Combined Structural and Biochemical Analysis of the H−T Complex in the Glycine Decarboxylase Cycle: Evidence for a Destabilization Mechanism of the H-Protein†

Laure Guilhaudis,‡ Jean-Pierre Simorre,‡ Martin Blackledge,‡ Dominique Marion,‡ Pierre Gans,*,‡ Michel Neuburger,§ and Roland Douce§

Laboratoire de Résonance Magnétique Nucléaire, Institut de Biologie Structurale CEA-CNRS "Jean-Pierre Ebel", 41 rue Jules Horowitz, 38027 Grenoble CEDEX 1, and Laboratoire de Physiologie Cellulaire Végétale, Département de Biologie Moléculaire et Structurale, CEA-Grenoble, 23 avenue des Martyrs, 38027 Grenoble CEDEX 1

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ABSTRACT: The lipoate containing H-protein plays a pivotal role in the catalytic cycle of the glycine decarboxylase complex (GDC), undergoing reducing methylamination, methylene transfer, and oxidation. The transfer of the CH_2 group is catalyzed by the T-protein, which forms a 1:1 complex with the methylamine-loaded H-protein (Hmet). The methylamine group is then deaminated and transferred to the tetrahydrofolate-polyglutamate (H_4FGlu_n) cofactor of T-protein, forming methylenetetrahydrofolate-polyglutamate. The methylamine group is buried inside the protein structure and highly stable. Experimental data show that the H_4FGlu_n alone does not induce transfer of the methylene group, and molecular modeling also indicates that the reaction cannot take place without significant structural perturbations of the H-protein. We have, therefore, investigated the effect of the presence of the T-protein on the stability of Hmet. Addition of T-protein without H_4FGlu_n greatly increases the rate of the unloading reaction of Hmet, reducing the activation energy by about 20 kcal mol^{-1}. Differences of the ^1H and ^{15}N chemical shifts of the H-protein in its isolated form and in the complex with the T-protein show that the interaction surface for the H-protein is localized on one side of the cleft where the lipoate arm is positioned. This suggests that the role of the T-protein is not only to locate the tetrahydrofolate cofactor in a position favorable for a nucleophilic attack on the methylene carbon but also to destabilize the H-protein in order to facilitate the unlocking of the arm and initiate the reaction.

In plant and mammalian mitochondria (*1, 2*), the glycine decarboxylase complex catalyses the oxidative decarboxylation of glycine in a multistep reaction. Like its mammalian counterpart, the complex of pea mitochondria consists of four different component enzymes designated as the P-protein (a homodimer containing pyridoxal phosphate, 200 kDa), the H-protein (a monomeric lipoamide-containing protein, 14 kDa), T-protein (a monomeric protein requiring tetrahydrofolate cofactor, 41 kDa), and L-protein, a dihydrolipoamide dehydrogenase, (a homodimer containing FAD and a redox active cystine residue, 100 kDa). In the catalytic cycle of the glycine decarboxylase complex (GDC),[1] the lipoate-dependent H-protein plays a pivotal role, acting as a mobile substrate that commutes successively between the other three proteins (*3, 4*). The P protein catalyses the decarboxylation of the glycine molecule, and the resulting methylamine moiety is passed onto the distal sulfur of lipoamide cofactor of the H-protein (*4*). The T-protein then interacts with the methylamine-loaded H-protein (Hmet) resulting in the deamination and transfer of the methylene carbon to its tetrahydrofolate-polyglutamate cofactor (H_4FGlu_n), forming methylenetetrahydrofolate-polyglutamate ($CH_2-H_4FGlu_n$) and reduction of the lipoamide arm. Finally, the L-protein catalyses the reoxidation of the lipoamide cofactor. All the components of the glycine decarboxylase system dissociate very easily and behave as independent proteins following mitochondrial inner membrane rupture. The overall activity of the complex as well as each catalytic step can thus be recovered in vitro by association of the purified proteins, permitting the study of the different interaction mechanisms between the various partners. It has been shown that the free T-protein exhibits a strong propensity to form high-molecular mass aggregates and was found to be unstable in solution. However, these aggregates were prevented in the presence of H-protein. Under these conditions, one molecule of T-protein associated with one molecule of H-protein to form a complex characterized by small angle X-ray scattering (*5*).

In the methylamine-loaded H-protein (Hmet), the lipoyl arm binds into a cleft at the surface of the H-protein (*6, 7*)

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‡ CEA-CNRS.

§ CEA-Grenoble.

* To whom correspondence should be addressed: Telephone: (33) 4 76 88 57 98. Fax: (33) 4 76 88 54 94. E-mail: pierre.gans@ibs.fr.

[1] Abbreviations: $CH_2H_4FGlu_n$, N_5N_{10}-methylenetetrahydrofolate-polyglutamate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothreitol; GDC, glycine decarboxylase complex; apoH, unlipoylated form of the H-protein; Hmet, H-protein loaded with methylamine; H-$^{[3H]}$-met, H-protein loaded with [^{3}H]methylamine; Hox, oxidized lipoylated form of the H-protein; Hred, reduced lipoylated form of the H-protein; H_4FGlu_n, tetrahydrofolate-polyglutamate; TCEP, tris(2-carboxyethyl)-phosphine; HSQC, heteronuclear single quantum coherence spectroscopy.

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and is locked in a stable conformation as indicated by the very high activation energy of the nonenzymatic hydrolysis of the methylamine moiety (*8*). As the methylene transfer is thought to be the result of a nucleophilic attack of the basic N_5 or the N_{10} atom of the pterin ring of H_4FGlu_n on the carbon atom of the methylamine group (*9, 10*), such a location raises questions about the role of T-protein in this transfer. Indeed, it has been suggested that the main role of T-protein is to provide a close contact between the H_4FGlu_n cofactor and the carbon atom of the methylamine group (*7*).

