homogeneity through four or five successive rounds of screening, and the cDNA inserts were subcloned into the *Eco*RI site of pUC18 for further characterization. The sizes of these recombinant phage clone inserts ranged from 0.6 to 1.7 kilobase pairs (kbp).

Restriction Endonuclease Map and Nucleotide Sequence Analysis. The restriction endonuclease map of a cDNA insert from the phage clone λb$E_1\beta$-2 and sequencing strategy for the insert are shown in Figure 4. The nucleotide sequence and deduced amino acid sequence are shown in Figure 5. The λb$E_1\beta$-2 insert is composed of 1668 bp consisting of a 186-bp 5′-untranslated sequence, a 1176-bp open reading frame, and a 306-bp 3′-untranslated sequence with a poly(A) tail. A type AATAAA polyadenylation signal (Birnstiel et al., 1985) is located 17 nucleotides upstream of the start of the poly(A) tail. The open reading frame could be translated into a 392 amino acid residue protein. Comparison of the amino acid

Amino acid sequence		-Lys-Met-Ile-Asn-Tyr-End
mRNA sequence	5'	AA(A/G)-AUG-AU(A/C/U)-AA(C/U)-UA(C/U)-UA 3'
Probe sequence	5'	AA(A/G)-ATG-AT(A/C/T)-AA(C/T)-TA(C/T)-TA 3'

FIGURE 3: Amino acid sequence (residues 338-342) used for synthesis of the 17-mer oligonucleotide probe, possible codons for the amino acids, and probe sequences designed to account for all possible codon combinations.

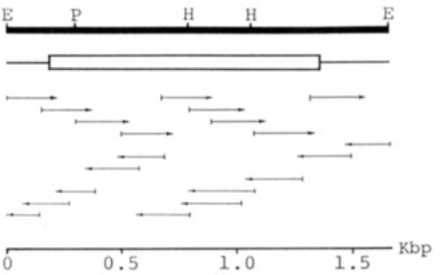

FIGURE 4: Restriction map and sequencing strategy for bovine BCKDH $E_1\beta$ cDNA. The open box and the line depict coding and noncoding regions, respectively. Restriction sites are indicated above the λb$E_1\beta$-2 insert (1668 bp) at the top of the figure, with the following restriction enzymes being used: *Eco*RI (E), *Pst*I (P), and *Hin*dIII (H). Solid horizontal arrows indicate orientation and region of sequencing.

