The mitochondrial glycine decarboxylase complex (GDC) consists of four component enzymes (P, H, T, and L proteins) involved in the breakdown of glycine. In order to investigate structural interactions involved in the stabilization of the methylamine-loaded H protein (a transient species in the GDC reaction), we designed several mutants of H apoprotein.

Structural analysis of the wild-type and mutants of H apoprotein emphasized the necessity to carefully assess, by biophysical techniques, the correct folding of mutated proteins prior to investigate their biochemical properties. The correctly folded wild-type and mutants of H apoprotein were in vitro lipoylated and then characterized in the context of GDC reaction by studying the reconstituted complex and partial reactions. We showed that Val82 and Ala64, surrounding the lipoyl-lysine, play an important role in the molecular events that govern the reaction between P and H protein but do not intervene in the recognition of the binding site of lipoic acid by lipoyl ligase. The biochemical results obtained with the HE14A mutant of H protein pointed out the major role of the Glu14 amino acid residue in the GDC catalysis and highlighted the importance of the ionic and hydrogen bonds in the hydrophobic cleft of H protein for the stabilization of the methylamine-loaded lipoyl arm.

Glycine decarboxylase complex (GDC) is a multienzyme system involved in the breakdown of glycine in almost all organisms (1, 2). GDC is found in large amount in the mitochondria of C3 plant leaves, where it plays a key role in the photosynthetic pathway (3). The enzymatic complex consists of four different component proteins: P, T, L, and H proteins. H protein is a 14.1-kDa monomer that plays a pivotal role in the reaction mechanism, since it interacts sequentially with each of the other three proteins of the complex through its cofactor lipoic acid bound to a lysine residue. The P protein (100 kDa) catalyzes the oxidative decarboxylation of glycine and the transfer of the resulting methylamine group to the oxidized lipoamide arm carried by H protein. The lipoamide-bound methylamine is then shuttled to the T protein (41 kDa), where the methylene carbon atom is bound to 5,6,7,8-tetrahydropteroylpolyglutamate (H4PteGlu$n$, where $n$ is the number of glutamate residues), yielding N$^n$N$^{10}$-methylene-H4PteGlu$n$, and the amino group is released as NH$_3$. Finally, the L protein (50 kDa), a dihydrolipoamide dehydrogenase, reoxidizes the reduced lipoamide of H protein by the sequential reduction of FAD and NAD$^+$, thereby completing the catalytic cycle of the H protein.

The three-dimensional structure of pea H protein revealed that the lysine bearing the lipoic acid was located at the tip of a hairpin configuration (4, 5). Upon reaction with P protein, the lipoamide-methylamine arm swivels toward a hydrophobic cleft on the surface of the protein (6). In this configuration, the fragile methylamine group should be stabilized and protected from nucleophilic attack by OH$^-$ ions until the reaction with H$_4$PteGlu$n$ and T protein takes place.

