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Lipoylation of H-protein of the glycine cleavage system

The effect of site-directed mutagenesis of amino acid residues around the lipoyllysine residue on the lipoate attachment

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H-protein of the glycine cleavage system has lipoic acid on the Lys^{59} residue. Comparison of amino acid sequences around the lipoate attachment site of H-proteins from various sources and acyltransferases of α-*keto* acid dehydrogenase complexes indicated that Gly^{43}, Glu^{56}, Glu^{63} and Gly^{70} of bovine H-protein are highly conserved among these proteins. Modification of these conserved residues by site-directed mutagenesis indicated that Glu^{56} and Gly^{70} are important for the lipoylation of H-protein and suggested that the proper conformation around the lipoic acid attachment site is required for the association of H-protein to the enzyme responsible for the lipoylation.

Glycine cleavage system; H-protein; Lipoylation; Post-translational modification; Site-directed mutagenesis

1. INTRODUCTION

The glycine cleavage system catalyzes the reversible oxidation of glycine, yielding carbon dioxide, ammonia, 5,10-methylenetetrahydrofolate, and a reduced pyridine nucleotide [1–5]. It consists of 4 component proteins named P-protein, H-protein, T-protein, and L-protein. P-protein catalyzes the pyridoxal phosphate-dependent decarboxylation of glycine. The carboxyl carbon is released as CO_2 and the remaining methylamine moiety of glycine is transferred to one of the sulfhydryl groups of the lipoic acid prosthetic group of H-protein. T-protein catalyzes the release of NH_3 from the intermediate bound to H-protein and the synthesis of 5,10-methylenetetrahydrofolate in the presence of tetrahydrofolate. L-protein is a lipoamide dehydrogenase and catalyzes the reoxidation of the resulting dihydrolipoic acid. The amino acid sequence of chicken H-protein has been determined chemically [6]. The acidic protein of 125 amino acid residues has lipoic acid with an amide linkage between the carboxyl group of lipoic acid and the amino group of Lys^{59}. Complementary DNAs for bovine [7], human [8,9], chicken [10], and pea [11,12] H-protein have recently been cloned, and the primary sequences of the proteins have been deduced. The sequence around the lipoate residue in H-protein shows significant homology with the corresponding region of acyltransferases (E2) of α-*keto* acid dehydrogenase complexes, which also utilize lipoic acid as a cofactor in their catalytic mechanism (Fig. 1 and [13]). The presence of conserved amino acids in the lipoate attachment site of these proteins suggests that the residues may play important roles in function and/or in their enzymatic lipoylation.

Little is known concerning the enzymes responsible for attachment of lipoic acid to proteins. In order to explore the mechanism of lipoate attachment, we have devised a method for detecting the lipoylation of H-protein and demonstrated that the enzymatic attachment of lipoic acid is an intramitochondrial event [7]. The method is based on the fact that lysylendopeptidase cleaves peptide bonds at the carboxyl-terminal side of lysine residues but not if the residue is modified [21]. In the present study, we have employed this method to examine the importance for the lipoylation of the conserved amino acid residues around the lipoate attachment site of H-protein. Site-directed mutagenesis and in vitro translation of mutant bovine H-proteins indicate that Glu^{56} and Gly^{70} are implicated in the efficient lipoylation.

2. MATERIALS AND METHODS

2.1. *Materials*

L-[4,5-^{3}H]leucine (120–190 Ci/mmol) was obtained from Amersham Corp. (UK). [α-^{32}P]dCTP (3000 Ci/mmol) was from Du Pont-New England Nuclear (USA). Restriction endonucleases were purchased from Toyobo (Tokyo, Japan) or Takara Shuzo (Kyoto, Japan). T7 RNA polymerase was from Promega (USA). RNase-free DNase was prepared from a preparation of pancreatic DNase I (Sigma) according to the method of Maxwell et al. [22]. Lysylendopeptidase was obtained

