Cloning and Expression of a cDNA Encoding Bovine Lipoyltransferase*

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Lipoic acid is a prosthetic group of H-proteins of the glycine cleavage system and the acyltransferase components of the pyruvate, α-ketoglutarate, and branched chain α-ketoacid dehydrogenase complexes (1–4). Lipoyltransferase I and II (LipTI and LipTII) from bovine liver (5) catalyze both Reactions 1 and 2 (8).

Reaction 1: lipoyl-AMP + apoH-protein → holoprotein + AMP

Reaction 2: lipoyl-AMP + ATP → lipoyl-AMP + PPi

In mammals, Reaction 1 is catalyzed by lipoyl-activating enzyme (5), and Reaction 2 is catalyzed by lipoyl-AMP:N* -lysine lipoyltransferase (lipoic transferase) (6). We have isolated two isoforms of lipoyltransferase, lipoyltransferase I (LipTI) and II (LipTII), from bovine liver mitochondria employing apoH-protein as an acceptor of lipoic acid. We have purified LipTI homogeneity, but homogeneous LipTI has not been obtained yet. Although the isoforms were separated by the chromatography on a hydroxylapatite column, they showed similar molecular mass (about 40 kDa), catalytic properties, and behavior on column chromatographies except on hydroxylapatite. They also catalyze the transfer of lipoic group from lipoyl-AMP to apolipoprotein domains of acyltransferase components of the pyruvate, α-ketoglutarate, and branched chain α-ketoacid dehydrogenase complexes (7). In contrast, lipoate-protein ligase of Escherichia coli catalyzes both Reactions 1 and 2 (8). To understand the structure and function of LipTI and its relationship to LipTII, we isolated and sequenced cDNA encoding bovine liver LipTI and expressed the protein in E. coli. Deducing amino acid sequence of LipTI determined by Edman degradation. An antibody raised against the recombinant protein cross-reacted with LipTI and LipTII in a similar manner. The evidence reported here suggests the possibility that LipTI and LipTII are derived from the same translated product but processed differently.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (6000 Ci/mmol) and [α-32P]dCTP (6000 Ci/mmol) were obtained from Amersham. DNA restriction and modifying enzymes were purchased from New England Biolabs, ToyoBio (Tokyo, Japan), or Takara Shuzo (Shiga, Japan). Oligonucleotides, SuperScript II reverse transcriptase, RNase H, RNase T1, and Glass Max carbox diketopiperazine were obtained from Life Technologies, Inc. Plasmidaceous RNase inhibitor was from Promega. Lysylendopeptidase and p-amidinophenylmethane-sulfonyl fluoride hydrochloride were from Wako Pure Chemicals (Osaka, Japan). Lipoyl-AMP and lipoyltransferase from bovine liver were prepared as described previously (6).

Isolation and Sequencing of cDNA Clone—DNA manipulations were accomplished by standard techniques (9). Two oligonucleotides encoding for two different regions of LipTI were used for screening. Probe 1 (5′-AA/CA/TC/GA/TC/GG/GA/A/GA/TA/TC/CA/GA/CT/AA-3′) encoded for residues 13–18, and probe 2 (5′-CAT/AG/TG/AG/TC/AG/TA/TC/AG/TA/CT/C-3′) encoded for residues 24–29 (see Fig. 1B). They were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and used to screen a λgt10 bovine liver cDNA library (CLONTECH). The hybridization and washing of Hybond N+ membranes (Amersham) were carried out as described previously except that the final washings were carried out at 41 and 44 °C with probe 1 and probe 2, respectively (10).

The abbreviations used are: LipTI, lipoyltransferase I; LipTII, lipoyltransferase II; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).
SacI. DNA fragments were resolved on a 0.7% agarose gel, treated with 0.25 M HCl for 20 min, and transferred to Hybond N* under alkaline conditions. The membranes were hybridized with a 1.1-kilobase pair SplII/HincII cDNA fragment (see Fig. 1A) labeled with [α-32P]dCTP using Multiprime DNA labeling system (Amersham). Hybridization and washing of the membranes were carried out in a buffer containing 0.1× SSPE, 0.1% SDS, 10 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4) at 60 °C for 5 min were carried out according to the protocol provided by Amersham.

