In mammals, lipoate-activating enzyme (LAE) catalyzes the activation of lipoate to lipoyl-nucleoside monophosphate. The lipoic moiety is then transferred to the specific lysine residue of lipoate-dependent enzymes by the action of lipoyltransferase. We purified LAE from bovine liver mitochondria to apparent homogeneity. LAE activated lipoate with GTP at a 1000-fold higher rate than with ATP. The reaction absolutely required lipoate, GTP, and Mg$^{2+}$ ion, and the reaction product was lipoyl-GMP. LAE activated both (R)- and (S)-lipoate to the respective lipoyl-GMP, although a preference for (R)-lipoate was observed. Similarly, lipoyltransferase equally transferred both the (R)- and (S)-lipoyl moieties from the respectively activated lipoates to apoH-protein. Interestingly, however, only H-protein carrying (R)-lipoate was active in the glycine cleavage reaction. cDNA clones encoding a precursor LAE with a mitochondrial presequence were isolated. The predicted cDNA clones encoding a precursor LAE with a mitochondrial presequence were isolated. The predicted amino acid sequence of LAE is identical with that of xenobiotic-metabolizing/medium-chain fatty acid-CoA ligase-III, but an amino acid substitution due to a single nucleotide polymorphism was found. These results indicate that the medium-chain acyl-CoA synthetase in mitochondria has a novel function, the activation of lipoate with GTP.

Lipoic acid is a prosthetic group of H-protein of the glycine cleavage system and the acyltransferase components (E2s) of the pyruvate, α-ketoglutarate, and branched-chain α-ketoacid dehydrogenase complexes (1–4). The lipoic moiety is attached to the specific lysine residue of the proteins via an amide linkage. The lipoyllysine arm is responsible for the shuttling of the reaction intermediate and reducing equivalents between the active sites of the complexes.

The covalent attachment of lipoic acid occurs in two steps as follows.

**Lipoic acid + ATP → lipoyl-AMP + PP;**

**REACTION 1**

**Lipoyl-AMP + apoprotein → holoprotein + AMP**

**REACTION 2**

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glyceraldehyde-3-phosphate (5 mM), ATP (5 mM), MgCl2 (5 mM), 10 mM Tris, and 10 mM NH2OH-
HCl at 32 °C for 1 h. After terminating the reaction by heating the mixture, the mixture was transferred to a Microcon YM-30 (Millipore) and centrifuged. The product, lipoyl-GMP, in the filtrate was analyzed by high performance liquid chromatography (HPLC). The column was developed with an acetonitrile gradient as described (12). Elution of lipoyl-GMP was monitored at 252 nm (λmax).

TABLE I

| Step            | Protein | Coupled method with GTP | Hydroxamate method with ATP |
|-----------------|---------|-------------------------|-----------------------------|
|                 |         | Total activity (mg) | Specific activity (μmol/h/mg) | Total activity (muol/h) | Specific activity (μmol/h/mg) |
| Extract         | 168.5   | 487.2                  | 0.25                        | 29.2                     | 0.017                      |
| DEAE-Sepharose  | 212     | 268.3                  | 1.27                        | 15.7                     | 0.074                      |
| Hydroxylapatite | 15.8    | 115.8                  | 7.33                        | 9.22                     | 0.58                       |
| Superdex 200    | 8.30    | 85.2                   | 10.27                       | 8.39                     | 1.01                       |
|                 | 6.87    | 67.7                   | 9.85                        | 7.09                     | 1.03                       |

RESULTS AND DISCUSSION

Purification of LAE—To isolate LAE, we employed the coupled method for the detection of the enzyme. Mitochondrial, microsomal, and cytosolic fractions were separated from bovine liver homogenate, and LAE activities in each fraction was determined. Surprisingly, high activity (3400 nmol/h/g of liver) was observed in mitochondria when GTP was employed as a high energy compound, and more than 99% of the total activity was confined in mitochondria. In contrast, total activity assayed with ATP was extremely low (8.78 nmol/h/g of liver).