In this paper, we address the role of the individual components of the reaction: the H_4FGlu_n cofactor and the T-protein on the Hmet-protein using biochemical and NMR experiments. First, we have investigated experimentally the direct effect of H_4FGlu_n on the unloading process and then calculated the accessibility of the methylene group to direct attack by this cofactor using restrained molecular dynamic simulations. Second, biochemical experiments have been performed to determine the effect of the T-protein alone on the Hmet-protein. Finally, NMR studies of the H−T complex in solution have allowed us to determine the interaction site of the T with different forms of the H-protein. The results are interpreted with respect to the possible mechanism of the methylene transfer in the catalytic cycle, and a model for the role of the T-protein is presented.

MATERIALS AND METHODS

Preparation of T-protein. Pea (*Pivum sativum* L. Var. Douce Provence) plants were grown from seeds in vermiculite for 15 days under a 12 h photoperiod at 26 °C (day) or 20 °C (night). Mitochondria were extracted and purified as described previously (*11*). T-protein was purified according to Bourguignon et al. (*12*), except for the use of ammonium acetate buffer instead of potassium phosphate buffer (T-protein did not aggregate when concentrated in the presence of ammonium acetate). T-protein was stored frozen in 50 mM ammonium acetate, 2 mM DTT, pH 7.5. We have verified that purified T-protein was entirely devoid of H_4FGlu_n by using our purification procedure. Indeed, reassociation of the different components of the glycine decarboxylase did not lead to glycine oxidation unless we added small amounts of H_4FGlu_n to the incubation medium. In addition, we have observed that this cofactor is loosely bound to the T-protein (*13*) and becomes fully detached during the various purification steps.

Expression and Purification of Recombinant ApoH-protein (apoH) and [15N] Labeled ApoH-protein ([15N]apoH). The apoH was obtained by overexpression in *Escherichia coli* in the absence of lipoic acid in the culture medium and purified as described previously by Macherel et al (*14*). Uniformly [^{15}N]apoH was obtained by growing the *E. coli* cells on a minimal medium (M9) containing 1 g/L of [^{15}N]-ammonium chloride (Isotec) as the sole nitrogen source.

Lipoylation of the ApoH and [15N]ApoH. Lipoylation of apoH or [^{15}N]apoH was carried out in vitro using *E. coli* lipoate−protein ligase. The *E. coli* clone containing the gene encoding the mature lipoate−protein ligase was a gift from Professor J. E. Cronan; the ligase has been overexpressed in *E. coli* and purified according to Green et al. (*15*). Hox and [^{15}N]Hox were obtained by incubation of 1 mM apoH or [^{15}N]apoH in 1 mL incubation medium, containing 10 mM potassium MOPS/10 mM TRIS·HCl (pH 7), 5 mM $MgSO_4$, 5 mM ATP, 10 mM lipoate, and 10 μM of lipoate protein ligase. The sample was incubated for 30 min at 37 °C. The full lipoylation was checked by mass spectrometry. Hox and [^{15}N]Hox were purified according to Neuburger et al (*16*).

Methylamination of Hox and [15N]Hox by P-protein. The methylamination of [^{15}N]Hox by P-protein using [^{13}C,^{15}N]-glycine was carried out in a medium containing 10 mM potassium phosphate/10 mM potassium MOPS (pH 7.5), 20 μM pyridoxal phosphate, 2 μM pea leaf P-protein prepared according to Bourguignon et al. (*12*), 1 mM [^{15}N]Hox and 40 mM [^{13}C,^{15}N]-glycine (Sigma) in a final volume of 0.5 mL. The sample was incubated for 15 min at 30 °C. The methylamination of Hox by P-protein using [2-^{3}H]glycine was carried out under the same conditions. [^{15}N] H-$^{[13C,15N]}$-met and H-$^{[3H]}$met were purified according to Neuburger et al (*16*).

Demethylamination of Hmet-protein. The release of the [^{3}H]methylamine moiety from H-$^{[3H]}$met was followed by measuring the rate of Hred formation and/or formaldehyde released. H-$^{[3H]}$met was incubated at various temperature in 50 mM potassium phosphate buffer, pH 7, containing or free of T-protein. Aliquots of the protein suspension were withdrawn for measurement of Hred and radioactive formaldehyde (^3H_2CO). The effect of the pH on the release of the [^{3}H]methylamine moiety was followed according to this procedure in absence or in the presence of T-protein (Hmet to T-protein ratio: 1/1).

Assay for Hred. Measurements were performed according to Gueguen et al. (*17*) at 30 °C in 50 mM potassium MOPS buffer (pH 7.5) containing the sample (Hred/Hmet mixture), 8 mM tris(2-carboxyethyl)phosphine (TCEP), a specific disulfide reducing agent, 3 mM NAD and 0.8 μM dihydrolipoamide dehydrogenase (pig heart, Sigma). The initial rate of NADH formation is related to the amount of Hred (or Hox) present in the assay medium (*17*). To quantify the Hred content of the sample, a calibration curve was first recorded by plotting the rate of the catalytic reaction as a function of the Hred concentration. The calibration curve was obtained using the same concentration of dihydrolipoamide dehydrogenase as that used for the assay.

Assay for ^3H_2CO. The amount of [^{3}H]$_2$CO deriving from H-$^{[3H]}$met was determined according to Taylor and Weissbach (*18*) with modifications. To separate the H-$^{[3H]}$met from $^3H_2$-CO, aliquot of the incubation medium was placed on a CM cellulose−DEAE cellulose mixed bed resin in a small Ultrafree centrifugation design (Millipore) kept at 4 °C. The resin (200 μL) was preequilibrated in 20 mM potassium phosphate buffer containing 20 mM ammonium acetate pH 7.5 prior to the addition of the sample. After the solution was mixed, the sample was centrifuged for 2 min at 2000 rpm at 4 °C. Under these conditions, only the formaldehyde was released in the through-flow; 0.3 mL of 1 M ammonium acetate, pH 4.5, 0.04 mL of 0.5 M carrier formaldehyde, and 0.3 mL of 0.4 M dimedon (Sigma) were successively added to the through-flow. The mixture was then heated for 2 min at 100 °C and the [^{3}H] formaldehyde−dimedon complex was extracted by vigorous shaking with 3 mL of toluene. A 2 mL sample of the organic phase was added to a liquid scintillation cocktail (Ready Safe, Beckman) and the radioactivity was measured on a β-liquid-scintillation

counter (β Matic, Kontron Instruments).

