Lipooylation of Aroyltransferase Components of \( \alpha \)-Ketoacid Dehydrogenase Complexes*

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Lipoic acid is a prosthetic group of the acyltransferase components of the pyruvate, \( \alpha \)-ketoglutarate, and branched chain \( \alpha \)-ketoacid dehydrogenase complexes, protein X of the eukaryotic pyruvate dehydrogenase complex, and H-protein of the glycine cleavage system. We have purified lipoyl-AMP-N\(^{\text{a}}\)-lysine lipoyltransferase I and II from bovine liver mitochondria employing apoh-protein as an acceptor of lipoic acid (Fujiwara, K., Okamura-Ikeda, K., and Motokawa, Y. (1994) J. Biol. Chem. 269, 16605–16609). In this study, we demonstrated the lipooylation of the lipoyl domains of the mammalian pyruvate (LE2\(p\)), \( \alpha \)-ketoglutarate (LE2\(k\)), and branched chain \( \alpha \)-keto acid (LE2\(b\)) dehydrogenase complexes using the purified lipoyltransferase I and II. Lipoyltransferase I and II lipoylated LE2\(p\) and LE2\(k\) as efficiently as H-protein, but the lipooylation rate of LE2\(b\) was extremely low. Comparison of amino acid sequences surrounding the lipooylation site of these proteins shows that the conserved glutamic acid residue situated 3 residues to the N-terminal side of the lipooylation site is replaced by glutamine (Gln-41) in LE2\(b\). When Gln-41 of LE2\(b\) was changed to Glu, the rate of lipooylation increased about 100-fold and became comparable to that of LE2\(p\) and LE2\(k\). The replacement of the glutamic acid residue of LE2\(p\) (Glu-169) and LE2\(k\) (Glu-40) by glutamine resulted in decrease in the lipooylation rate more than 100-fold. These results suggest that the glutamic acid residue plays an important role in the lipooylation reaction possibly functioning as a recognition signal. Gly-27 and Gly-54 of LE2\(k\) are also well conserved among the lipoyl domains of the \( \alpha \)-ketoacid dehydrogenase complexes and H-protein. The mutagenesis experiments of these residues indicated that the glycine residue situated 11 residues to the C-terminal side of the lipooylation site (Gly-54 of LE2\(k\)) is important for the folding of lipoyl domain, and that existence of a small residue such as Gly or Cys at the position is essential for the lipooylation of these proteins.

Five lipoate-containing proteins are known in vertebrate: acyltransferase components of the pyruvate, \( \alpha \)-ketoglutarate, and branched chain \( \alpha \)-ketoacid dehydrogenase complexes (E2\(p\), E2\(k\), and E2\(b\), respectively), protein X of the pyruvate dehydrogenase complex, and H-protein of the glycine cleavage system. Lipoyl attaches to the ε-amino group of the specific lysine residue of the proteins via an amide linkage. The lipoyllysine residue functions as a carrier of intermediates of the reactions and reducing equivalents interacting with the active sites of the components of the complexes (1–4). Early studies of the lipooylation of apohE2\(p\) using crude enzyme preparations from Escherichia coli and Streptococcus faecalis showed that lipoyl-AMP is an activated intermediate of the reaction (5). We have purified lipoyl-AMP-N\(^{\text{a}}\)-lysine lipoyltransferase (lipoyltransferase) from bovine liver mitochondria using apoh-protein as an acceptor of lipoate (6). Two isoforms, lipoyltransferase I and II, 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 catalyze the transfer of the lipoyl moiety from lipoyl-AMP to apoh-protein, but have no ability to activate lipoate to lipoyl-AMP. Lipoylation of H-protein in bovine liver, therefore, requires lipoate-activating enzyme that has been partially purified from bovine liver (7). On the contrary, lipooylation of E2\(p\) in E. coli is catalyzed by a single enzyme, lipoate-protein ligase (8, 9).

Our current interest is to determine whether the lipoyltransferases purified from bovine liver are involved in the lipooylation of E2 components of the \( \alpha \)-ketoacid dehydrogenase complexes. To address the question, we used the apolipo domain of E2 components translated in vitro with a reticulocyte lysate system as a protein substrate. The results presented here show that lipoyltransferase I and II can lipoylate the lipoyl domain of E2 components as well as H-protein. Site-directed mutagenesis experiments indicated that the glutamic acid residue situated 3 residues on the N-terminal side of the lipooylation site is important for the lipooylation of E2 components and the glycine residue situated 11 residues on the C-terminal side of the lipooylation site is responsible for the folding of the lipoyl domain in accordance with the previous observations with H-protein (10).