Lipoic acid is also the prosthetic group of the E2 protein component (dihydrolipoamide S-acyltransferase) of 2-oxoacid dehydrogenases, a family of multienzyme complexes involved in the decarboxylation of pyruvate, 2-oxoglutarate, and branched chain 2-oxoacids. The E2 protein component forms the structural core of the complexes, on the surface of which oxoacid decarboxylase (E1 protein) and dihydrolipoamide dehydrogenase (E3 protein) are bound in a noncovalent manner. In E2 proteins, the lipoyl domains show both striking sequence and structure similarity with H protein (7–9). Considerable work has been done on the lipoylation, structure, and activity of recombinant lipoyl domains engineered from the oxoacid dehydrogenases (10–12). In contrast, few studies have been undertaken at the structural level to highlight amino acids involved in catalytic site of the enzymes. H protein is a small lipoic acid protein that offers an interesting alternative to analyze the functioning of the lipoyl-lysine group in this type of multienzyme complex. A major feature of H protein catalysis lies in the stabilization of the methylamine-loaded form through the burying of the lipoyl arm within a hydrophobic cleft (Fig. 1). Close inspection of the structure of H protein indicates that the NH$_3$ moiety of the methylamine group is tightly linked, by an ionic bond, to the lateral chain carbonyl group of Glu14, and by hydrogen bonds to the carbonyl group of Ser12 and Asp7. The two residues Ser12 and Glu14 are also linked by hydrogen bonds between the hydroxyl group of serine and the carbonyl group of glutamate (Ref. 9, Fig. 1b). The only significant interaction of...
the carbon in the methylamine group occurs through hydrophobic contact with Ile27. Since a system allowing the production of recombinant H protein (apoform or lipoylated form) was available (13), we sought to further investigate the interactions between amino acids Ser12, Glu14, and Ile27 of the hydrophobic cavity and methylamine bound to the lipoamide using site-directed mutagenesis. We constructed four mutants of H protein: HE14D (Glu14 was replaced by Asp), HE14A (Glu14 was replaced by Ala), HI27A (Ile27 was replaced by Ala), and HS12K (Ser12 was replaced by Lys) (Fig. 1c). We were also interested in the structural features of the hairpin loop carrying the lipoate cofactor that could intervene in catalysis but also in the lipoylation of the apoprotein. There is indeed a noticeable similarity between lipoate-dependent proteins and biotin-dependent proteins. The biotin-moiety is attached to a specific lysine residue in the highly conserved motif MKM, which is structurally similar to the lipoyl domains or H protein (7,15). In Escherichia coli, the lipoate protein ligase (LPL) is responsible for the ATP-dependent attachment of free lipoic acid to a specific lysine residue in the lipoyl domain of E2 protein (16). We have previously reported the three-dimensional structure of the recombinant H apoprotein at a 0.25-nm resolution and demonstrated that the apoprotein, which exhibits the same conformation as the lipoylated protein, was an excellent substrate for LPL (13). Therefore, we decided to make a fifth double mutant, HV62M/A64M (Val62 and Ala64 both replaced by Met residues), where the lipoylation motif VKA is replaced by the biotinylation motif (Fig. 1). Such a mutant was expected to shed light both on the reaction mechanism of H protein and on the post-translational modification of the apoprotein by LPL.

We report here the detailed structural analysis of these five H protein mutants and the effect of these mutations regarding the lipoylation process and the reaction mechanism of the GDC.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis**

The plasmid pET-H encoding the mature pea H protein (13) was used as a template to generate mutations in the DNA sequence with the unique site elimination (USE) mutagenesis kit (Amersham Pharmacia Biotech) that utilizes a two-primer system to generate site-specific mutations. The second primer (target mutagenic primer) is directed to the sequence of the mature pea H protein cDNA and carries the desired mutation. The sequences of the mutagenic oligodeoxynucleotides E14A, E14D, I27A, S12K, V62M/A64M are listed in Table I. The mutations are designated with the position of the mutated residue in the mature H protein sequence preceded by the original amino acid. The mutagenized plasmids, designated pET-HE14A, pET-HE14D, pET-HI27A, pET-HS12K, and pET-HV62M/A64M, carried DNA inserts encoding mature H protein with the mutations. All constructions were sequenced on both strands (Génome Express, Grenoble) to verify that the desired mutation was introduced within the H protein sequence.

**Expression and Purification of the Recombinant H Apoproteins**

The transformation of the different pET plasmids in BL21 (DE3) E. coli, the culture conditions for overexpression of the recombinant apoproteins, the cell lysis, and the protein ammonium sulfate fractionation were performed as described earlier (13). The H apoprotein solutions (15 ml, 200 mg) were treated with RNase and DNase (20 μg/ml each) for 20 min at 30 °C and were loaded on a Q-Hyperdex column (Amersham Pharmacia Biotech) equilibrated with buffer A (40 mM Tris, pH 7.4, 0.1 mM EDTA) and connected to an Amersham Pharmacia Biotech FPLC system. Proteins were eluted with a continuously increasing KCl gradient (0–1 M) (flow rate, 1 ml/min; fraction size of 2 ml). The fractions containing H apoproteins (analyzed by SDS-PAGE) were pooled, dialyzed extensively against buffer A, and concentrated on 3K-macrosep (Filtron) by centrifugation (7,000 × g) to a concentration of about 10 mg/ml.