Thermal Activation. The incubation medium for the demethylamination of the Hmet-protein was set at different temperatures (from 10 to 40 °C). The reaction was initiated by adding H-[3H]met, and its progress was monitored by measuring the release of ^3H_2CO. An Arrhenius plot [ln(k) vs $1/T(K^{-1})$] allows the determination of the activation energy E_a according to the equation: $E_a = -R \times p$ where p is the slope of the Arrhenius plot and R the gas constant.

Accessibility of the C Atom of the Methylamine Group to H_4FGlu_n. To investigate the possibility of direct nucleophilic attack of the N_5 or N_{10} atoms of the H_4FGlu_n on the carbon atom of the methylamine group in the buried conformation, we have performed simple restrained molecular dynamics simulations. All calculations were performed using the program DISCOVER with the AMBER4 force field (*19*), modified to incorporate parameters characterizing the lipoate and tetrahydrofolate. Protons were added to the coordinates of the crystal structure of Hmet, which was then energy-minimized with the heavy atoms fixed.

Distance restraints between the methylene carbon and either the N_5 or N_{10} atoms of H_4FGlu_n were set at $d_{C-N} < 2.5$ Å using a force constant of 1.0 kcal $mol^{-1}Å^{-2}$. The backbone atoms of the Hmet, the heavy side-chain atoms of Lys63 and the heavy atoms of the lipoate arm, were tethered to their initial positions by incorporating the additional energy term

$$E_{teth} = K_{teth}\sum_i \sqrt{(x_i - x_i^0)^2/N}$$

into the potential energy function; x_i are the Cartesian coordinates of the atoms to be tethered and x_i^0 the target coordinates. A force constant of $K_{teth} = 500.0$ kcal $mol^{-1}Å^{-1}$ was used to restrain the $N = 542$ atoms to the coordinates of the crystal structure. The H_4FGlu_n was free to move, as were all remaining side-chain and protons of the Hmet-protein. Solvent effects were implicitly included using a distance-dependent dielectric constant and reduced charges on solvent-exposed polar side-chains (*20*).

The initial position of the two molecules was chosen arbitrarily to allow apparently direct access of the tetrahydrofolate group to the CH_2 of the methylamine moiety. The system was equilibrated at 300 K for 5 ps, using direct velocity scaling, followed by a 50 ps sampling period during which the temperature was controlled using weak coupling to a thermal bath using a time constant of 0.1 ps (*21*). The system was then slowly cooled to 100 K over a period of 10 ps. This was followed by energy minimization in the same force field. Nonbonded interactions were ignored beyond 11 Å, and a switching function applied from 9.5 to 11 Å and a time-step of 1 fs was used for all molecular dynamics simulations. The calculation was repeated using different initial velocity distributions and relative starting positions of the Hmet and H_4FGlu_n for both $C-N_5$ and $C-N_{10}$ constraining calculations. In total, 20 complexes were calculated for each ensemble.

NMR. 2D ^1H-^{15}N HSQC (*22*) experiments were carried out for Hox at 18 °C on a Bruker AMX-600 NMR spectrometer and for Hmet at 5 °C on a Varian INOVA-600 NMR spectrometer. Each spectrometer was equipped with a triple-resonance (^1H,^{13}C,^{15}N) probe including shielded z-gradients. All spectra were processed using FELIX version 97.0 (MSI technologies). Proton chemical shifts were reported at pH 5.5 with respect to the H_2O signal relative to DSS taken as 4.848 ppm at 18 °C and 5.003 ppm at 5 °C. The ^{15}N chemical shifts were referenced indirectly using the $^1H/X$ frequency ratios of the zero-point: 0.101329118 (*23*).

Hmet and Hox Assignments. For Hmet and Hox, assignments were previously made at 18 °C (*24*). A series of gradient enhanced ^1H-^{15}N HSQC (*25*) spectra were recorded at 18, 14, 10, and 5 °C to monitor the chemical shift variations with the temperature and thus assign the spectra at 5 °C.

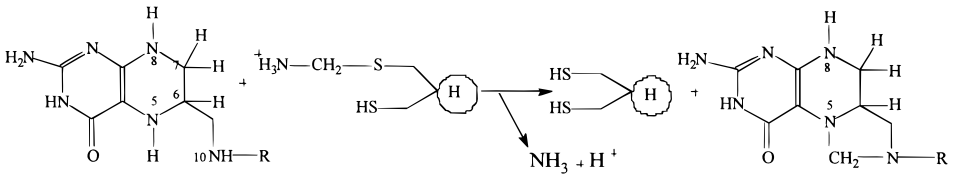
Hox/T Interaction. [^{15}N]Hox (0.11 μmol) was suspended in 50 mM potassium phosphate, pH 5.5 containing 0.1 mM EDTA, 0.2% sodium azide, and 10%(v/v) D_2O in a total volume of 0.450 mL. The T-protein used for titration was added to the Hox suspension as followed: small aliquots of the T-protein (stock solution, 1.5 mM) were added successively to the [^{15}N]Hox sample to get the following final concentrations: 0.03, 0.075, 0.1, and 0.15 mM. At the end of the titration, the final volume of the sample was 0.5 mL. 2D ^1H-^{15}N HSQC experiments were performed after each addition of T-protein and carried out with a spectral resolution of 13.9 Hz in t_1 and 9.4 Hz in t_2. The experimental times were, respectively, 1.5 h (no T-protein), 3 h (0.03 mM of T-protein), 6 h (0.075 mM of T-protein), 9 h (0.1 mM of T-protein), and 24 h (0.15 mM of T-protein). In our experimental conditions, the H−T complex was stable.