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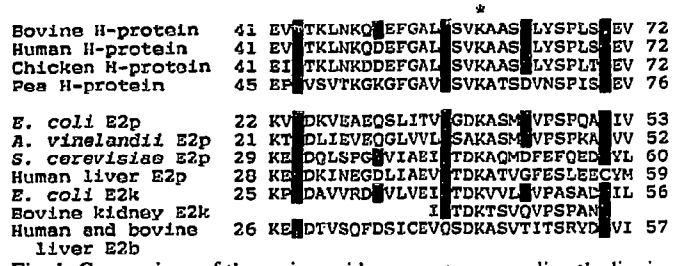

Fig. 1. Comparison of the amino acid sequence surrounding the lipoic acid attachment site. The sequences of bovine [7], human [8,9], chicken [6,10], and pea [11,12] H-proteins were compared to the sequences of acetyltransferases (E2p) of *Escherichia coli* [14], *Azotobacter vinerandii* [15], *Saccharomyces cerevisiae* [16], and human liver [17], to the sequences of succinyltransferases (E2k) of *E. coli* [18] and bovine kidney [13], and to the sequences of human [19,20] and bovine liver [19] acyltransferases of the branched-chain α-*keto* acid dehydrogenase complex (E2b). The sequences of the first lipoyl domain of acetyltransferases are presented. The asterisk indicates the residue involved in lipoic acid attachment. The shadowed amino acids show the conserved residues which were chosen for mutagenesis. The numbers on the right and left refer to the positions of the amino acids of these proteins.

from Wako Pure Chemicals (Osaka, Japan). The oligonucleotides used as primers for preparing the mutants of H-protein were synthesized on an Applied Biosystems 381A DNA synthesizer.

2.2. *Site-directed mutagenesis*

A 0.8 kb *Eco*RI-*Eco*RI fragment containing the coding sequence of the bovine H-protein cDNA clone BH8A [7] was subcloned into phagemid vector pTZ18U. Mutagenesis was performed according to the method developed by Kunkel et al. [23] using a Bio-Rad kit. Fig. 2 shows the oligonucleotides used in the preparation of each of the mutants. Mutants were identified by DNA sequence analysis using the dideoxy method as previously described [7]. The mutagenesis efficiency was greater than 80%.

2.3. *mRNA synthesis and analysis*

The phagemid vectors containing the *Eco*RI fragment of BH8A or the mutated coding regions of bovine H-protein were linearized with *Bam*HI and purified by phenol/chloroform extraction and ethanol precipitation. The linearized phagemid DNA (1 μg) was transcribed in vitro as described previously [7] except that incubation was carried out at 37°C with 20 units of T7 RNA polymerase. Then 5 μg of DNase was added, and the incubation was continued for 25 min. The in vitro synthesized RNA was purified by phenol/chloroform extraction and ethanol precipitation in the presence of 0.85 M ammonium acetate and dissolved in sterile water. Usually 5–7 μg of RNA transcripts were obtained from 1 μg of linearized template DNA. The transcripts, 30 ng each, were electrophoresed in a 1.4% agarose-formaldehyde gel [24] with RNA ladder markers (0.24–9.49 kb) (Bethesda Research Laboratories) as size markers and transferred onto Hybond-N+ membrane (Amersham Corp.). The membrane was hybridized with ^{32}P-labeled BH8A cDNA as described previously [7] and exposed for 2 h. Size markers were stained with ethidium bromide.