**Expression and Purification of the E. coli LPL**

E. coli strain TM 202 harboring the recombinant plasmid pET-TM 70 containing the lipA gene encoding LPL was kindly supplied by Prof. J. E. Cronan (Illinois University). E. coli LPL was overexpressed as described by Green et al. (16). Bacteria resuspended in buffer B (20 mM Tris, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol) were disrupted by three
cycles of freezing and thawing, and the resulting suspension was centrifuged (35,000 × g, 10 min). Ammonium sulfate was added to the supernatant to 35% saturation, and the solution was stirred on ice for 1 h. After centrifugation (24,000 × g, 10 min), the pellet was suspended in buffer B (5–10 ml) and dialyzed overnight against buffer B. After centrifugation and filtration of the supernatant through a 0.22-μm filter, the protein solution was then applied to a MonoQ-HR10/10 column (Amersham Pharmacia Biotech) preequilibrated with buffer B. Proteins were eluted with a continuously increasing NaCl gradient (0–500 mM) (flow rate, 1 ml/min; fraction size of 1 ml). The fractions containing LPL, as deduced from SDS-PAGE analysis, were pooled, dialyzed extensively against buffer B, and concentrated (10 mg/ml).

**Lipoylation of Purified H Apoproteins**

For the purpose of lipoylating proteins on a preparative scale, the experiment was performed at 37 °C for 1 h in a reaction medium containing 10 mM Tris, 10 mM Mops, pH 7.5, 5 mM MgSO₄, 5 mM ATP, 200 μM ni-t-lipoic acid, 0.25 mM E. coli LPL, and 43 μM H apoprotein, usual in a final volume of 850 μl. Different forms of H protein were resolved by matrix-assisted laser desorption ionization mass spectrometry. The proportion of lipoylated protein always exceeded 98%.

**Functional Characterization of the Different H Proteins**

**Dihydrolipoamide Dehydrogenase (TCEP) Assay**—The quantitative determination of lipoylated H protein by dihydrolipoamide dehydrogenase (H₂LipDH) was measured by a new enzymatic assay using TCEP (Roche Diagnostics) as a reductant for bound lipoic acid. This new enzymatic assay is described in the legend of reaction 1. The reaction medium contained the following in a total volume of 500 μl: 20 μM porcine heart H₂LipDH (Sigma), 8 mM TCEP, and various amounts of lipoylated H protein in a fully oxidized state, 0.2 μM pea P protein dimer, and 20 mM lipoylated H proteins in a final volume of 500 μl. The reaction was started with 2 mM NAD⁺. The rate of NADH formation was followed spectrophotometrically at 340 nm by measuring the absorbance change of NADH at this wavelength. The initial GDC rates were expressed as nmol of NADH formed/min.

**Table 1**

| Primer | Sequence |
|--------|----------|
| E14A   | 5'GGACCTTCAGCTGATGGCTAGACCTAGT |
| E14D   | 5'GGACCTTCAGCTGATGGCTAGACCTAGT |
| I27A   | 5'GAGCTTGGCACTGACCATGCCC |
| S12K   | 5'CAATGACCAAAATATGGGTC |
| V62MA/A64M | 5'GGAGCTTGGAATATGCAACTGACATGCAACTGATGTAAC |

**P Protein Activity**—The methylation of H protein by P protein was monitored by following the disappearance of free lipoylated H protein in the incubation medium (see Fig. 2). The reaction medium contained 10 mM Tris, 10 mM Mops, pH 7.4, 1 mM EDTA, 1 mM MgCl₂, 20 μM pyridoxal phosphate, 1.25 mM NAD⁺, 0.3 μM porcine heart H₂LipDH (additions of NAD⁺ and H₂LipDH are required to provide H protein in a fully oxidized state), 0.2 μM pea P protein dimer, and 20 μM lipoylated H proteins in a final volume of 500 μl. The reaction was performed at 30 °C was initiated by 20 mM glycine. Aliquots of 50 μl were taken from the reaction medium at different times (0, 5, 10, 15, 20 min) and added to 50 μl of a solution containing 10 mM carboxymethylamine (an inhibitor of P protein (17)) to stop the reaction. This reaction was then analyzed using the H₂LipDH/TCEP assay described above to quantify the residual free lipoylated H protein and thus deduce the amount of methylation-loaded H protein. The methylation-loaded H protein was converted, when needed, into dihydrolipoyl H protein by the addition of 1 μM T protein and 200 μM H₂PteGlu (see Fig. 2).