In another set of experiments, we verified that the successive addition of aliquots of T-protein buffer to the ^{15}N−Hox solution (0.11 μmol in 0.45 mL) prepared as stated above did not induce chemical shift changes in the 2D ^1H-^{15}N HSQC spectra.

Hmet/T Interaction. As addition of T-protein to the Hmet suspension increases the rate of conversion of Hmet into Hred, in the absence of H_4FGlu_n, the NMR study was performed at low temperature (5 °C) and with a T/Hmet ratio lower than 0.5 to slow the demethylamination process. Under these conditions, Hmet remained over 90% loaded during the NMR experimental time (about 10 h). [^{15}N]Hmet (0.11 μmol) was suspended in 50 mM potassium phosphate (pH 5.5), containing 0.1 mM EDTA, 0.2% sodium azide and 10% (v/v) D_2O in a total volume of 0.450 mL. 2D ^1H-^{15}N HSQC spectra were recorded with a spectral resolution of 11.7 Hz in t_1 and 9.76 Hz in t_2. A first spectrum of the ^{15}N−Hmet was recorded with an experimental time of 2 h. T-protein (40 μL of the stock solution) was added to the Hmet solution (final concentration of T-protein: 0.073 mM). Three spectra were then recorded. The experimental times were, respectively, 4 h for the first spectrum and 2 h for the last two spectra. The three experiments were added for the chemical shift analysis. Reproducibility of the experiment was checked using two different samples of ^{15}N−Hmet and T-proteins.

RESULTS.

Effect of H_4Fglu_n on the Stability of the Hmet-protein. T-protein catalyzes the transfer of the CH_2 unit of the methylamine moiety to the H_4FGlu_n, leading to the formation of $CH_2-H_4FGlu_n$ with concomitant release of NH_3 and Hred according to the following equation:



(1)

When Hmet (28 nmol) was incubated alone in 1 mL of 50 mM potassium phosphate buffer at pH 7.5, at 30 °C under anaerobic conditions for 20 min a very slow formation of Hred (approx 2 nmol/h) was observed (*8*). Under these conditions, addition of a large excess of the O_2-sensitive H_4-$FGlu_1$ (0.20 mM) did not change the rate of Hred formation. This slow demethylamination is probably initiated by a nucleophilic attack of OH^- on the methylamine group:

$$\text{Hmet} + OH^- \rightarrow \text{Hred} + H_2CO + NH_3 \qquad (2)$$

In contrast, addition of 0.5 μM T-protein to the (Hmet + H_4FGlu_1) mixture induced a very fast unloading of the Hmet with a rate 5 orders of magnitude greater than that observed before. This might suggest that the nucleophilicity of the H_4FGlu_1 is increased by the binding to T-protein. It has, nevertheless, been shown that H_4FGlu_1 is able to directly react with formaldehyde by a nucleophilic process (*10*). In addition, previous studies have shown that the C atom of the methylamine group is buried in a cleft at the surface of the H-protein (*6*−*8*). This leads us to expect that the lack of reactivity of the H_4FGlu_1 observed here, in the absence of T-protein, is due to the inability of this bulky molecule to approach this C atom. To confirm this hypothesis, we have investigated the accessibility of the H_4FGlu_n to the C atom of the methylamine group using restrained molecular simulations.

Accessibility of Methylene Group to H_4FGlu_n by Restrained Molecular Dynamics. Formation of 5,10-CH_2−H_4-$FGlu_n$ is the result of a nucleophilic attack by the basic N_5 atom of H_4FGlu_n in H_4FGlu_n-binding enzymes such as serinehydroxymethyltransferase (*10*). However, the reaction mechanism is still unknown in the case of GDC and an attack by the N_{10} atom cannot be excluded. We have, therefore, investigated the possibility of interaction between Hmet and H_4FGlu_n involving either the N_5 or N_{10} atoms of the cofactor. A typical example of a restrained molecular dynamics calculation is shown in Figure 1. Despite broad sampling of conformational space by the H_4FGlu_n molecule relative to the Hmet, neither the N_5 nor the N_{10} atoms are able to approach the C atom of the methylamine group to within a range necessary for successful nucleophilic attack if the lipoate arm remains in the conformation found in the crystal structure (*6*) and verified in a recent study of the molecule in solution (*8*). The relevant N atoms are unable to approach closer than 4.3 Å for N_5 and 4.8 Å for N_{10} if the protein backbone and lipoate arm are tethered to the known structure. By doubling the distance restraining force relative to the overall tethering force, the appropriate distances can be reduced, although not to within the range necessary for nucleophilic attack (closest distance of 3.8 Å). Inspection of these structures shows that the C atom of the methylamine group leaves the pocket, by performing a 180° crankshaft dihedral jump about the main lipoate chain. While these calculations are simplistic, they nevertheless illustrate the steric hindrance impeding direct interaction of the H_4FGlu_n group with the known configuration of the lipoate arm of the Hmet protein. In reality, the accessibility of the CH_2 group is probably an upper limit due to the additional presence of water molecules, which were not explicitly taken into account by the calculation.

It appears, therefore, that the network of hydrogen bonding and hydrophobic contacts stabilizing the methylamine group of the lipoamide arm (*7*) must be ruptured in order to allow interaction between the C atom of the methylamine group and the H_4FGlu_n nitrogen. Since H_4FGlu_n alone (see above) did not destabilize the methylamine group, we are forced to postulate that the main function of the T-protein is to increase the accessibility of the methylamine to the N atom of the H_4FGlu_n.

Biochemical Study of the Interaction Between H-protein and T-protein. To further investigate the influence of the T-protein on the stability of the methylamine group, we have studied the demethylamination of Hmet in the absence of tetrahydrofolate.