**Northern Blot Analysis—**Total RNA was prepared from bovine liver using TRIzol Reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Poly(A)+ RNA was purified from the total RNA using employing Message Maker Reagent Kit (Life Technologies, Inc.) 5 µg of poly(A)+ RNA was electrophoresed on a formaldehyde/agarose gel with 0.24–0.5-kilobase RNA ladder markers (Life Technologies, Inc.) and transferred to Hybond N* (Novagene). Conditions of hybridization with the 32P-labeled SplII/HincII cDNA fragment and washing of the membrane were as described (10).

**RT-PCR—**RT-PCR reaction was performed to isolate a variant cDNA for lipoyltransferase employing poly(A)+ RNA prepared as above. A mixture of 500 ng of poly(A)+ RNA and 2.5 pmol of gene-specific reverse primer, 5′-CATCAAAACTGTCAACATTAAG-3′ (nucleotides 1247–1226; see Fig. 1B), in 15 µl was heated at 70 °C for 10 min and then cooled on ice. First strand cDNA was synthesized at 44 °C for 50 min in a 25 µl reaction mixture containing the above mixture of poly(A)+ RNA and reverse primer, 20 µM dNTPs, 30 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 58 units plasmid RNaase inhibitor, and 200 units Super-Script II reverse transcriptase. The reaction was terminated by heating at 70 °C for 15 min, and RNase H and RNase T1 were added to the PCR mixture carried out according to a protocol provided by the manufacturer. The PCR reaction mixture contained (in a total volume of 50 µl) a fifth of the first strand product, 40 pmol forward primer, 5′-ATTCATAGAACACAGT- TTAAAAAGTGGGACTC-3′ (XbaI site underlined) then nucleotides 165–185 (see Fig. 1B), 40 pmol reverse primer, 5′-AATCCATATTAA-GGAATTCCTTGAATATTAT-3′ (XhoI site underlined) then nucleotides 1228–1209 (see Fig. 1B), 250 µM dNTPs, 2.5 µl TaKaRa LA Taq DNA polymerase, and LA PCR buffer (Takara Shuzo) containing 2 mM MgCl2. PCR amplification was performed for 30 cycles of 94 °C for 1 min, 51 °C for 2 min, and 72 °C for 3 min. After agarose gel electrophoresis, an amplified product was isolated with QIAEX (QIAGEN), digested with XbaI and BamHI, and employed for the construction of pLipT(T). The nucleotide sequence of the resultant expression vector, pLipT(T), was determined using an Applied Biosystems 477A protein sequencer and a Hewlett Packard G100A protein sequencer. The lipoylpeptides were obtained by digestion of carboxymethylated LipTII with lysylendopeptidase and separation of the peptides by high performance liquid chromatography on an ODS column as described previously (2).

**Results**

**Cloning and Sequencing of Bovine Liver Lipoyltransferase II—**The N-terminal amino acid sequence of LipTI and amino acid sequences of several lysylpeptides derived from LipTII were examined (Fig. 1B). To isolate cDNA encoding LipTII, two degenerated oligonucleotide probes were synthesized based on the amino acid sequence of the N-terminal region of LipTII. Approximately 2.4 × 106 independent clones in a λgt10 bovine liver cDNA library were screened, and only one positive clone that hybridized with both probes was obtained. It may reflect a low abundance mRNA of lipoyltransferase as expected from the yield of the purified enzyme (6).