EXPERIMENTAL PROCEDURES

Materials—T4 DNA ligase and TaKaRa Ex Taq DNA polymerase were purchased from Takara Shuzo (Shiga, Japan). Restriction endonucleases were from Toyobo (Tokyo, Japan). L-[\( ^{35} \)S]Methionine (0.055 Ci/mmol) was purchased from Amersham Corp. [\( ^{14} \)C]Octanoic acid (0.055 Ci/mmol) was purchased from DuPont NEN. Lipoyl-AMP was prepared as described previously (6). \( [\ ^{13} \)C]Octanoyl-AMP was synthesized essentially as described (6) using 250 μCi of \( [\ ^{13} \)C]Octanoic acid and isolated by high performance liquid chromatography on an ODS-120T column (4.5 × 250 mm; Tosoh, Tokyo, Japan) with a linear gradient of 0.05 mM sodium phosphate buffer, pH 5.5 (buffer A) and acetonitrile (buffer B). The gradient was developed from 10 to 45% B, and the elution of the product was monitored at 259 nm. Octanoyl-AMP was eluted at an acetonitrile concentration of 29%. Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer, cDNAs for rat E2\(p\) (11) and E2\(k\) (12) subcloned into pUC18 were kindly provided by Dr. Sada yuki Matuda (Department of Biology, Kanoya National Institute of Fitness and Sports, Kanoya, Japan). Lipoyltransferases were purified as described previously (6). The final preparations of lipoyltransferase I and II showed specific activities of 135 and 144 units/mg of protein, respectively. The activity of both enzyme preparations was adjusted to 1.67 × 10\(^{14}\) units/μl. Activity of lipoyltransferase was assayed as described previously (6).
Lipoylation of E2 Components—In order to test whether lipoyltransferase I and II that catalyze lipoylation of apoH-protein can lipoylate E2 components of the pyruvate, α-ketoglutarate, and branched chain α-ketoacid dehydrogenase complexes, we constructed the plasmids containing cDNA for the lipoyl domain of each E2 component as described under "Experimental Procedures." The plasmids were transcribed and translated in vitro. The translated products named LE2p, LE2k, and LE2b, respectively, were subjected to lipoylation with the purified lipoyltransferases. We have previously demonstrated that H-protein when lipoylated with lipoyltransferase moves faster than apoH-protein on non-denaturing polyacrylamide gel electrophoresis, since the apoprotein has an additional positive charge on the unmodified lysine residue (4, 6). Similarly, the lipoyl-AMP- and lipoyltransferase-dependent alterations of mobility of lipoyl domains were observed in this experiment (Fig. 1), indicating that apolipoyl domains of acyltransferases are lipoylated as well with the purified lipoyltransferases that lipoylate H-protein of the glycine cleavage system. The fact that the faster migrating lipoyl domain on non-denaturing polyacrylamide gel electrophoresis is the lipoylated form has been demonstrated with the overexpressed human E2p (18). Fully lipoylated proteins were obtained with apolipoyl domains of E2p and E2k and apoH-protein as substrates when 6.7 × 10^−4 unit of either lipoyltransferase I or II was employed (Fig. 1, A, B, and D), whereas much more amounts of lipoyltransferase were required to lipoylate the apolipoyl domain of E2b (Fig. 1C). Again, lipote plus could not substitute lipoyl-AMP in the lipoylation of apolipoyl domains (results not shown) as reported previously with apoH-protein (6). A minor component was detected when the in vitro translation products of pLE2p were subjected to non-denaturing polyacrylamide gel electrophoresis (Fig. 1A). pLE2p encodes the inner lipoyl domain of rat E2p spanning from Ser-127 to Pro-253 (11). The peptide has three methionine residues (Met-132, Met-143, and Met-145) in addition to the inserted initiator methionine. Either the codon for Met-143 or Met-145 is probably functional as an alternate initiator to produce the minor component, since the nucleotide sequences around the ATG codons conform the consensus sequence of Kazak (19). The minor component receives lipote, since the mobility changed when incubated with lipoyl-AMP and lipoyltransferase (Fig. 1A).

The Role of the Glutamic Acid Residue Situated 3 Residues on the N-terminal Side of the Lipoyllysine Residue—Comparison of amino acid sequences around the lipote attachment site of H-protein and acyltransferases of the α-ketoacid dehydrogenase complexes from various sources indicated that Gly-43, Glu-56, and Gln-70 of bovine H-protein are highly conserved among these proteins (10). Inspection of the sequences around the lipoylation site of E2 components indicates that among...
Anionic charge appears to have a role in the recognition by lipoyltransferase. The important for the lipoylation with lipoyltransferase possibly residues to the N-terminal side of the lipoyllysine residue is protein (10) indicate that the glutamic acid residue situated 3 results obtained from the intramitochondrial lipoylation of H-wild type (Table I and Fig. 4). These results together with the LE2k-E40Q decreased dramatically when compared with the glutamine. The lipoylation rate of mutants LE2p-E169Q and residue, Glu-169 of LE2p and Glu-40 of LE2k were replaced by nearly the same initial velocity.