In another set of experiments, we investigated the stability of the methylation-loaded lipoamide of H protein (wild type and mutants) as a function of time. First, the methylation of H protein was carried out as described above for 30 min in a total volume of 500 μl. The reaction was then stopped by adding 500 μl of a solution containing 10 mM carboxymethylamine. The protein solution was then incubated at 30 °C, and aliquots of 100 μl were analyzed at different times with the H₂LipDH/TCEP assay in order to monitor the appearance of the unloaded form of H protein.

**Structural Analysis of H Proteins**

**Mass Spectrometry**—Mass spectrometry analysis was performed as described by Bourguignon et al. (18).

**Small Angle X-ray Scattering**—H proteins were analyzed by small angle x-ray scattering. Data were processed by linear regression. According to Guinier (21), two experimental parameters can be determined: the forward scattered intensity, which is proportional to an apparent molecular mass, and an apparent radius of gyration of electron density for the particles. The H proteins were diluted in 20 mM K₂HPO₄, pH 7.4, 1 mM EDTA at a concentration of 10 mg/ml for HE14A protein and 5 mg/ml for the other proteins.

**Nuclear Magnetic Resonance**—NMR experiments were performed with samples containing 0.5–0.8 mM H proteins in 20 mM phosphate buffer, pH 7.2, 90% H₂O, and 10% D₂O. Spectra were acquired at 27 °C using a Bruker AM-X spectrometer operating at 400 MHz. One-dimensional experiments were recorded at 27 °C. NMR spectra were the result of adding 512 scans. Chemical shifts were referenced relative to water resonance fixed at 4.75 ppm at 27 °C. Data were processed with the Felix program (MSI, Inc.).

**Circular Dichroism Spectroscopy**—Spectral acquisition was performed using a Bruker AM-X spectrometer operating at 400 MHz. One-dimensional experiments were recorded at 27 °C. NMR spectra were the result of adding 512 scans. Chemical shifts were referenced relative to water resonance fixed at 4.75 ppm at 27 °C. Data were processed with the Felix program (MSI, Inc.).
Characterization of H Protein Mutants of GDC

RESULTS

Overexpression and Purification of H Aipoprotein Mutants—The pET-H plasmid carrying the cDNA encoding the mature form of pea mitochondrial H protein was mutagenized to produce the site-directed mutations within domains interacting with the lipoamide-methylamine arm or around the lipoic acid residue. The wild-type pea H protein was expressed to high yields with the lipoamide-methylamine arm or around the lipoyl-acceptor site. The wild-type H protein with its reoxidation catalyzed by dihydrolipoamide dehydrogenase (H2LipDH) in the presence of NAD⁺ was purified in E. coli, as reported before (13), and all mutant proteins, designated HE14A, HE14D, HI27A, HS12K, and HV62M/A64M were expressed in E. coli in the presence of SDS (a) and non-denaturing conditions (b). 3 μg (a) or 0.5 μg (b) of proteins were analyzed as described under “Materials and Methods” and revealed by Coomassie Blue staining. The molecular masses of the protein bands are indicated on the right (in kilodaltons).

Electrophoretic Analyses of Proteins—Protein samples were analyzed by SDS-PAGE (15% acrylamide, 0.2% bisacrylamide) as described by Sambrook et al. (19). H apoproteins were also subjected to PAGE analysis in non-denaturing conditions (12% acrylamide, 0.2% bisacrylamide). The concentrations of proteins were estimated by the method of Lowry (20) with bovine serum albumin as standard.

Structural Analysis of H Aipoprotein Mutants—In order to characterize the biochemical properties of HE14A and HV62M/A64M mutants in comparison with the wild-type H protein, the apoforms of these proteins were lipoylated. This post-translation modification of pea H protein that occurred normally in the mitochondria was done in vitro using recombinant E. coli LPL, which was shown earlier to carry out the ATP-dependent attachment of the lipoate to the amine group of the lateral chain of K63 of the pea H protein (13, 16). We developed a new enzymatic assay to follow spectrophotometrically the lipoylation of apoproteins by LPL in a coupled assay that detected the presence of lipoylated H protein (see “Materials and Methods”). This assay combines the TCEP chemical reduction of lipoamide into dihydrolipoamide bound to H protein with its reoxidation catalyzed by dihydrolipoamide dehydrogenase (H₂LipDH) in the presence of NAD⁺ (see Fig. 2). In this experiment, the amount of lipoylated H protein (or mutants) was estimated with the H₂LipDH/TCEP assay according to specific calibration curves made with known amounts of fully lipoylated protein (H, HE14A, and HV62M/A64M). Fig. 7 showed that the H apoprotein lipoylation proceeded at a similar rate as that of HV62M/A64M and HE14A.
apoproteins. The lipoylation states of the different proteins (at 30 min) were also confirmed by mass spectrometry analyses (matrix-assisted laser desorption ionization). We could estimate the proportions of lipoylated protein to between 95 and 100% (results not shown). These results demonstrated that neither mutations of Val62 and Ala64 residues adjacent to the catalytic lysine nor that of Glu14 in the hydrophobic cavity of the H protein hampered the lipoylation of apoproteins by the *E. coli* LPL. When the different H apoproteins were lipoylated for biochemical analyses, reaction time and LPL concentration were increased in order to obtain 100% of lipoylated H proteins (see "Materials and Methods").