Figure 2 shows the formation of Hred as a function of time when Hmet was incubated either alone (Figure 2a) or

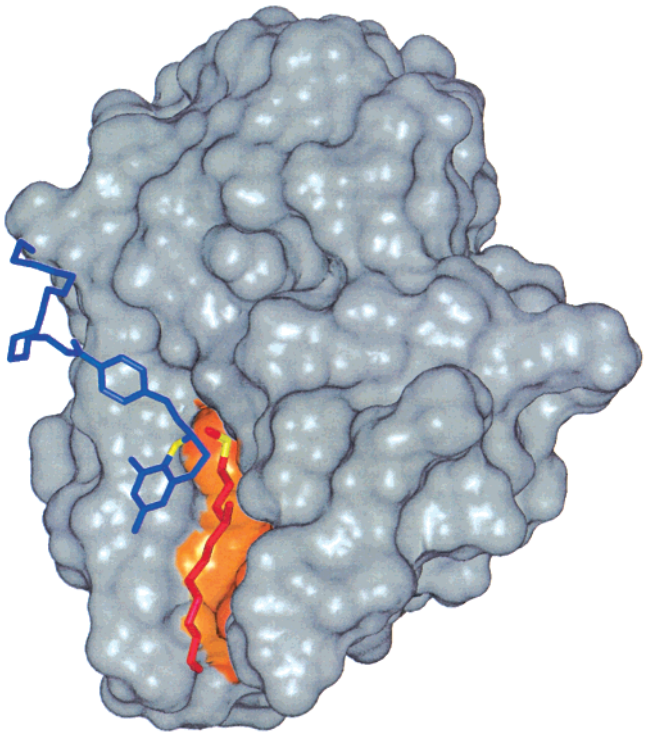


FIGURE 1: Example of a restrained molecular dynamics calculation designed to test the accessibility of the CH_2 group of the methylamine moiety to the N_5 atom of tetrahydrofolate (blue), while locked in the pocket. The two atoms involved in the reaction are shown in yellow, the cleft containing the lipoate arm (red) is shown in orange. To simplify the figure only one glutamate of the tetrahydrofolate is represented. The two atoms cannot approach closer than 4.3 Å (4.66 ± 0.15). Calculations revealed similar results for the N_{10}−CH_2 interaction (4.52 ± 0.02).

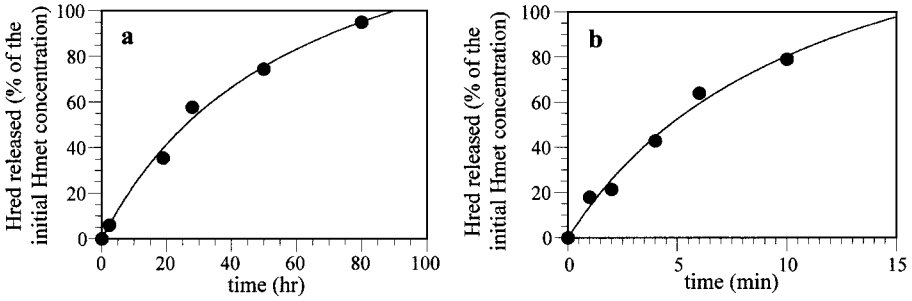


FIGURE 2: Kinetic studies of the demethylamination reaction of Hmet as a function of time. (a) Hmet alone; (b) in the presence of T-protein. Assays were performed using 20 μM Hmet protein alone or in the presence of 20 μM T-protein in a medium containing 50 mM potassium phosphate buffer at 30 °C, pH 7.0 (final volume, 0.5 mL). At different times, aliquots of 50 μL were removed and cooled on ice. The rate of the demethylamination reaction was followed by checking the release of Hred protein, expressed as a percentage of the total Hmet-protein concentration (See Material and Methods).

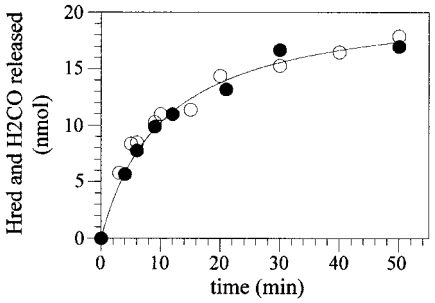


FIGURE 3: Formadehyde and Hred production as a function of time, during incubation of H-[3H]met in the presence of T-protein (molar ratio of 1.0 Hmet/0.33 T). Assays were performed at 30 °C in 50 mM potassium phosphate buffer, pH 7.5, containing 15 μM Hmet and 5 μM T-protein (final volume 1.4 mL). At selected intervals, aliquots of the medium were withdrawn for H_2CO and Hred analysis (see Material and Methods). (●) formaldehyde, (○) Hred.

in the presence of T-protein (Figure 2b). At 30 °C and at pH 7.0, the full demethylamination of Hmet, in the absence of T-protein, requires about 100 h. When Hmet was incubated in the presence of T-protein, we observed a marked increase of the rate of Hred formation, which led to a complete demethylamination in less than 20 min. In the presence of T-protein, the formation of Hred is also associated with a stoichiometric release of formaldehyde (Figure 3) as observed for Hmet alone (not shown). Furthermore, the rates of Hred production in the presence and absence of T-protein show similar pH-dependent behavior (Figure 4): the rate of formaldehyde released increased rapidly as the pH of the assay increased. These results indicate that the T-protein, in the absence of its cofactor, exerts a direct influence on the stability of the methylamine group. These results also suggest that, in the absence of H_4FGlu_n, the demethylamination of the Hmet is attributable to a nucleophilic attack by hydroxyl ions.

The activation energy (E_a) for the reaction in the presence of T-protein was determined by following the release of formaldehyde at different temperatures (Figure 5). From the Arrhenius plot shown in Figure 5b, the derived value (17 ± 2 kcal mol^{-1}) is significantly lower than that determined for Hmet in absence of the T-protein (37 ± 4 kcal/mol) (*8*). The marked decrease in the activation energy may be due to a change in the accessibility of the methylamine group of Hmet induced by the T-protein.

