Activation and competition of lipoylation of H protein and its hydrolysis in a reaction cascade catalyzed by the multifunctional enzyme lipoate-protein ligase A

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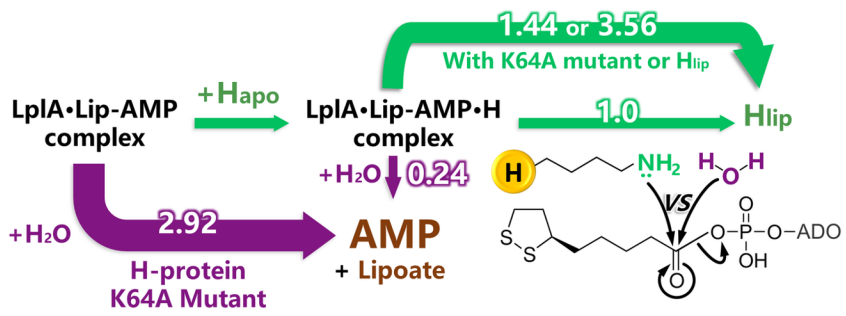
Abstract

Protein lipoylation is essential for the function of many key enzymes, but barely studied kinetically. Here, the two-step reaction cascade of H protein lipoylation catalyzed by the multifunctional enzyme lipoate-protein ligase A (LplA) was quantitatively and differentially studied. We discovered new phenomena and unusual kinetics of the cascade: (1) the speed of the first reaction is faster than the second one by two orders of magnitude, leading to high accumulation of the intermediate Lip-AMP; (2) Lip-AMP is hydrolyzed, but only significantly at the presence of H protein and in competition with the lipoylation; (3) both the lipoylation of H protein and its hydrolysis are enhanced by the apo and lipoylated forms of H protein and a mutant without the lipoylation site. A conceptual mechanistic model is proposed to explain these experimental observations in which conformational change of LplA upon interaction with H protein and competitive nucleophilic attacks play key roles.

GRAPHICAL ABSTRACT:



KEYWORDS:

H protein, lipoate-protein ligase A, lipoylation, hydrolysis, protein interaction

INTRODUCTION

Lipoic acid is an essential cofactor that is covalently bound to its cognate enzyme proteins, a process known as lipoylation, and conveys activated reaction intermediates between different active sites of several multienzyme complexes involved in the central metabolism of organisms (Cronan, 2016). These enzyme systems include pyruvate dehydrogenase complex (PDHC) and 2-oxoglutarate dehydrogenase complex (KDHC) for the entry

of carbon into the tricarboxylic acid (TCA) cycle and the progression of carbon through the TCA cycle, respectively, the branched-chain alpha-ketoacid dehydrogenase complex (BCKDH) in amino acid metabolism and the glycine cleavage system (GCS) in one-carbon (C1) metabolism(Cronan, 2018; Perham, 2000). Recently, lipoylation of the GCS H protein has gained interest in the engineering synthetic C1 metabolism. In the so-called reductive glycine pathway, the reverse GCS reaction serves in the assimilation of the C1 carbon source CO_2 into biomass (Bang and Lee, 2018; Bar-Even, 2016; Bar-Even et al., 2010; Bar-Even et al., 2012; Doring et al., 2018; Tashiro et al., 2018; Yishai et al., 2018). However, our recent studies showed that the degree of the intracellular H protein lipoylation is in general quite low and can represent a limiting step for C1-based biosynthesis (Zhang et al., 2019).

Lipoylation of the H protein has received much interest in the past (Cronan, 2016; Fujiwara et al., 1990; Fujiwara et al., 1991; Fujiwara et al., 1992; Macherel et al., 1996; Solmonson and DeBerardinis, 2018). It is now clear that in *E. coli* , lipoate-protein ligase A (LplA) catalyzes the lipoylation of the apo-H protein (H_{apo}) in the presence of lipoic acid and ATP (Fig. 1a). The reaction is divided into two steps: (1) the lipoate adenylation reaction, where lipoic acid is activated by ATP, forming the intermediate lipoyl-AMP (Lip-AMP); (2) the lipoate transfer reaction, where the lipoyl part is transferred onto a specific lysine residue of H_{apo} (Fujiwara et al., 2005). Recent years, the functionality of LplA as a connection tool was further explored (Fernández-Suárez et al., 2007; Uttamapinant et al., 2010). Uttamapinant, C. *et al.* first engineered LplA as a fluorophore ligase. The new protein labeling method, called probe incorporation mediated by enzymes (PRIME) will provide a much-needed alternative to GFP, and provide life scientists with a way to label proteins of interest in a minimally invasive and extremely specific manner.

The crystal structures of *E. coli* LplA and its complexes with lipoic acid (lipoate), Lip-AMP and H protein have been determined by Fujiwara *et al.* (Fujiwara et al., 2010; Fujiwara et al., 2005). LplA contains a large N-terminal domain and a small C-terminal domain. Based on information from the structural analysis and some kinetic studies of potential key residues involved in the binding of Lip-AMP and the lipoate transfer, Fujiwara*et al.* (Fujiwara et al., 2010) proposed a reaction mechanism model of the H protein lipoylation catalyzed by *E. coli* LplA. It involves large conformational changes of LplA as summarized in Fig.1b. At the beginning of the lipoylation process the C-terminal domain of unliganded LplA adopts a bending conformation. Lipoic acid attaches to the hydrophobic cavity in the N-terminal domain by hydrophobic interactions without changing the bending conformation of LplA first (Fujiwara et al., 2005). After the adenylation of the lipoic acid (Step 1 in Fig.1a), LplA undergoes a dramatic conformational change: the adenylate-binding and lipoate-binding loops take certain movements to bind the reaction intermediate Lip-AMP, while the C-terminal domain rotates by about 180° and adopts a stretched conformation. The large conformational change is suggested to be a prerequisite for LplA to accommodate H_{apo} for the second reaction step (Fujiwara et al., 2010). However, little information is available about the quantitative kinetics of the lipoylation reaction, i.e. the kinetics of the individual reaction steps as well as the influence of structural factors affecting the reaction rates are basically unknown. This is probably among others due to lack of suitable analytic methods (Hong et al., 2020). While the product of the first adenylation step, Lip-AMP, can be measured relatively easily using HPLC (Fujiwara et al., 2010), assays available for the second lipoate transfer reaction are complicated to perform and less suitable for quantitative kinetic studies. They are relied on radioactive isotope labeling either directly by using [^{35}S] lipoic acid (Morris et al., 1994), or indirectly by determining the activity of the glycine-$^{14}CO_2$ exchange reaction catalyzed by P protein of GCS (Fujiwara et al., 1992). The latter can only be carried out under low concentrations of H_{apo}, because the glycine-CO_2 exchange reaction is inhibited when the concentration of H_{apo} is higher than 5μM (Fujiwara et al., 2005).