**Effect of the H Protein Mutations on GDC Activity**—To further analyze the impact of these mutations on the domain interacting with the methylamine-loaded lipoamide, the glycine cleavage system was reconstituted using various concentrations of H protein mutants. This assay was carried out with saturating stoichiometric amounts of P and T proteins from pea, H2LipDH from pig, and the required cofactors: pyridoxal phosphate, H4PteGlu, and NAD+. The glycine oxidation reaction was initiated by the addition of glycine, and the resulting production of NADH was followed spectrophotometrically. The initial rates of GDC activity (measured between *t* = 0 and *t* = 20 s) were expressed as a function of H protein concentration, as shown in Fig. 8. In the reconstituted system, H protein behaves as a substrate as can be judged from the aspect of the Henri-Michaelis-Menten saturation curve pattern. Fig. 8 shows clearly that the two mutations of the H protein affect the functioning of the reconstituted GDC. The reconstituted system displayed an apparent *Km* value for the wild-type H protein close to that determined with the HE14A mutant (apparent *Km* values of 6 and 3.4 μM, respectively). Likewise, the reconstituted system had a *Vmax* value with wild-type H protein higher than with the HE14A mutant (*Vmax* values of 6.2 and 2.8 μmol of NADH produced/min/mg of P protein, respectively). On the other hand, the HV62M/A64M mutant affected dramatically the activity of the reconstituted system.

To gain insight into the effect of mutations toward the GDC
mechanism, we analyzed partial reactions of H protein with its partners L, P, and T proteins. We started with the reaction catalyzed by H2LipDH, which was measured using the H2LipDH/TCEP assay (see "Materials and Methods" and Fig. 2) with H, HE14A, and HV62M/A64M proteins. The curves, displaying the NADH production measured as a function of the H protein or mutant amounts (0–40 μM), revealed some variations in the behaviors of the different H proteins with H2LipDH (4 pM). All of the kinetic parameters are summarized in Table II. The affinity of the H2LipDH for the HV62M/A64M mutant was higher than for the wild type H protein (Km values of 14 and 25.5 μM, respectively). The Vmax value of H2LipDH with the HV62M/A64M mutant is quite similar to that with the wild type H protein (Vmax values of 180 and 163 μmol of NADH).
produced/min/mg of H2LipDH, respectively), indicating that the HV62M/A64M mutant is as active as the wild-type H protein in the assay. On the other hand, the affinity and the Vₘ value of H2LipDH for the HE14A mutant were reduced (Kₘ value of 44 μM and a Vₘ of 100 nmol of NADH produced/min/mg of H2LipDH). Table II also shows that the Kₘ cat values are quite similar in the cases of H and HV62M/A64M (271.5 and 300 s⁻¹ respectively) and lower in the case of HE14A (166.5 s⁻¹). However, the catalytic efficiency of HV62M/A64M was slightly higher (2.15 × 10⁷ M⁻¹ s⁻¹) than that of HE14A (3.8 × 10⁶ M⁻¹ s⁻¹) and wild type H protein (1 × 10⁷ M⁻¹ s⁻¹). As a whole, these results pointed out a mild effect of the H protein mutations toward H2LipDH activity, which is, however, unlikely to explain the differences observed between the wild type H protein and mutants in the reconstituted system.