The 1:1 stoechiometry of the Hmet−T complex described previously (*5*) is also relevant for the demethylamination process. Indeed, this reaction rate increases as a function of the T-protein concentration in the incubation medium and reaches a plateau when the T:H ratio becomes close to 1 (Figure 6). It is, therefore, reasonable to suppose that the Hmet protein structure is altered in this complex, affecting the stabilization of the methylamine group. The structural changes in the H-protein induced by the interaction with T have, therefore, been studied using 2D-NMR spectroscopy.

Structural Interaction Between H- and T-proteins. The interaction between the proteins is expected to induce structural and dynamic changes giving rise to chemical shift and line-width variations of the signals. The residues of the H-protein affected by the interaction with T-protein have thus been identified from heteronuclear 2D spectra. About 80% of the $^{15}N/^{1}H$ backbone amide chemical shifts remain unaffected by complex formation, indicating that the global structure of the Hmet-protein is not strongly modified when complexed to the T-protein (Figure 7). However, the formation of the large H:T complex (55 kDa) resulted in a general decrease of the cross-peak intensities. Figure 8 shows the major variations induced by the addition of T-protein for Hox and Hmet. They are mainly localized in the regions of the H-protein including the segment S61−T65 to which the lipoamide arm is attached (K63) and the segment L35−E42. The T-protein seems, therefore, to interact with the same zone of both forms of the H-protein.

Addition of Hox to the Hmet−T complex would be expected to decrease the observed rate of the demethylamination due to a competitive Hox−T interaction. We have, therefore, experimentally compared the demethylamination of Hmet in the absence and presence of Hox. Addition of a 10-fold excess of Hox over Hmet led to near-complete inhibition, suggesting a similar interaction site for the two forms, an observation which is supported by the NMR data.

In the Hmet−T complex observed by NMR (Figure 8b), significant chemical shift variations are observed in the region of the peptide chain around the lipoamide arm attachment and on the side of the cleft where the arm is located (S61−T65) (*6*). In contrast, no major chemical shift changes are detected for the pocket where the methylamine group interacts with the protein (residues S12−E14, I27, D67) or for the short helix D29−L35, which forms the opposite side of the cleft. This implies that, in the first step of the methylene transfer reaction, i.e., the formation of the Hmet−T complex, the methylamine group remains in the cleft. The T-protein then acts by destabilizing the lipoamide arm cleft, for example, via an opening of the bottom of the pocket (relative to the orientation shown in Figure 8b),

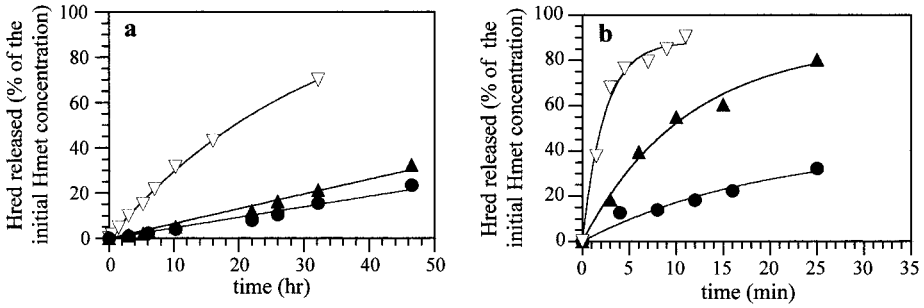


FIGURE 4: Kinetic studies of the demethylamination reaction of Hmet as a function of pH. (a) Hmet alone. (b) in the presence of T-protein. Assays were performed using 40 μM Hmet-protein or 10 μM Hmet protein and 10 μM T-protein in a medium containing 50 mM potassium phosphate buffer at 30 °C, at various pH, 5.5 (●), 6.5 (▲) and 8.0 (▽) (final volume 0.5 mL). At different times, aliquots (50−80 μL) were removed and cooled on ice. The rate of the demethylamination reaction was followed by checking the release of Hred protein, expressed as a percentage of the total Hmet-protein concentration (See Material and Methods).

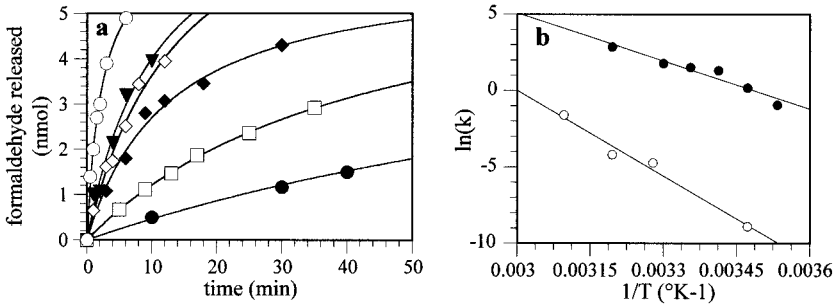


FIGURE 5: (a) Kinetic studies of the demethylamination reaction of Hmet in the presence of T-protein as a function of temperature. Assays were performed using 5 μM H-[3H]met and 5 μM T in 1.2 mL 20 mM potassium phosphate buffer at pH 7. The samples were incubated at various temperatures 10 (●), 15 (□), 20 (◆), 25 (◇), 30 (▼), and 40 °C (○). At different times, aliquots of 0.2 mL were removed and checked for ^{3}H-formaldehyde formation as described in Material and Methods. (b) Corresponding Arrhenius plot for the demethylamination reaction in the presence of T-protein (●). For Hmet alone (○), Arrhenius plot data were from ref 8.