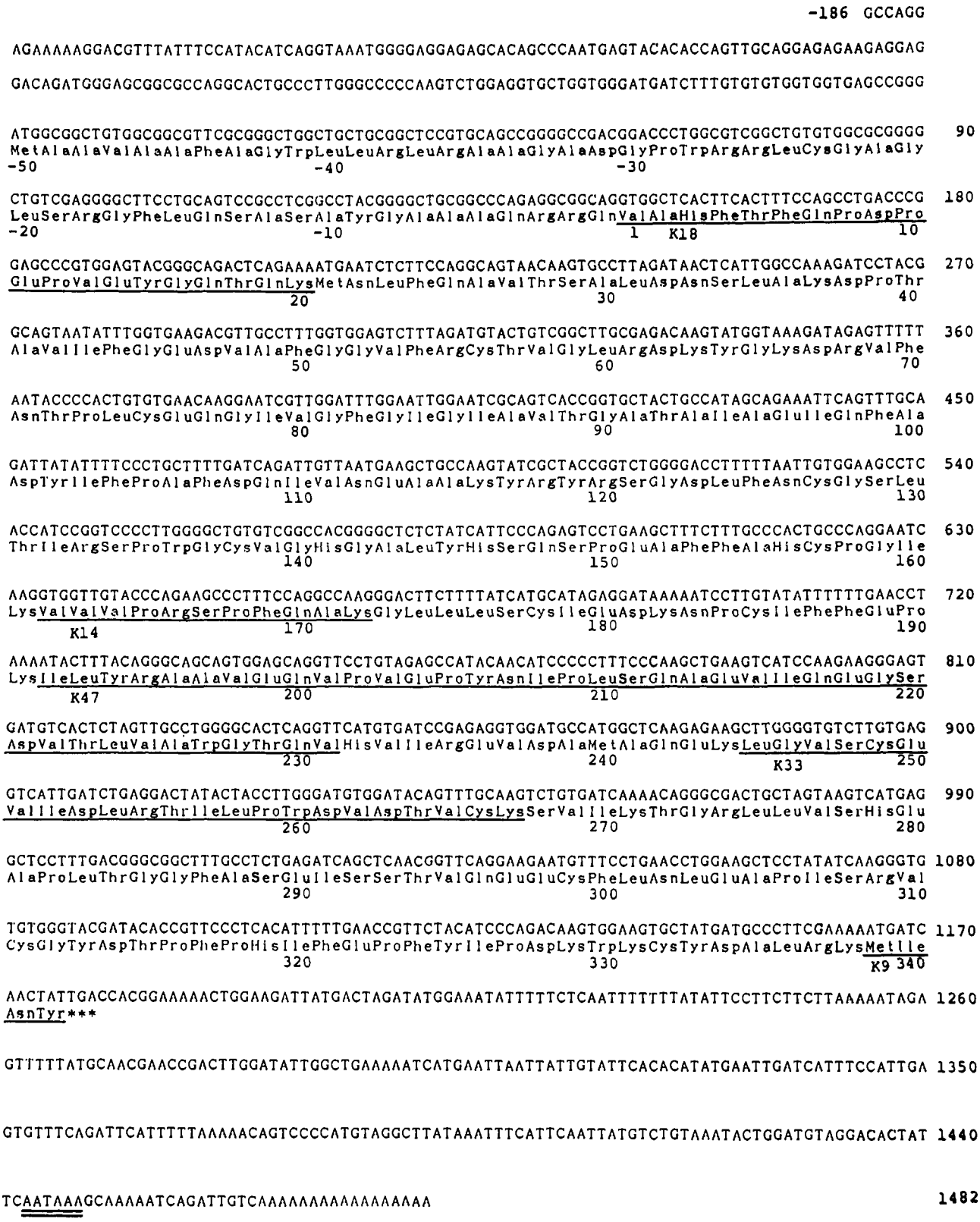

FIGURE 5: Nucleotide sequence of the λbE$_1$β-2 insert and deduced amino acid sequence of the bovine BCKDH E$_1$β precursor. Numbers on the far right correspond to the ordinates of the last nucleotide in each row. Nucleotides are numbered in the 5′ → 3′ direction, beginning with the first residue of the ATG triplet encoding the putative initiator methionine. Numbers below the amino acid sequence refer to residues beginning with the N-terminus of the mature enzyme determined by Edman degradation. Regions of the amino acid identifying partial amino acid sequences (K18, K14, K47, K33, and K9) are underlined by solid lines. The polyadenylation signal (AATAAA) is double underlined.

sequence predicted from the nucleotide sequence of the clone cDNA insert with the partial amino acid sequence determined by Edman degradation revealed that the sequences of peptides K18, K14, K47, K33, and K9 matched those of five regions (residues 1–20, 162–172, 192–231, 245–267, and 339–342) of the predicted sequence (Figure 5). This finding suggests that the cDNA insert of clone λbE$_1$β-2 encodes for bovine BCKDH E$_1$β. Four of the five isolated clones proved to be false positive by nucleotide sequence analysis.

Northern Blot Analysis. By Northern blot analysis, a single species of mRNA (1.8–1.9 kb) hybridized with the λbE$_1$β-2 insert (Figure 6).