To monitor the reaction with P protein, oxidized wild-type or mutant H proteins (1 nmol of each) were allowed to react with P protein in the presence of glycine, which results in its decarboxylation and the reductive transfer of the remaining methylamine group to the lipoamide arm covalently bound to the H protein. At various times, the reaction was stopped with carboxymethoxylamine (an analog of glycine and a potent inhibitor of P protein), and the disappearance of free lipoylated H protein was monitored with H2LipDH using the H2LipDH/TCEP assay (see “Materials and Methods”). Indeed, the H protein loaded with methylamine does not react either with TCEP or with the H2LipDH. Its concentration was calculated as the difference between the initial concentration of H protein in the medium (before the P protein addition) and the residual unloaded H protein. The kinetics of appearance of H protein and mutants to proceed enough (25 min) to reach a plateau, and then we added 1 μM of T protein (concentration utilized for the measurement of GDC activity; see “Materials and Methods”) with H4PteGlu to trigger the unloading of the methylamine group and the concomitant release of unloaded lipoylated H protein (Fig. 9a). This latter reaction proceeded so fast that we could not detect any significant differences between the H protein and the mutants.

In order to further characterize the impact of site-directed mutagenesis on the stability of the methylamine-loaded H protein, an experiment equivalent to that described for Fig. 9a was performed. Indeed, the methylamine group bound to lipoamide interacts via ionic bond to Glu¹⁴ in the wild-type protein (see Fig. 1), and the replacement of this amino acid residue by Ala (HE14A mutant) should deeply affect the anchoring of the methylamine-bound methionine in the hydrophobic cleft of the H protein. As a consequence, the increased mobility of the methylamine-loaded lipoamide arm should facilitate the nucleophilic attack of the carbon atom of the methylamine group by OH⁻ ions. When the final concentration of methylamine-loaded H protein reached a plateau, the P protein was inhibited by the addition of a large excess of carboxymethoxylamine to prevent further loading of H protein with methylamine. The stability of the methylamine-loaded H proteins in the medium was then followed at 30 °C as a function of the time (Fig. 9b) by measuring the release of free lipoylated H protein using the H2LipDH/TCEP assay. This figure indicated that the wild-type H protein and the HV62M/A64M mutant loaded with methylamine remained stable with time. In marked contrast, methylamine-loaded HE14A mutant was rather unstable, since free lipoylated H protein was detected soon after the carboxymethoxylamine addition. The presence of free lipoylated H protein increased then steadily until all of the lipoamide-bound methionine was converted into free lipoamide. In presence of T protein and without H4PteGlu, we have also noticed that the methylamine-loaded form of HE14A protein was rapidly converted in the lipoylated form (4 min), whereas the methylamine-loaded wild-type H protein remained rather stable. Altogether, these results pinpoint the key role of Glu¹⁴ in the stabilization of the lipoamide-bound methionine.

**DISCUSSION**

All of the H protein mutants that have been constructed (HE14D, HI27A, HS12K, HV62M/A64M, and HE14A) could
successfully be expressed at a very high level and in a soluble form in E. coli using the pET system. Unfortunately, a series of biophysical analyses of all six apoproteins thus obtained was coherent with a lack of defined structure for HE14D, HI27A, and HS12K proteins, possibly corresponding to a random coil conformation. However, the HV62M/A64M and HE14A mutants were shown to have structures close to that of the native protein. Careful examination of the NMR spectra of HE14A and H proteins revealed significant spectral variations, suggesting that some subtle structural modifications might have been introduced by the mutation. This will have to be further investigated by two-dimensional NMR. The strong disorganization of the overall folding of H protein introduced by the single mutation E14D, S12K, or I27A was very hard to predict. The residues Glu14 and Ile27 are conserved in H protein of plant, animal, and bacterial sources (6) and thus may have a major structural and/or functional role. Nevertheless, we expected that the replacement of Glu by Ala would affect much more the overall folding of H protein than its replacement by Asp, which has a shorter lateral chain to weaken the ionic interaction with Glu (HS12K mutant). Ile27 was replaced by Ala, whose small size should prevent hydrophobic contact with the methylene group (HI27A mutant). Unfortunately, we could not detect the incidence of the E14D, S12K, and I27A mutations on the GDC activity, because these mutants were not correctly folded as discussed above. Nevertheless, the lipoated HE14A mutant could be characterized in the context of GDC reaction, using a reconstituted complex and partial reactions. The mutation in HE14A protein resulted in a marked decrease in GDC activity measured with HE14A mutant was 2-fold lower than the wild type, could never be reached. This may be attributed to the fact that the methylamine-loaded HE14A mutant was unstable, leading to an equilibrium between the loaded and unloaded forms. It is worthy of note that the unloading can proceed even in the absence of T protein and H2PteGLu, indicating a chemical process. Moreover, the methylamine-loaded HE14A protein was totally destabilized in presence of T protein alone (result not shown), confirming the weakness of the interactions between the methylamine group.
and the hydrophobic cavity of the HE14A protein. Thus, we demonstrated for the first time by a biochemical analysis the pertinence of the interactions of Glu14 with the NH2 group of methyamine. It is possible that the loading-unloading equilibrium was still favorable, because other residues (Ser12 and Asp67) also stabilize the methyamine group by hydrogen bonds and could compensate for the lack of ionic interaction between Glu14 and the methyamine group. Furthermore, we cannot exclude the possibility that the mutation induces slight structural modifications, as suggested by NMR spectra, that affects the recognition of H protein by P protein and L protein.