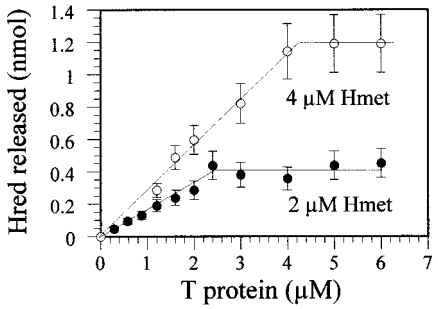


FIGURE 6: Kinetic studies of the demethylamination of Hmet as a function of T-protein concentration. Assays were performed using 2 μM (●) or 4 μM (○) Hmet and various concentrations of T-protein (0.25−6 μM) in 0.5 mL of 50 mM potassium MOPS buffer, pH 7.5 at 30 °C. The sample was incubated for 5 min and the release of Hred-protein was checked using the TCEP assay. We have verified that the rate of Hred released was linear at least during the first five minutes of the reaction whatever the concentration of T-protein used in the incubation medium. The data plotted on the graph were the mean value of three replicates and error bars have been plotted. The maximum velocities of demethylamination observed at saturation of T-protein were, respectively, 0.45 ± 0.10 nmol of H released/5 min at 2 μM of Hmet and 1.2± 0.2 nmol/5 min at 4 μM Hmet.

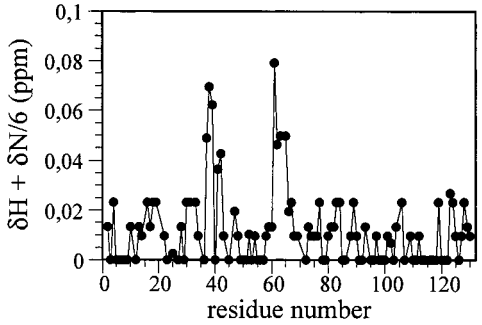


FIGURE 7: Variation of the chemical shifts of the Hmet-protein on complex formation with T-protein at a molar ratio of 1.0 H/0.37 T. The difference of the chemical shifts (abs(δH) + abs(δN/6)) observed in the ^{15}N−HSQC Hmet-T and Hmet spectra is displayed as a function of the sequence number. The factor 6 corresponds to the ratio of the average mean δN and δH: δN/δH.

resulting in increased accessibility of the methylene group. In the transfer of the methylene group, this destabilization is probably the mechanistic step facilitating the nucleophilic attack by the tetrahydrofolate.

DISCUSSION

The different proteins forming the glycine decarboxylase complex dissociate following mitochondrial inner membrane rupture, a feature that facilitates the study of the different interaction mechanisms constituting each catalytic step. It has thus previously been suggested that the H-protein acts as a chaperone in protecting T-protein from aggregation and irreversible inactivation (*5*). The formation of this complex H−T is also particularly interesting, because the reactive methylamine group is buried in a cleft of the H-protein, and apparently unavailable for direct methylamine transfer to the H_4FGlu_n bound to the T-protein.

The aim of this study was to identify the exact role played by the T-protein and the tetrahydrofolate cofactor, respectively, in the catalytic step leading to the demethylamination of the Hmet and resulting in the transfer of the CH_2 group. This has been done using a combined biochemical, spectroscopic, and molecular dynamic approach. When H_4FGlu_n was

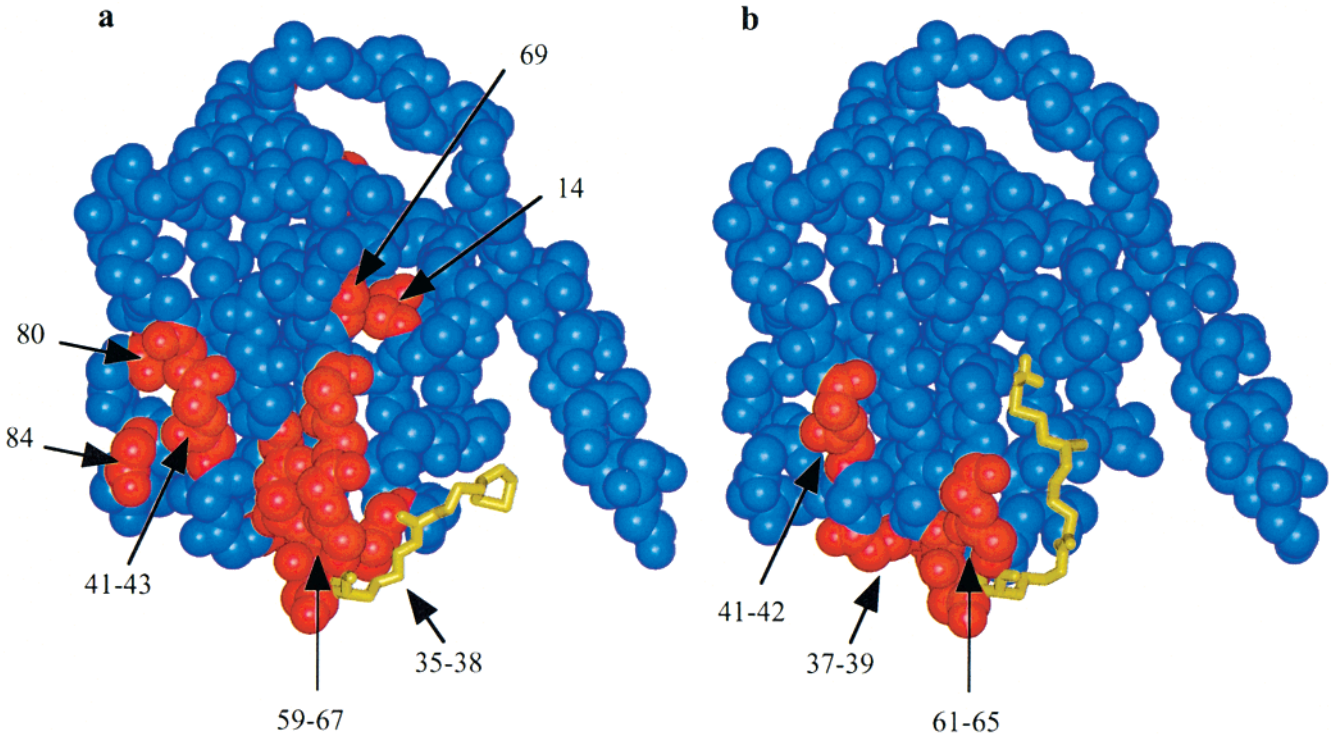


FIGURE 8: Spatial distribution of the chemical shift variations observed between the H and the H complexed with T-proteins at a molar ratio of 1.0 H/0.37 T is mapped onto the crystallographic structures (*6, 7*). (a) Hox-protein. (b) Hmet-protein. The significant chemical shift variations ($\delta H + \delta N/6 > 0.04$ ppm for Hox and 0.03 ppm for Hmet) are shown in red and the lipoamide arm is displayed in yellow. As the two interaction studies were made at different temperatures due to the unloading of Hmet in the presence of T-protein, only a qualitative comparison is possible.