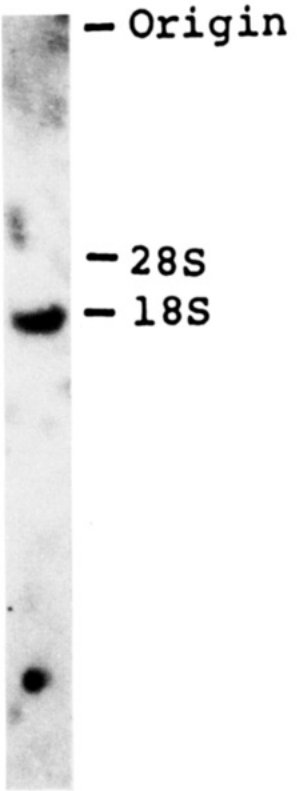

FIGURE 6: Northern blot analysis of bovine liver mRNA. A total of 12 μg of poly(A+) RNA prepared from bovine liver was denatured and electrophoresed on a 1.0% agarose gel. The separated RNA was transferred to nitrocellulose paper and probed with the random primer labeled λbE$_1\beta$-2 cDNA insert.

Secondary Structure Predictions and Hydropathy Profile. The presence of α-helices (23.1%), β-sheets (48.2%), and 18 potential β-turns was predicted for the 342-residue sequence of BCKDH E$_1\beta$ (data not shown). Some of the predicted α-helical and β-sheet structures were short and may not exist as such. The hydropathy profile indicated that BCKDH E$_1\beta$ contains neither an extremely hydrophobic nor an extremely hydrophilic region but that small hydrophobic and hydrophilic regions are evenly distributed throughout the subunit (data not shown).

Discussion

Two independent cDNA expression libraries were initially screened with a specific rabbit antibody raised against bovine BCKDH E$_1\beta$ or with a synthetic oligonucleotide mixture as the probe, corresponding to the N-terminal amino acid sequence of BCKDH E$_1\beta$. All of more than 10 positive clones obtained were found to be false positive by nucleotide sequencing.

We isolated from the bovine liver cDNA library a 1.7-kb cDNA clone encoding the entire BCKDH E$_1\beta$ by screening with a mixture of synthetic oligonucleotides corresponding to the C-terminal amino acid sequence of the mature E$_1\beta$ subunit. The nucleotide sequence surrounding the tentative initiator codon, CCGGGAUGG, is similar to the consensus sequence of CC$^{A}_{G}$CCAUGG (Kozak, 1984).

The N-terminal 50 amino acid residues of the putative precursor protein are missing in the mature E$_1\beta$ subunit and presumably represent the leader sequence. On the basis of these findings, the molecular weight of the E$_1\beta$ precursor is estimated as 42 904 and that of mature E$_1\beta$ as 37 745, which is in good agreement with a molecular weight of 37 000 estimated by SDS–PAGE.

BCKDH E$_1\beta$ is a nuclear-coded mitochondrial protein, the precursor of which seems to contain a leader sequence of 50 amino acid residues. Comparison of the putative E$_1\beta$ leader

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BCKDH    3 HFTFQPDPEPVEYGQTQKMNLFQAVTSALDNSLAKDPTAVIFGEDVA.FG 51
PDH     24 HWT...APAAVQ......VTVRDAINQGMDEELERDEKVFLLGEEVAQYD 64

BCKDH   52 GVFRCTVGLRDKYGKDRVFNTPLCEQGIVGFGIGIAVTGATAIAEIQFAD 101
PDH     65 GAYKVSRGLWKKYGDKRIIDTPISEMGFAGIAVGAAMAGLRPICEFMTFN 114

BCKDH  102 YIFPAFDQIVNEAAKYRYRSGDLFNCGSLTIRSPWGCVGHGALYHSQSPQ 151
PDH    115 FSMQAIDQVINSAAKTYYMSGGL.QPVPIVFRGPNGASAGVAAQHSQCFA 163