lipoyltransferase I or II than the wild-type LE2b to obtain 100-fold, since LE2b-Q41E required 100-fold less amounts of lipoylation observed above in the lipoylation reduced rate of lipoylation observed above in the lipoylation site-directed mutagenesis (10). It is plausible that the presence of the glutamine residue at position 41 of E2b is the cause of the altered position of lipoylation observed above in the lipoylation with the purified lipoyltransferase. To test the possibility, LE2b-Q41E, a mutant lipoyl domain of E2b in which Gln-41 is replaced by Glu was generated in an in vitro translation reaction and subjected to lipoylation. As shown in Fig. 3 and Table I, the lipoylation of LE2b-Q41E was greatly improved when compared with the wild-type LE2b. Initial velocity studies of lipoylation of the wild-type and the mutant LE2b indicated that the alteration of Gln-41 to Glu increased the rate about 100-fold, since LE2b-Q41E required 100-fold less amounts of lipoyltransferase I or II than the wild-type LE2b to obtain nearly the same initial velocity.

To investigate further the critical role of the glutamic acid residue, Glu-169 of LE2p and Glu-40 of LE2k were replaced by glutamine. The lipoylation rate of mutants LE2p-E169Q and LE2k-E40Q decreased dramatically when compared with the wild type (Table I and Fig. 4). These results together with the results obtained from the intramitochondrial lipoylation of H-protein (10) indicate that the glutamic acid residue situated 3 residues to the N-terminal side of the lipoyllysine residue is important for the lipoylation with lipoyltransferase possibly functioning as a recognition signal for lipoyltransferase. The anionic charge appears to have a role in the recognition by lipoyltransferase. The side chain length of the glutamic acid residue appears to be also responsible, since replacement of Glu-56 of H-protein with Asp reduced the rate of lipoylation significantly (10). Griffin et al. (20) reported that the bovine mature E2b overexpressed in E. coli cells grown in a medium containing [2-3H]lipolate received no radioactivity, whereas the inner lipoyl domain of human E2p was lipoylated in E. coli (18). These observations suggest that the mechanism of the interaction of the substrate and lipoyltransferase is similar in animals and bacteria. In the three-dimensional structure of lipoyl domain of E2p from E. coli (21), the glutamic acid residue is
situated at the end of a β-strand preceding the tight β-turn in which the lysine residue to be lipoylated is located. No amino acid residues responsible for recognition for lipoyltransferase other than the glutamic acid residue have been reported. The conserved aspartic acid and alanine residues of E2 domain flanking the lipoyllysine residue, on the N- and C-terminal sides, appear to have no role for the recognition (22).

The Role of Conserved Glycine Residues—Highly conserved glycine residues reside 16 residues to the N-terminal side and 11 residues to the C-terminal side of the lipoylation site in E2 components and H-protein (Fig. 2). To investigate the role of these glycine residues in lipoylation of E2 components, these residues of LE2k (Gly-27 and Gly-54) have been replaced by serine or asparagine. On nondenaturing polyacrylamide gel electrophoresis, the mobilities of apoforms of LE2k-G54S and LE2k-G54N were greatly reduced when compared with the wild type (Fig. 5, A, C, and E), suggesting that the substitution caused a conformational change. The Gly to Ser substitution affected the lipoylation moderately, but the Gly to Asn substitution abolished the lipoylation (Table I and Fig. 5, A, C, and E). On the other hand, the replacements of Gly-27 by Ser or Asn did not show any effects on the mobility on the nondenaturing polyacrylamide gel electrophoresis or the rate of lipoylation (Table I and Fig. 5, B and D). To confirm these observations, the incorporation of the radiolabeled octanoyl moiety from [14C]octanoyl-AMP to wild-type and mutant LE2ks was examined. We employed [14C]octanoyl-AMP as an alternative substrate for lipoyltransferase since radiolabeled lipoic acid is not available commercially, and lipoyltransferase can octanoylate apoE2k (Fig. 6) as well as apoH-protein (6). As shown in Fig. 6, LE2k, LE2k-G27S, and LE2k-G27N are equally octanoylated by lipoyltransferase II. The octanoylated LE2k-G54S shows a broad band with lowered mobility as compared with the wild-type LE2k on nondenaturing polyacrylamide gels in accordance with the results shown in Fig. 5C. As expected, LE2k-E40Q and LE2k-G54N were not octanoylated (Fig. 6). The same results were obtained with lipoyltransferase I (not shown). These results are consistent with the above observations with lipoyl-AMP. The results presented here indicate that Gly-54 has some role for the folding of the lipoyl domain. The effects of Gly-54 mutations on lipoylation apparently depend on the substituting amino acid residue. Therefore, Gly-54 appears not to be involved in the recognition by lipoyltransferase. Indeed, the amino acid residue corresponding to Gly-54 of the outer lipoyl domain of rat E2p is cysteine (Fig. 2). We previously reported the similar results with H-protein when Gly-70 corresponding to Gly-54 of rat E2k was replaced by Ser or Asn (10). The mutations apparently destabilized H-protein and reduced the