2.4. *In vitro translation and mitochondrial import*

In vitro translation was carried out in the reaction mixture of 25 μl containing 0.2 μg of RNA transcripts, 20 μl of rabbit reticulocyte lysate (Amersham Corp.), and 20 μCi of L-[4,5-^{3}H]leucine, and the mixture was incubated for 90 min at 30°C. One μl aliquot of translated products was removed and subjected to electrophoresis on a 14% polyacrylamide gel containing SDS [25]. The gel was fixed, treated with Amplify (Amersham Corp.), dried, and fluorographed. The remaining translation mixture was incubated with an equal volume of mitochondria (40 mg/ml) isolated as described previously [7] for 60 min at 27°C. The mixture was transferred to an ice bath, incubated with trypsin (final concentration, 40 μg/ml) for 10 min and then with soybean trypsin inhibitor (final concentration, 100 μg/ml) for 20 min. After centrifugation at 15 000 × *g* for 20 min, the mitochondrial pellet was suspended in 25 μl of 0.15 M NaCl, 0.01 M EDTA, 0.5% Triton X-100. Two μl of the suspension was subjected to SDS-polyacrylamide gel electrophoresis as described above, and the remaining products were saved for lipoylation analyses.

2.5. *Analysis of lipoylation*

The translation products prepared above were immunoprecipitated with antibody against chicken H-protein as described previously [7]. The precipitated ^{3}H-labeled H-protein (20 000–30 000 dpm/tube) was carboxymethylated and digested with lysylendopeptidase, and the resulting peptides were resolved by HPLC [7]. As reported previously, a peptide containing lipoic acid (designated K5, residues 49–77) and a peptide named *C* (presumably a modified form of K5) were obtained from the holoform, while 2 shorter peptides (designated *A* and *B*, residues 49–59 and 60–77, respectively) were cleaved from the apoform [7]. These peptides were collected and their radioactivities were measured. The percentage of the in vitro translated H-protein that had been lipoylated was calculated from the equation where A, B, C and K5 stand for the radioactivity of the respective peptide:

$$\text{Lipoylation (\%)} = \frac{C + K5}{A + B + C + K5} \times 100$$

The amino acid substitution employed in this study caused little change of the retention time of the mutated peptides on HPLC analysis.

3. RESULTS AND DISCUSSION

3.1. *Site-directed mutagenesis and in vitro transcription and translation of the mutants*

In order to determine which residues around the lipoate attachment site of H-protein are essential for lipoylation reaction, we changed codons for Gly^{43} and Gly^{70}

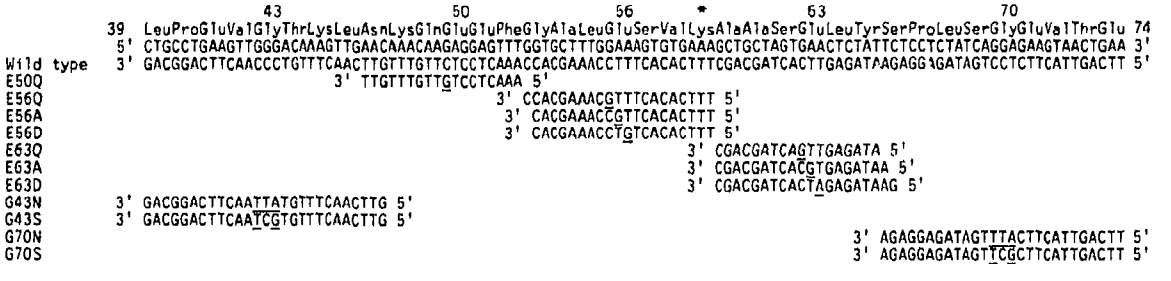

Fig. 2. Sequences of synthetic primers used for site-directed mutagenesis. Mutagenic oligonucleotides used in the construction of mutant H-proteins are compared with the native bovine sequence. Mismatched bases are underlined. The wild-type H-protein sequence is numbered from Ser^1 [7]. The asterisk indicates the residue involved in lipoic acid attachment. Oligonucleotides are named according to the amino acid replacement using the designation wild-type amino acid in single letter code, position, amino acid replacement.