Tsunoda and Yasunobu (6) demonstrated the presence of LAE in the postmitochondrial supernatant fraction of bovine liver, but we were not able to find significant activity in the cytosol from bovine liver. The mitochondrial localization of LAE is consistent with the observation that lipoyltransferase and the proteins to be lipoylated are localized in mitochondria. Therefore, we purified the GTP-dependent LAE from mitochondria. The mitochondrial extracts were applied to a DEAE-Sepharose column, and the column was developed with a NaCl gradient. Most of the activity (268.3 μmol/h) was recovered in fractions eluted at about 40 mM NaCl, whereas a very small amount of the activity was detected in the flow-through fractions (0.236 μmol/h). About half of the activity in the flow-through fraction was precipitated by centrifugation at 144,000 × g for 1 h, suggesting that the activity is still bound to fragments of mitochondrial membranes. Further characterization of the activity in the flow-through fraction has not been performed because the activity is negligible compared with that in the 40-mM NaCl fraction. LAE in the 40-mM NaCl fraction was purified further by successive chromatographies on hydroxylapatite, Blue-Sepharose, and Superdex 200 columns. Throughout these purification steps, a single peak of LAE activity was observed, and elution profiles obtained by the coupled method and the hydroxamate method were parallel (not shown). A summary of the purification is shown in Table I. Activities by the coupled method with ATP were less than 1% of those by the hydroxamate method throughout the purification (data not shown).

The mitochondrial extracts were digested with EcoRV, BamHI, HindIII, KpnI, or SacI, separated on a 0.7% agarose gel, and transferred to Hybond N+ as described previously (11). The membranes were hybridized with a 32P-labeled 333-base pair cDNA fragment (nucleotides 1733–2065 (GenBank accession number AB048289)) synthesized by polymerase chain reaction. The hybridization and washing of the membrane were carried out according to the protocol provided by Amersham Pharmacia Biotech.

Other Methods—The medium-chain acyl-CoA synthetase activity was measured by the standard radioisotope method described by Vessey et al. (15), and medium-chain acyl-CoA synthesis was determined by the HPLC method for LAE described above. The N-terminal amino acid sequences of the purified LAE and lysylpeptides were determined as described previously (16). Assay of lipoyltranserase was carried out as described (7). Chromatofocusing of LAE was carried out with PBE™ 94 and Polybuffer 74 according to a protocol provided by the manufacturers. Protein concentrations were determined by the method of Bradford (17). Native-PAGE and SDS-PAGE were carried out as described (2, 18).

Southern Blot Analysis—Bovine genomic DNA (2.5 μg) was digested with EcoRV, BamHI, HindIII, KpnI, or SacI, separated on a 0.7% agarose gel, and transferred to Hybond N+ as described previously (11). The membranes were hybridized with a 32P-labeled 333-base pair cDNA fragment (nucleotides 1733–2065 (GenBank accession number AB048289)) synthesized by polymerase chain reaction. The hybridization and washing of the membrane were carried out according to the protocol provided by Amersham Pharmacia Biotech.
determined by the coupled method with ATP and the hydroxamate method suggests that the lipoate-AMP produced is hardly released from the enzyme. The final purified preparation represented a 39-fold purification with a yield of 16% over the extracts when measured by the coupled method and was apparently homogeneous on SDS-PAGE (not shown). The molecular mass of LAE was determined to be 61 kDa by SDS-PAGE, whereas it was 49 kDa by gel filtration chromatography on Superdex 200. An isoelectric point of 5.67 was obtained by chromatofocusing. An optimal pH of the LAE reaction was determined to be 6.6 by the HPLC method. However, the reaction in the coupled method was carried out at a higher pH, because lipoate transferase has a strict optimal pH at 7.9 (7).