BCKDH  152 AFFAHCPGIKVVVPRSPFQAKGLLLSCIEDKNPCIFFEPKILYRAAVE.. 199
PDH    164 AWYGHCPGLKVVSPWNSEDAKGLIKSAIRDNNPVVVLENELMYGVPFEFL 213

BCKDH  200 .QVPVEPYNIPLSQAEVIQEGSDVTLVAWGTQV.HVIREVDAMAQEKLGV 247
PDH    214 RKLSQKILLIPIGKAKIERQGTHITVVSHSRPVGHCLEAAAVLSKE..GV 261

BCKDH  248 SCEVIDLRTILPWDVDTVCKSVIKTGRLLVSHEAPLTG.GFASEISSTVQ 296
PDH    262 ECEVINMRTIRPMDMETIEASVMKTNH.LVTVEGGWPQFGVGAEICARIN 310

BCKDH  297 EECFLN.LEAPISRVCGYDTPFPH..IFEPFYIPDKWKCYDALRKMINY 342
PDH    311 GSPAFNFLDAPAVRVTGADVPMPYAKILEDNSIPQVKDIIFAIKKTLNI 359
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FIGURE 7: Comparison of amino acid sequences of bovine BCKDH E$_1\beta$ (present study) and human PDH E$_1\beta$ (Koike et al., 1988).

sequence (negatively numbered amino acid residues in Figure 5) with those of other mitochondrial proteins reveals a number of common features. The putative $E_1\beta$ leader sequence contains periodically spaced basic amino acids, is rich in Ser, Leu, and Arg, and has few acidic residues (only one Asp at residue −31). These findings are compatible with those proposed for the leader sequence of mitochondrial targeting enzymes (von Heijne, 1986; Roise & Schatz, 1988).

A high degree of homology between the amino acid sequences of bovine BCKDH $E_1\beta$ and human PDH $E_1\beta$ was evident. Figure 7 depicts the alignment of homologous regions of BCKDH $E_1\beta$ and PDH $E_1\beta$ (Koike et al., 1988). Approximately 35% of the amino acid residues of BCKDH $E_1\beta$ in these regions are identical with the corresponding residues of PDH $E_1\beta$. It has been demonstrated that mammalian α-ketoacid dehydrogenase complexes such as PDH, BCKDH, and KGDH are functionally and structurally homologous (Yeaman, 1989). The amino acid sequence of the mammalian BCKDH $E_1\alpha$ subunit is highly homologous to that of mammalian PDH $E_1\alpha$ (Zhang et al., 1988); the same is true of BCKDH E_2 and PDH E_2 (Lau et al., 1988; Griffin et al., 1988; Danner et al., 1989). The present study showed that BCKDH $E_1\beta$ is similarly homologous to PDH $E_1\beta$.

The complete primary structure of bovine BCKDH $E_1\beta$ elucidated by molecular cloning, assisted by protein sequencing, would be useful for revealing the structural and functional relationships of the enzyme complex. This approach is being used in ongoing investigations of the molecular basis of MSUD.

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Registry No. DNA (ox clone λb$E_1\beta$-2 branched-chain α-ketoacid dehydrogenase $E_1\beta$ subunit messenger RNA-complementary), 124316-28-5; branched-chain α-ketoacid dehydrogenase (ox clone λb$E_1\beta$-2 $E_1\beta$ subunit precursor protein moiety reduced), 124316-30-9; branched-chain α-ketoacid dehydrogenase (ox clone λb$E_1\beta$-2 $E_1\beta$ subunit protein moiety reduced), 124316-29-6; branched-chain α-ketoacid dehydrogenase E_1 component, 63653-19-0; pyruvate dehydrogenase, 9014-20-4.