**Southern Blot Analysis and Immunoprecipitation—**10 ng of LipT(T), LipTI, and LipTII were separated by 12.5% SDS-PAGE, electroblotted onto Immobilon-P (Millipore), and incubated with anti-LipTII antibody diluted 2000-fold with 0.05% Tween 20, 20 µM Tris-HCI, pH 7.5, 0.15 M NaCl. The antibodies were obtained with Protoblot AP system (Promega) as described (2). Immunoprecipitation was carried out in a mixture of 14-µl phosphate-buffered saline containing the indicated amount of IgG and 2.5 µg of LipTI or LipTII, respectively. The amount of protein was kept constant by the addition of bovine serum albumin. The control experiment was carried out with nonimmune IgG. After incubation at 4 °C for 16 h, the supernatant fractions were obtained by centrifugation at 15,000 × g for 20 min, and a quarter of each supernatant fraction was employed for the assay of lipoyltransferase activity.

**Amino Acid Sequence Analysis—**The N-terminal amino acid sequence of LipTII and amino acid sequences of several lysylpeptides derived from LipTII were examined (Fig. 1B). To isolate cDNA encoding LipTII, two degenerated oligonucleotide probes were synthesized based on the amino acid sequence of the N-terminal region of LipTII. Approximately 2.4 × 106 independent clones in an Agt10 bovine liver cDNA library were screened, and only one positive clone that hybridized with both probes was obtained. It may reflect a low abundance mRNA of lipoyltransferase as expected from the yield of the purified enzyme (6).

The insert cDNA of the clone is 1326-bp long and consists of an 89-bp 5′-untranslated region, a 1115-bp open reading frame, and a 118-bp 3′-untranslated region (Fig. 1). The sequence AGCATG surrounding the first inframe ATG codon is in good agreement with the optimum translation initiation sequence (ACCATTG) described by Kozak (16). The open reading frame encodes a 373-amino acid protein. The predicted amino acid sequence completely matches the N-terminal amino acid sequence of purified LipTII and amino acid sequences of lysylpeptides (Fig. 1B). Thus, a mitochondrial targeting signal of 26 amino acids is predicted. The predicted size of the mature protein is 37 amino acids (with a calculated molecular mass of 39,137 Da) is in good agreement with the size previously determined by SDS-PAGE (40 kDa) (6). A potential N-linked glycosylation site, ATTAA, is located 20 bp upstream from the poly(A) tail. The N-terminal amino acid sequence of LipTII was determined to be NTYKSGLILQSISSNDVYHNL-. This sequence is identical to the residues —1 to 19 of LipTII (Fig. 1B), suggesting that LipTI and LipTII are produced from the same translated product by alternative processing in mitochondria.
Amino acid sequences determined by protein sequencing are numbered starting at the N-terminal amino acid of mature LipTII. The coding regions. Nucleotide numbering starts at the 5'-end of the insert. The putative polyadenylation signal is doubly underlined.

Protein sequence similarity between LipTII and known proteins was analyzed by searching the data library of the DNA Data Bank of Japan using the FASTA program (version 3.0). LipTII showed 35, 32, and 28% identity with E. coli lipoate-protein ligase A (8), yeast methionyl-aminopeptidase (accession number, Swiss-Prot P47051), and Mycoplasma genitalium probable lipoate-protein ligase A (P47512), respectively (Fig. 2). In particular, amino acids 8–86 of LipTII shares high homology with residues 6–85 of E. coli enzyme (59% identity), suggesting that the N-terminal domain of these proteins may be responsible for the lipoate-transferring activity. In contrast, the C-terminal part of these proteins is less homologous. Presumably, the C-terminal half of E. coli lipoate-protein ligase A may be involved in the lipoate activating activity that bovine lipo-transferase lacks.

Southern and Northern Blot Analyses—Because LipTII and LipTIII share the identical amino acid sequence around the N-terminal region, Southern blot analysis was carried out to examine whether more than one gene encoding lipo-transferase was present employing the LipTII cDNA as a probe. A single band was detected in each enzyme digest (Fig. 3A). The result suggests the presence of a single copy gene encoding lipo-transferase.

A Northern blot that contains size fractionated bovine liver poly(A)^+ RNA was probed with the ^32P-labeled SphII/HinII cDNA fragment. A single mRNA species of about 1.5 kilobases long was detected (Fig. 3B). The size of the message corresponds to that of the cloned cDNA.