Catalytic Properties—The requirements for LAE reaction were determined by the coupled method. The removal of (R)-lipoate, GTP, or MgCl₂ from the reaction mixture resulted in no holoH-protein formation, indicating that the reaction was absolutely dependent on (R)-lipoate, GTP, and MgCl₂. With (S)-lipoate, holoH-protein formation was less than 1% of that with (R)-lipoate, the naturally occurring enantiomer. Removal of lipoate transferase also resulted in no holoH-protein formation, indicating that LAE has no ability to transfer the activated lipoate to apoH-protein.

Steady state kinetic analyses were carried out with various nucleoside triphosphates. The reaction exhibited typical Michaelis-Menten kinetics with respect to both substrates. As shown in Table II, \( V_{\text{max(app)}} \) values with GTP, CTP, and UTP were about 1000-fold greater than with ATP. ATP is strongly involved in the reaction, because lipoyltransferase has a strict optimal pH at 7.9 (7).

**Table II**

| Varied substrate | \( K_{\text{m(app)}} \) \(^a\) | \( V_{\text{max(app)}} \) \(^a\) | \( k_{\text{cat(app)}} \) \(^a\) |
|------------------|-----------------|-----------------|-----------------|
| ATP              | 1.3             | 14.4            | 0.88            |
| (R)-lipoate      | 0.0047          | 14.3            | 0.87            |
| GTP              | 0.37            | 13800           | 842             |
| (R)-lipoate      | 0.041           | 15300           | 933             |
| CTP              | 1.2             | 13500           | 824             |
| (R)-lipoate      | 0.039           | 12700           | 775             |
| UTP              | 8.8             | 14100           | 860             |
| (R)-lipoate      | 0.18            | 13000           | 793             |

\(^a\) Values are expressed as the average of two experiments with a standard error of less than 15%.

The product of LAE reaction was analyzed by the HPLC method. Authentic lipoyl-GMP was subjected to the lipoyltransferase reaction. The lipoylated H-protein exhibited the glycine-\(^14\)CO₂ exchange activity of 7.51 nmol/30 min, corresponding to 9940 nmol/h/mg of protein. The value was in good agreement with that obtained by the coupled method (Tables I and II). The formation of (S)-lipoyl-GMP (1597 pmol) was determined to be 64.5% of the (R)-lipoyl-GMP formation. The peak fractions were collected, and an aliquot of (R)-lipoyl-GMP or (S)-lipoyl-GMP was subjected to the glycine-\(^14\)CO₂ exchange reaction. The lipoylated H-protein was then analyzed by native-PAGE and the glycine-\(^14\)CO₂ exchange activity. The lipoylated H-protein migrates faster than apoH-protein on native-PAGE because of reduction of a positive charge of the lysine residue to be lipoylated (2). As shown in Fig. 1B, apoH-protein (7.1 pmol) is completely lipoylated by lipoate transferase with 20 pmol of (R)-lipoi-GMP. The lipoylated H-protein exhibited the glycine-\(^14\)CO₂ exchange activity of 7.51 nmol/30 min, corresponding to 9.74 pmol of lipoylated H-protein (Fig. 1C). Interestingly, lipoate transferase could transfer the lipoate moiety from (S)-lipoyl-GMP to H-protein as well as from (R)-lipoyl-GMP (Fig. 1B). However, it was clearly demonstrated for the first time that only H-protein having the (R)-lipoyl moiety was active in the glycine cleavage system (Fig. 1C). The very low exchange activity with (S)-lipoylated H-protein may be attributed to the contamination of (R)-lipoate.