to codons for asparagine or serine and codons for Glu[56] and Glu[63] to codons for glutamine, aspartic acid, or alanine by site-directed mutagenesis. These amino acid residues were chosen because they are highly conserved among H-proteins and acyltransferases (Fig. 1). In addition, Glu[50], which is less conserved than the above residues, was changed to glutamine for comparison. The linearized phagemids were transcribed in vitro using T7 RNA polymerase. On Northern blot analysis, transcription products from cDNA for BH8A and for these mutants migrated as a single band at 800-base long and hybridized with the ^{32}P-labeled *Eco*RI fragment of BH8A with the same intensity (Fig. 3A). The transcripts were translated in a cell-free translation system in the presence of L-[^{3}H]leucine, and the products were analyzed by SDS-polyacrylamide gel electrophoresis. All the products from mutated transcripts co-migrated with the precursor form of H-protein, but their amounts were variable (Fig. 3B). The low yield of the protein products from G70N and G70S mutants may be the result of the stimulated degradation of the translated products or the reduced translational efficiency due to unsuitable secondary structure of RNAs introduced by mutagenesis. The latter seems unlikely because the predicted secondary structure of the RNAs transcribed from G70S and G70N mutants by the method of Zuker and Stiegler [26] were very similar to that of wild-type (data not shown). Since Gly[70] is situated in a β-turn segment predicted by the method of Chou and Fasman [27] as reported [7], the substitution of the residue with asparagine or serine may introduce a large conformational change into H-protein and accelerate the degradation of the mutated protein. The mutation, however, did not affect the transport into mitochondria and proteolytic processing of precursors. As shown in Fig. 3B and C, the amount of each mature protein detected in mitochondria is proportional to the amount of each precursor protein synthesized. The data about E50Q, E56Q, and E63Q mutants were excluded from Fig. 3. The amounts and behaviors on electrophoresis of transcription and translation products of these mutant cDNAs were identical to those of the wild-type.

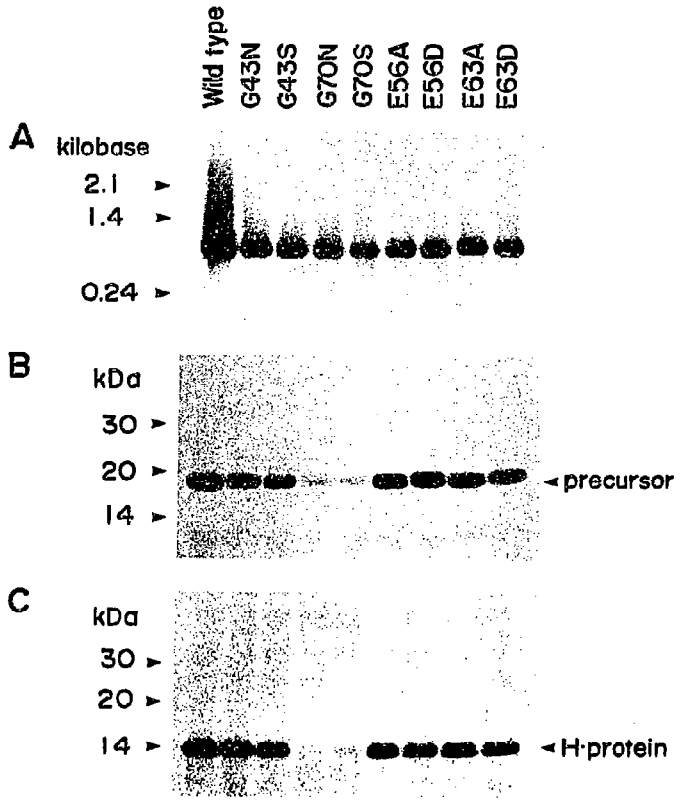

Fig. 3. Products of in vitro transcription and translation of the mutated cDNA. (A) Northern blot analysis of in vitro transcribed RNA. Synthesized RNAs (30 ng/lane) were electrophoresed, transferred to Hybond N+ membrane, and hybridized with a ^{32}P-labeled *Eco*RI fragment of BH8A. (B) 0.2 μg of synthesized RNAs were translated in 25 μl of the reaction mixture containing [^{3}H]leucine for 90 min at 30°C. One μl aliquots were removed and electrophoresed. (C) Translated products (24 μl) were mixed with the same volume of mitochondrial suspension and incubated for 60 min at 27°C. After centrifugation, mitochondria were suspended in a solution containing Triton X-100, and 2 μl aliquots were electrophoresed.