RT-PCR Analysis—Although Northern blot analysis showed the presence of a single mRNA species for lipo-transferase, a possibility exists that the mRNA includes a variant for LipTI with minor differences in the internal region of the sequence produced by alternative splicing. To examine the possibility, RT-PCR was utilized with primers that were synthesized based on the findings that LipTI has an N-terminal amino acid sequence identical with that of LipTII and a molecular mass similar to LipTII. If mRNA for LipTI co-exists, two kinds of cDNA should be isolated with identical ratio by the cloning of the RT-PCR products, because the levels of LipTI and LipTII in bovine liver are nearly equal (6). The PCR products showed only a single band of about 1100 bp on agarose gel electrophoresis (data not shown). Nucleotide sequences of the cDNAs from 24 independent clones were identical to that of LipTII (Fig. 1B), except that a few of them showed a single base replacement at the 3'-end of the insert. Amino acid numbering starts at the N-terminal amino acid of mature LipTII. Because LipTI and LipTII share the identical amino acid sequence around the N-terminal region, Southern and Northern blot analyses were carried out to examine whether more than one gene encoding lipo-transferase was present employing the LipTII cDNA as a probe. A single band was detected in each enzyme digest (Fig. 3A). The result suggests the presence of a single copy gene encoding lipo-transferase.

Expression and Purification of Recombinant LipTI and Kinetic Analysis—LipT(T) was expressed in E. coli as described under “Experimental Procedures.” The induction of BL21(DE3)-pLysS cells harboring pLipT(T) with isopropyl-B-D-thiogalactopyranoside resulted in high level expression of a 40-kDa protein (Fig. 4A). Although most of the expressed protein was sequestered in inclusion bodies, the supernatant fraction obtained from the cell extracts exhibited about 100-fold higher lipo-transferase activity (5.97 units/mg protein) than that from control cells harboring pET-3a (0.04 unit/mg protein). The protein from inclusion bodies solubilized with 6 M urea had a low lipo-transferase activity. Attempts to recover fully active LipT(T) from the inclusion bodies through the use of urea, followed by dialysis for 2 days during which urea was incrementally diluted, were unsuccessful. We purified LipT(T) from the supernatant fraction to homogeneity by the successive chromatographies on hydroxylapatite, DEAE-Sepharose, and apoH-protein-Sepharose affinity columns (Fig. 4B). Table I summarizes a typical purification of LipT(T) from a 400-ml culture. LipT(T) emerged from the hydroxylapatite column at about 230 mM phosphate, nearly the same concentration at which LipTII was eluted from the column (6). The N-terminal amino acid sequence of LipT(T) showed that the initiation Met residue was cleaved off by an E. coli methionyl-aminopeptidase in agreement with the rule for the methionine removal from protein in E. coli (17).

FIG. 1. Structure of cDNA encoding bovine lipo-transferase II. A, restriction map. Open box, open reading frame; solid lines, non-coding regions. B, nucleotide and predicted amino acid sequences. The nucleotide numbering starts at the 5'-end of the insert. Amino acid numbering starts at the N-terminal amino acid of mature LipTII. Amino acid sequences determined by protein sequencing are underlined. The asterisk represents the stop codon. The putative polyadenylation signal is doubly underlined.
parable with those of LipTII, but the \( V_{\text{max}} \) value was more than 3-fold that of LipTII (Table II). The difference may be due in part to the inactivation of LipTII during the purification because the purification of LipTII required another two steps of column chromatography (6). LipT(T) could not lipoylate apoH-protein with lipoate plus MgATP, confirming the previous observation that bovine lipoyltransferase has no ability to activate lipoate to lipoyl-AMP (6).

We attempted to express and purify a protein that has an additional Asn residue on the N terminus of LipT(T). The protein designated LipT(N) was expressed in BL21(DE3)pLysS cells. Again the substantial amounts of LipT(N) were segregated in inclusion bodies. The cell extract showed a lipoylation activity of 5.31 units/mg protein comparable with that of LipT(T). LipT(N) was eluted at about 190 mM phosphate from a hydroxylapatite column. The behavior was quite similar to that of LipTII. However, most of the LipT(N) protein passed through the column of DEAE-Sepharose in a condition where LipTII and LipTII activities can be retained on the column. Amino acid sequence analysis of the partially purified LipT(N) revealed that the protein has an initiation Met residue on the N terminus.