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Excimer Fluorescence of Pyrenyliodoacetamide-Labeled Tropomyosin: A Probe of the State of Tropomyosin in Reconstituted Muscle Thin Filaments†

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ABSTRACT: Rabbit skeletal tropomyosin (Tm) specifically labeled at cysteine groups with *N*-(1-pyrenyl)-iodoacetamide (PIA) exhibits excimer fluorescence. The excimer fluorescence was sensitive to the local conformation of Tm, to actin binding, and, in reconstituted thin filaments, to the Tm state change induced by binding of myosin subfragment 1 (S1). The properties of PIATm were similar to previously studied pyrenylmaleimide-labeled Tm (PMTm) [Ishii, Y., & Lehrer, S. S. (1985) *Biochemistry 24*, 6631] except that S1 binding to actin–Tm increased the excimer fluorescence in contrast to the time-dependent decrease seen for PMTm. The fluorescence properties of PIATm are sensitive to the Tm chain–chain interaction via equilibria among pyrene configurations and nonfluorescent dimer as well as the monomer and excimer-forming configurations. The effect of bound troponin (Tn) on the excimer fluorescence of PIATm in the reconstituted systems was dependent on ionic strength with a slight Ca^{2+} dependence. S1 titrations in the absence and presence of Tn and Ca^{2+} indicated that the excimer fluorescence probes the state change of Tm from the weak S1 binding state to the strong S1 binding state which is facilitated by Ca^{2+} [Hill et al. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186]. Binding of MgADP–S1 and MgAMPPNP–S1 produced the same total excimer fluorescence change as for nucleotide–free S1, showing that the strong S1 binding state of Tm–actin is independent of nucleotide. TnT1, the N-terminal fragment of troponin T, did not affect the S1-induced Tm state change profile although it strengthened the binding of Tm to actin due to enhanced end-to-end interaction. Thus, changes in Tm chain–chain interactions are associated with the Tm state change important in regulation.

Tropomyosin (Tm),[1] a two-chain coiled-coil α-helical molecule, is an essential component of Ca^{2+}-dependent thin filament regulation of striated muscle contraction (Leavis & Gergely, 1984). Biochemical studies have shown that reconstituted thin filaments of actin–Tm and actin–troponin (Tn)–Tm equilibrate between two states, a weak myosin binding state or an inhibited state and a strong myosin binding state or an activated state, which results in cooperative myosin subfragment 1 (S1) binding (Greene & Eisenberg, 1980) and cooperative S1 dependence of ATPase activity (Lehrer & Morris, 1982; Nagashima & Asakura, 1982). The state change induced by the S1 binding has been directly monitored with fluorescence probes on Cys-190 of Tm sensitive to environmental changes (Ishii & Lehrer, 1985, 1987; Lehrer & Ishii, 1988) and to changes in the geometrical relationship between Tm and actin (Lehrer & Ishii, 1988).

Previous studies with pyrenylmaleimide-labeled Tm (PMTm) showed excited-state dimer (excimer) fluorescence from pyrenes attached to Cys-190 on each chain (Betcher-Lange & Lehrer, 1978). The excimer fluorescence from PMTm is sensitive to localized conformational changes (Graceffa & Lehrer, 1980), to binding to actin, and to binding of S1 to the PMTm–actin complex (Ishii & Lehrer, 1985). Some problems were noted with the PM probe on Tm. Although the monomer fluorescence could be used to study the S1 binding profile, the excimer fluorescence could not be used due to time-dependent changes, and the PM probe caused an appreciable perturbation of the Tm conformation.

In this study, we used *N*-(1-pyrenyl)iodoacetamide-labeled Tm (PIATm) which contains the pyrene fluorophore covalently

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[1] Abbreviations: Tm, tropomyosin; S1, myosin subfragment 1; Tn troponin; TnT1, N-terminal half of the chymotryptic fragment of the troponin T component; PIATm, Tm labeled with *N*-(1-pyrenyl)iodoacetamide; PMTm, Tm labeled with *N*-(1-pyrenyl)maleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; AMPPNP, 5′-adenylyl imidodiphosphate.

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