**Fig. 1. Analyses of reaction products.** A, HPLC analysis of the reaction product by LAE with GTP. The LAE reaction was carried out with (R)-lipoate (c) or (S)-lipoate (d) in the presence of 0.249 μg of LAE or with (R)-lipoate in the absence of LAE (b) as described under "Experimental Procedures." The reaction products were resolved on an ODS column. The arrows indicate retention times of the standard lipoyl-GMP (a), ——, absorbance at 252 nm; – – –, concentration of acetonitrile. B, native-PAGE analysis of H-protein lipoylated with lipoyl-GMP obtained in panel A. ApoH-protein (7.1 pmol) was lipoylated by 0.33 μg of lipoate transferase without lipoyl-GMP (lane 1) or with 20 pmol of (R)-lipoyl-GMP (lane 2) or (S)-lipoyl-GMP (lane 3) and analyzed. The gel was stained with a Silver Staining Kit, Protein (Amersham Pharmacia Biotech). C, the glycine-\(^14\)CO₂ exchange activity of H-protein lipoylated by 0.33 μg of lipoate transferase with lipoyl-GMP obtained in panel A. ApoH-protein (142 pmol) was lipoylated without lipoyl-GMP (column 1) or with 20 pmol of (R)-lipoyl-GMP (column 2) or (S)-lipoyl-GMP (column 3).
Kinetic Properties of Lipoyltransferase—The high rate of lipoylation with GTP in the coupled method described above strongly indicates that lipoyltransferase is able to utilize lipoyl-GMP as a substrate. To confirm this, we examined the kinetic properties of lipoyltransferase using chemically synthesized lipoyl-GMP. As shown in Table III, lipoyltransferase utilized lipoyl-GMP as well as lipoyl-AMP, although the affinity for lipoyl-GMP was about three times lower than that for lipoyl-AMP.

Cloning and Sequencing of cDNAs for LAE—The N-terminal amino acid sequence analysis showed that the purified LAE had heterogeneous N termini. They start from leucine (58%), amino acid 32 (GenBank accession number AB048289), serine (8%), amino acid 33), glycine (9%, amino acid 34), and alanine (25%, amino acid 35). We then obtained several lysylpeptides and analyzed the N-terminal amino acid sequence to synthesize oligonucleotide probes based on the sequences. The oligonucleotides were employed for the screening of a λgt10 bovine liver cDNA library. Approximately 4 × 10⁵ independent clones were screened, and 18 positive clones were obtained. Five clones containing full-length cDNA for LAE were chosen randomly, and the nucleotide sequences were analyzed. The longest cDNA consists of 2114 base pairs and contains an open reading frame of 1731 base pairs. The cDNA encodes for 577 amino acids, including a mitochondrial presequence of 31 amino acid residues and a mature LAE of 546 amino acid residues. The predicted presequence shows a characteristic feature, being rich in basic amino acid residues, and an absence of acidic amino acid residues. The nucleotide sequence has been submitted to the DDBJ/EMBL/GenBank with accession number AB048289. The calculated molecular mass of the mature LAE of 61,146 Da is in good agreement with that determined by SDS-PAGE. The sequence CCACATGCG surrounding the first in-frame ATG codon matches the optimum translation initiation sequence, CC(A/G)CCATG, described by Kozak (20). A putative polyadenylation signal, AATAAA, is located 18 base pairs upstream from the poly(A) tail. The amino acid sequence of LAE contains a putative AMP/ATP binding motif common to acyl-CoA synthetase (21). Amino acid sequence homology search by FASTA and BLAST showed that the sequence of LAE is identical to that of xenobiotic-metabolizing/medium-chain fatty acid:CoA ligase-III from bovine liver, although the mitochondrial presequence has not been predicted (10). The cDNA obtained extends by 28 nucleotides from the reported 5′ end. Furthermore, the following differences from the sequence reported were observed. First, a single nucleotide substitution of G for A at nucleotide position 1244 (GenBank accession number AB048289) was found in four of five clones analyzed, and consequently an amino acid substitution of alanine for threonine was predicted at position 372. Amino acid sequence analysis of a lysylpeptide obtained from purified LAE showed the amino acid residue at position 372 to be alanine. Second, an insertion of 39 nucleotides (nucleotides 80–118 (GenBank accession number AB048289)) due to an alternative splicing was found in two of five clones.