Also, the structural analogies between H protein and the biotinyl component of biotin carboxy carrier protein led us to design a mutant in which the lipoate motif VKM was changed to the consensus of biotinylolation MKM (HV62M/A64M mutant). The purpose was to determine whether biotin or lipoate could be attached to the mutated apoprotein either in vivo or in vitro. Despite bearing the biotinylolation consensus sequence, the HV62M/A64M mutant was correctly lipoylated and did not show any sign of biotinolation even when biotin was added to the culture medium of bacteria. The so-called biotinylation motif MKM did not appear to act as a signal for the recognition of the hairpin loop, either by LPL or by the biotin ligase. A similar result was obtained on the lipoyl domain of the E2 component of the Bacillus stearothermophilus pyruvate dehydrogenase complex (10). However, since the post-translational modification studies of the biotinyl domain of the biotin carboxy carrier protein revealed some contradictory data, further investigation will be necessary to understand the role of the methinione residues surrounding the biotinyl-lysine in the biotinylation process (23–25). A major question that remains is how the respective lipoyl and biotinyl ligases select their partners and what are the structural determinants required for the recognition. It has been clearly established that the position of the lysine at the tip of the hairpin configuration is essential both for lipoylation and biotinylation of the respective domains (10, 25). The superposition of H protein with the biotin domain of biotin carboxy carrier protein revealed a mirror inversion between the hairpin loops in the two structures, and such a structural feature might be of crucial importance for the interaction with the ligases or even catalysis (9). Successfully lipoylated HV62M/A64M mutant was then characterized both in the GDC reconstituted complex and in partial reactions. The HV62M/A64M mutant has the same overall structure than H protein but proved to be functionally distinct, since it was inactive in the reconstituted GDC, thus demonstrating the importance of Val62 and Ala64 that surround the lipoyl-lysine in the GDC reaction. Further analysis of partial reactions revealed that these residues were key determinants in the reaction between P and H protein, since the HV62M/A64M mutant was difficult to load with methylamine. The rate of methylamidation of the HV62M/A64M protein was 27-fold lower compared with the wild-type H protein, and this could explain the fact that HV62M/A64M mutant is inactive in the reconstituted GDC. In lipoyl domains of E2 components of 2-oxo acid dehydrogenases, Wallis and Perham (10) have shown that if the position of the target lysine was determinative for lipoylation, the surrounding residues were not involved in lipoylation but were essential for the reductive acylation of the lipoyl group by the E1 component. Hence, the Val62 and Ala64 residues of H protein may be directly involved in the catalytic site, or the bulky methionine residues could hamper essential movements of the lipoyl arm.

In conclusion, the biochemical work presented in this paper points out for the first time the major role of the Glu14 amino acid residue in the GDC catalysis. Indeed, the results obtained with the HE14A mutant highlight the importance of ionic and hydrogen bonds in the hydrophobic cleft of H protein for the stabilization of the methylamine-loaded arm. Finally, the amino acids surrounding lysine 63 of the H protein were shown not to interfere with the lipoylation process by LPL, whereas they are essential for the interaction with P protein. This strengthens an emerging vision of lipoyl or biotin ligases that recognize their respective targets according to their overall structure. These biochemical results represent dynamic evidence that supports the pivotal movement of the methylamine-loaded lipoyl arm, a feature that was originally deduced from the more static crystallographic analysis of H protein forms (5, 6).

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