Reactivity with Anti-LipT(T) Antibody—To further elucidate...
the relationship between LipTI and LipTII, we raised an antibody against the purified LipT(T) and examined the reactivity of LipTI and LipTII with the antibody. Western blotting (Fig. 5A) showed that both LipTI and LipTII were equally recognized by anti-LipT(T) antibody. An immunoprecipitation experiment showed similar inactivation curves of the activities of LipTI and LipTII with the increase of the amount of antibody (Fig. 5B). These results suggest that LipTI and LipTII are structurally related proteins.

**DISCUSSION**

This report describes the cloning of the full-length cDNA for bovine lipoyltransferase II. The cDNA contained a 1119-bp open reading frame encoding a peptide of 373 amino acids. The protein consists of a mitochondrial targeting signal of 26 amino acids and a mature protein of 347 amino acids. The mitochondrial targeting signal showed characteristic properties such as a high content of basic and hydrophobic amino acid residues, an absence of acidic residues, and amphiphilicity (18). This confirms the localization of lipoyltransferase in mitochondria as concluded previously from the distribution of the activity and translocation experiments of H-protein (2).

Although the cloning of LipTII has not resulted in very high levels of expressed soluble enzyme because the majority of recombinant LipTI (LipT(T)) is produced as inclusion bodies, it has facilitated the isolation and purification of the enzyme to homogeneity in an active form in quantities sufficient for the characterization of the enzyme. LipT(T) expressed in *E. coli* showed similar properties as compared with the native enzyme but a higher $V_{\text{max}}$ value. LipTI was unable to activate lipoic acid in agreement with the previous observation with the native enzyme. Thus it is confirmed that bovine lipoyltransferase catalyzes only lipoate transfer from lipoyl-AMP to apoproteins. Comparison of the primary sequence of LipTI and *E. coli* lipoate-protein ligase A, which catalyzes both the activation of lipoic acid and the transfer of lipoate, revealed a strong homology in the terminal region, suggesting that the active site for the transfer of lipoate to proteins is located within the N-terminal half of these proteins. *E. coli* biotin-protein ligase (BirA) and human holocarboxylase synthetase catalyze the attachment of biotin to biotin-dependent enzymes by two-step reactions similar to the lipoylation reaction. The enzymes contain the sequence GXGXXG predicted as a consensus sequence associated with ATP binding (19–21). Inspection of amino acid sequence of bovine LipTII revealed no similar sequence as expected, because LipTII catalyzes no ATP-dependent activation of lipoic acid.

An intriguing finding was that a stretch of the predicted amino acid sequence of LipTII (amino acids 1 to 19, Fig. 1B) completely matched the N-terminal amino acid sequence of LipTI. Although the internal amino acid sequence of LipTII has not been examined, it seems likely that LipTI and LipTII are derived from the same translated product. Several pieces of evidence favor this possibility: (i) The behaviors of Lip(TT) and LipT(T) on a hydroxylapatite column were similar to that of native LipTI and LipTII, respectively. (ii) LipTI and LipTII were equally recognized by anti-Lip(TT) antibody. (iii) Southern blot analysis suggested that bovine lipoyltransferase appears to be encoded by a single copy gene. (iv) Northern blot analysis indicated the presence of a single transcript of 1.5 kilobases. (v) A specific mRNA for LipTI could not be detected by RT-PCR analysis.

We cloned and expressed a cDNA encoding mammalian lipoyltransferase for the first time. Purification of lipoyltransferase from animal liver is laborious because of the low content of the enzyme. Here we present a simple purification method to obtain milligram quantities of recombinant bovine lipoyltransferase II. The recombinant enzyme showed the same properties as the native enzyme. The availability of cDNA clone coding for lipoyltransferase should facilitate studies of structural and functional aspects of this enzyme.

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