Southern blot analysis was carried out to examine whether more than one gene encoding LAE was present in a genome, because two distinct cDNA containing a different nucleotide at the position 1244 were isolated from a cDNA library. As shown in Fig. 2, a single band was detected in each of the enzyme digests, indicating the presence of a single copy of the gene encoding LAE. The result suggests that the difference of the nucleotide at position 1244 should be due to a single nucleotide polymorphic mutation.

Relationship between Medium-chain Acyl-CoA Synthesis and Lipoyl (S-lipoate) Activation—We next examined whether the purified LAE can catalyze acyl-CoA synthesis. Table IV shows that the purified enzyme exhibited activities of medium-chain acyl-CoA synthesis with the highest specific activity with hexanoic acid. The data are consistent with those of bovine mitochondrial xenobiotic-metabolizing/medium-chain fatty acid:CoA ligase-III reported previously (15, 22). Because LAE utilizes GTP, acyl-GMP formation was examined. A broad substrate specificity was observed with respect to the chain length of fatty acids (Table IV). The formation of lipoyl-GMP was comparable with that of decanoyl-GMP.

To explore the relationship between the lipoylation activation and the acyl-CoA synthesis, the effects of lipoyl and GTP on the acyl-CoA synthesis (with ATP) were investigated. When hexanoate was the fatty acid substrate, synthesis of hexanoyl-CoA was reduced by 65% (with 0.1 mM lipoyl) and 52% (with 2 mM GTP). The inhibitions by lipoyl and GTP were competitive with respect to hexanoate and ATP, respectively (K_i for lipoyl, 3.9 ± 0.3 μM, K_m(app) for hexanoate, 5.9 ± 1.3 μM, K_i for GTP, 0.58 ± 0.13 mM; K_m(app) for ATP, 0.32 ± 0.10 mM; n = 3). Similarly, lipoyl-GMP formation was inhibited by substrates.
for acyl-CoA synthesis. The addition of 0.25 mM CoA and 0.1 mM hexanoate inhibited the reaction by 52 and 15%, respectively. The addition of 2 mM ATP inhibited the reaction nearly completely (residual activity was 1.4% of that of control) because the lipoyl-AMP formed could hardly dissociate from LAE. The inhibition by ATP was slightly relieved in the presence of 0.25 mM CoA to 5.6% residual activity (586 nmol/h/mg of protein) with concomitant synthesis of lipoyl-CoA (13269 nmol/h/mg of protein). Although the rate of lipoyl-GMP formation in this situation is very low, it is about 40-fold greater than that of lipoyl-AMP formation (Table II). These results suggest that GTP and lipoate share the binding site with ATP and fatty acid, respectively, on LAE and that LAE can catalyze the lipoyl-GMP formation in mitochondria where ATP, CoA, and GTP coexist. It is unclear at present whether there is some biochemical mechanism responsible for the preferential production of lipoyl-GMP in mitochondria.

Conclusions—This work characterizes the LAE reaction and demonstrates a novel function of the medium-chain acyl-CoA synthetase in mitochondria. Although ATP has been recognized as a substrate for lipoate activation, the present study demonstrates that LAE catalyzes the activation of lipoate utilizing GTP and easily releases the product, lipoyl-GMP, from the active site to provide a substrate for lipoyltransferase. In contrast, ATP is an essential substrate for the acyl-CoA synthesis because the reaction intermediate, acyl-AMP, has to be retained on the active site of the enzyme to react with CoA. We could not detect a significant amount of lipoyl-activating activity other than the purified LAE in mitochondria, and we therefore conclude that the enzyme reported here is primarily responsible for the activation of lipoate in mitochondria.

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Purification, Characterization, and cDNA Cloning of Lipoate-activating Enzyme from Bovine Liver
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