incubated with Hmet, in the absence of T-protein, it was unable to catalyze the unloading process. In addition, molecular dynamics calculations demonstrate that the presence of H_4FGlu_n alone does not allow direct transfer of the methylene group as the reactive nucleophilic N_5 (or N_{10}) atom of the pteridine ring could not sufficiently approach the C atom of the methylamine group. Fujiwara et al. (*26*) have observed that T-protein alone can catalyze an extremely low release of ammonia and formaldehyde from the Hmet protein. Our results confirm this and demonstrate that the addition of T-protein to Hmet strongly increases the rate of formaldehyde production compared to that observed for Hmet alone. The characterization of the temperature dependence of the unloading reaction in the absence or presence of T-protein shows a decrease of activation energy of the global reaction from 37 to 17 kcal in the presence of T. It appears, therefore, that the tetrahydrofolate cofactor is not essential for the release of the methylamine group and that the T-protein acts specifically to destabilize the methylamine group inside the H-protein cavity.

We have investigated the localization of the interaction site of the two partners using chemical shift perturbation mapped onto the known structure of the isolated H-protein, providing novel evidence for the mechanism of molecular interaction. Surprisingly, the binding zone of the T-protein on the H-protein is not localized in the cleft stabilizing the methylamine. We have previously observed, using ^{15}N relaxation experiments, that loading of the methylamine group induces a stabilization of both the cleft and the C-terminal helix (*8*). It could, therefore, be expected that the binding of the T-protein affects these regions, inducing a destabilization of the arm. In fact, the binding zone is highly localized around residues 63−65, which form one side of the cleft holding the lipoamide arm. Theoretical studies of the conformation of the H-protein lipoic arm have shown that when loaded with the methylamine the arm is stabilized in the cavity by ionic and hydrogen bond interactions (*27*). The interaction energy (−42 kcal mol^{-1}) results from an electrostatic interaction of the NH_3^+ at the end of the arm with the side-chain carboxyl group of the Glu14 and the carbonyl of Asp67 as well as electrostatic interactions of the side-chain amide group of the lipoyl-lysine with the protein. The specific localization of the surface interaction and the lack of change observed for Glu14 suggest that the destabilizing effect of the T-protein does not result from a primary rupture of the methylamine group interaction. In contrast, the β-sheet structure that forms one side of the cleft is collectively destabilized during the interaction, probably leading to an increased access of the H_4FGlu_n to the CH_2 group, thus allowing the transfer to take place efficiently.

As the rate of formaldehyde production increased as a function of the pH, we assume that the hydrolysis of the methylamine group takes place following the partial removal of the lipoamide arm from the cavity, either due to increased temperature or the presence of T-protein. The increased concentration of OH^- as a function of the pH may account for the nucleophilic attack of the methylene unit. This would be similar to the reaction in the presence of the nucleophilic H_4FGlu_n cofactor. Interestingly, when the pH increased from 6.5 to 8, we observed that the rate of formaldehyde produced increases in the same ratio in the absence or presence of the T-protein. This seems to exclude an effect of some additive nucleophile in the presence of the T-protein (histidine residue). Therefore, the same process can be expected to govern hydrolysis in both cases. Nevertheless, below pH 6.5, the process is probably more complex because low pH may

affect the net charge of the acidic H-protein (pI = 4.5−5), and consequently, alter the ionic interaction with the basic T-protein (pI > 8.5). In the case of the Hmet alone, the low increased rate of demethylamination observed between pH 5.5 and 6.5 suggests some stabilizing effect on the lipoamide arm in acidic conditions. Although the rate of demethylamination is rapid in the presence of stoichiometric amounts of T-protein (about 1 nmole), it is far below that observed in the presence of H_4FGlu_n. This may be explained by the low nucleophilicity of hydroxyl ions whose concentration at pH 8 is close to 1 μM. At this concentration, the H_4FGlu_5 (K_m for the T-protein < 0.5 μM) (*13*), which strongly binds the T-protein, accelerates the reaction by a factor close to 10^4. It is clear that the tetrahydrofolate is a better nucleophile than OH^- and that the in vivo release due to hydrolysis is probably negligible with respect to the catalytic reaction.

After the previous results were considered, we propose a model whereby the function of the T-protein in the reaction mechanism is 2-fold: first, in modifying the stability of the Hmet leading to a release of the methylamine moiety, and second, as previously suggested (*7*) in locating the tetrahydrofolate cofactor in a position favorable to a nucleophilic attack of the methylene carbon.

Finally, NMR studies demonstrated that T-protein interacts in a similar manner with the Hox and Hmet. Interestingly, the interaction region has also been implicated in the *E. coli* GDC; using cross-linking experiments, the Asp43 of the reduced form of the H-protein has been shown to be involved in an electrostatic interaction with a surface lysine of the corresponding T-protein (*28*). Since the structures of Hox and Hmet studied here differ in the position of the extremity of the lipoamide arm (*6*, *7*, *8*), the exact configuration of the lipoamide cofactor appears to be unimportant for the recognition of the two proteins. On a mechanistic point of view, the observed competitive binding of Hox and Hmet for T-protein must be important in-vivo, because the concentration of H (3−4mM) largely exceeds that of the T-protein (0.8−1.0 mM). This implies that at the start of the photorespiratory cycle, the demethylamination reaction should be a limiting step for the global reaction. It is, therefore, of interest to study the dependence of the reaction kinetics on the respective concentration of the different forms of the H-protein and their different association constants. This subject is currently under investigation in our laboratory.

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