### Table I

| Lipoyl domain | Lipoyltransferase I | Lipoyltransferase II |
|---------------|---------------------|----------------------|
| LE2b          | 100                 | 100                  |
| LE2b-Q41E     | 8700                | 7800                 |
| LE2k          | 100                 | 100                  |
| LE2k-E40Q     | 0.3                 | 0.3                  |
| LE2k-G27S     | 98.6                | 104                  |
| LE2k-G27N     | 113                 | 110                  |
| LE2k-G54S     | 80.4                | 76.2                 |
| LE2k-G54N     | <0.2                | <0.2                 |
| LE2p          | 100                 | 100                  |
| LE2p-E169Q    | <0.2                | <0.2                 |

### Fig. 4

**Lipoylation of wild-type LE2k and mutant LE2k-E40Q.** Wild-type LE2k (A) and mutant LE2k-E40Q (B) were lipoylated with lipoyltransferase I (lanes 2–5), lipoyltransferase II (lanes 6–9), or without enzyme (lane 1). The enzyme preparation of 0.02 μl (lanes 2 and 6), 0.08 μl (lanes 3 and 7), 0.4 μl (lanes 4 and 8), and 2 μl (lanes 5 and 9) was used. The autoradiographs were obtained as described in the legend to Fig. 1. The percent lipoylation was determined by quantitating radioactivities of lipoyl domains in accordance with the results shown in Fig. 5. A, apo LE2k; ●, LE2k-E40Q. Apo and lip indicate apof orm and lipoylated form, respectively.

### Fig. 5

**Lipoylation of LE2k and its mutants.** Wild-type LE2k (A) and its mutants, LE2k-G27S (B), LE2k-G54S (C), LE2k-G27N (D), and LE2k-G54N (E) were lipoylated with 0 μl (lane 1), 0.02 μl (lane 2), 0.08 μl (lane 3), 0.4 μl (lane 4), and 2 μl (lane 5) of lipoyltransferase II. The autoradiographs were obtained as described in the legend to Fig. 1. A, apo and lip indicate apof orm and lipoylated form, respectively.
rate of lipoylation. The Gly-54 is located at the opposite site of the lipoyllysine residue in the lipoyl domain (21). A bulky residue at the position may prevent the access of the lysine residue to be lipoylated to the catalytic site of lipoyltransferase. Interestingly, a glycine residue corresponding to Gly-54 of E2k is also present in biotin enzymes. Mutations of the glycine residue of E. coli biotin carboxyl carrier protein of acetyl-CoA carboxylase and α-subunit of human propionyl-CoA carboxylase to serine caused a less efficient biotinylation (23, 24). The biotinylation of the mutant biotin carboxyl carrier protein is temperature-sensitive and the defect is more severe at 42°C than at 30°C (23), suggesting the incomplete folding of the protein to be biotinylated.

Conclusion—We demonstrated that lipoyltransferase I and II purified from bovine liver can lipoylate not only H-protein of the glycine cleavage system but also lipoyl domains of acyl-CoA dehydrogenase complexes. No difference in the substratespecificity was found with lipoyltransferase I and II. The lipoyl domain of bovine E2b is not a good substrate for the lipoyltransferases. It remains to be established whether the in vivo lipoylation of E2b is catalyzed by the lipoyltransferases already described or whether the lipoylation requires a novel, as yet unidentified lipoyltransferase. The study of lipoylation of mutant lipoyl domains indicated that the glutamic acid residue situated 3 residues to the N-terminal side of the lipoylation site appears to be essential, but not exclusive, for the recognition by lipoyltransferase, and that the glycine residue located 11 residues to the C-terminal side of the lipoyllysine residue appears to be important for the folding of the lipoyl domain of E2 components.

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Fig. 6. Octanoylation of LE2k and its mutants. Wild-type LE2k (lane 1), and its mutants, LE2k-E40Q (lane 2), LE2k-G27S (lane 3), LE2k-G27N (lane 4), LE2k-G54S (lane 5), and LE2k-G54N (lane 6) were incubated with [14C]octanoyl-AMP and lipoyltransferase II, and the products were separated by 20% nondenaturing polyacrylamide gel electrophoresis as described under "Experimental Procedures." Oct-1 indicates octanoylated LE2k, LE2k-G27S, and LE2k-G27N, and oct-2 indicates octanoylated LE2k-G54S.
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