3.2. *Lipoylation of mutant H-proteins*

The in vitro translated products were incubated with mitochondria, and the proteins incorporated and processed were immunoprecipitated and analyzed for their lipoylation. Of 3 glutamic acid residues examined, only Glu[56] is responsible for the lipoylation (Fig. 4). Replacement of Glu[56] by glutamine and alanine, which lack a negative charge, resulted in a great decrease in lipoylation (12% and 17% of that of the wild-type H-protein). Substitution by the negatively charged aspartic acid residue inhibited the lipoylation by 60%. Replacement of Glu[63] with glutamine reduced the lipoylation to 26% of that of the wild-type H-protein. Replacement of Glu[63] by aspartic acid had no effect. The anionic charge of Glu[63] may not be essential because the E63A mutant showed only a minor decrease in lipoylation (76% of that of the wild-type H-protein). The fact that the residues corresponding to Glu[63] are replaced by aspartic acid or other amino acids in pea H-protein and several acyltransferases indicates that Glu[63] is indeed not responsible for lipoylation. The region where the lipoyllysine residue (Lys[59]) is situated is predicted to form an α-helical structure and Glu[56] and Lys[59] are expected to reside at the same side of the helix [7]. Possibly Glu[56] interacts with the enzyme catalyzing lipoylation and the anionic charge may play a role in the interaction.

Gly[43] seems to be not essential for lipoylation (Fig. 4), although it is a completely conserved residue in all reported H-protein and acyltransferase sequences (Fig. 1). On the other hand the lipoylation rate was reduced to 14% of that of the wild-type H-protein when Gly[70] was replaced by the asparagine residue. Conversion of the glycine residue to serine, a smaller residue than the asparagine redisue, restored the lipoylation partially (54% of the wild-type). Gly[70] is predicted to be situated

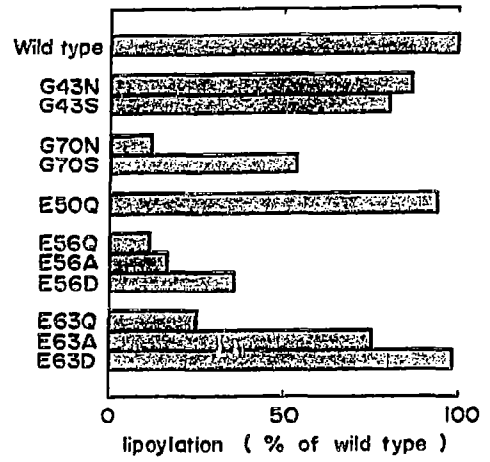

Fig. 4. The efficiency of lipoylation of mutants. The percent lipoylation of in vitro translated wild-type and mutant H-proteins labeled with [^{3}H]leucine was calculated as described under Materials and Methods. Data are expressed as the percent of values obtained with the wild-type H-protein. The percentage of the wild-type lipoylated ranged from 76–92% depending on the mitochondrial preparations used. The values given are means of 1 or 2 experiments done in duplicate except with G70N and G70S mutants, where the translated mixtures of the duplicate tube were combined and subjected to analysis because of the low radioactivity.

in a β-turn structure [7]. The presence of glycine at position 70 may be required to facilitate the lipoylation of the specific lysyl residue by providing a flexible loop. Interestingly, a glycine residue corresponding to Gly^{70} is also found in biotin enzymes [28].

These results demonstrated that 2 well conserved amino acid residues, Glu^{56} and Gly^{70}, are important, if not crucial, for the efficient lipoylation of H-protein and the preservation of a proper conformation around the lipoic acid site may be essential for the enzymatic lipoylation of the protein.

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