Recently, we developed an efficient direct assay method for H_{apo} and H_{lip} (the lipoylated form of H protein) using HPLC (Zhang et al., 2019). In this work, we have further developed an efficient HPLC method for a fast and reliable quantification of lipoic acid, Lip-AMP, AMP, ADP and ATP, enabling us thereby quantitative studies of the overall reaction and the individual steps of the LplA-catalyzed lipoylation of H protein. The quantitative and differentiated studies of the overall reaction and the individual steps revealed unusual the kinetic behaviors of H protein lipoylation. In particular, we discovered autocatalytic and feedback activations of lipoylation by the different forms of H protein and unexpected hydrolysis of Lip-AMP. The latter is a

major phenomenon associated with the second step of the lipoylation. Based on these findings, a revised mechanistic model of H protein lipoylation is proposed.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

NaCl, Tris, and HCl were of analytical grade and purchased from Sinopharm Chemical Reagent Co. LTD (Beijing, China). ATP, ADP, AMP and lipoic acid were purchased from Sigma-Aldrich (Shanghai, China). The bicinchoninic acid (BCA) Protein Assay Kit was purchased from Beijing Solarbio Science & Technology Co. LTD (Beijing, China). Acetonitrile and trifluoroacetic acid (TFA) were of chromatographic grade and purchased from J&K Scientific Ltd. (Beijing, China). Chemically competent cells of *E. coli* TOP10 and *E. coli* BL21(DE3) were purchased from Weidishengwu Ltd. (Beijing, China). In-fusion cloning was used for the ligation of sequence fragments to vector with the In-fusion HD Cloning Kit (Clontech Laboratories, Inc, US). Luria-Bertani (LB) liquid medium (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) and solid medium (1.5% agar) with kanamycin (100 μg/mL), ampicillin (100 μg/mL) were used for transformation, screening, and cell growth.

2.2 Plasmid construction

The genes encoding H protein and LplA protein were amplified from*E. coli* MG1655 cells by PCR with His-tag and cloned into the pET28a$^+$ vector, yielding the plasmids pET28-H and pET28-LplA, respectively. The lysine residue at position 64 of the H protein for binding lipoic acid was mutated into alanine, resulting in the plasmid pET28-H_{K64A}. The plasmids were transferred into competent cells of *E. coli* BL21(DE3) for protein expression. Oligonucleotide sequences of primers used for the cloning of genes coding for the target proteins were given in Table 1.

2.3 Expression of LplA, H protein and the variant H_{K64A}

Cells harboring the plasmids pET28-H, pET28-LplA, and pET28a-H_{K64A} were grown at 37 °C in LB medium containing suitable antibiotics, respectively. For obtaining H_{lip}, 200 μM lipoic acid were added to the corresponding culture to directly convert the expressed H_{apo} into H_{lip}. Induction of the target protein expression was started by adding 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density of the culture at 600 nm reached 0.6. Then, the culture was incubated for an additional 12 h at 30 °C. After the medium removal by centrifugation (10,000 x g, 5 minutes, 4 °C), the bacterial pellet was re-suspended in Tris-HCl buffer (50 mM, pH 7.5) and lysed using a Xinzhi JY92-IIN Ultrasonic Homogenizer. The supernatant (lysate) was collected by centrifugation at 10,000 x g for 5 min at 4 °C and stored at 4 °C for the following protein purification step. Protein expression was examined using SDS-PAGE.

2.4 Protein purification

Each lysate generated as described above was cleared by centrifugation, and the target protein was purified by nucleophilic chromatography on a column of chelating Sepharose Fast Flow (GE Healthcare Bio-Sciences Corp.) charged with Ni^{2+} ion. The column was pre-equilibrated with the Lysis Buffer (50 mM Tris, 10 mM imidazole, 300 mM NaCl, pH 7.8), loaded with 30 mL of a lysate at a flow rate of 1.0 mL/min, and then washed with a Wash Buffer containing 30 mM imidazole, 0.3 M NaCl, and 50 mM Tris-HCl (pH 7.8), eluted with an Elution Buffer containing 300 mM imidazole, 0.3 M NaCl, and 50 mM Tris-HCl (pH 7.8) to obtain a purified fraction containing the target protein. After dialyzing against Tris-HCl (50 mM, pH 7.5), the purified protein fraction was collected and stored at -80°C. Protein concentration was measured using the BCA Protein Quantitation Kit.

2.5 Analysis of the overall reaction and the individual reaction steps of lipoylation

1. **Overall reaction activity.** The overall reaction activity was determined at constant concentrations of R-(+)-lipoic acid (2 mM), ATP (2 mM), $MgCl_2$ (2 mM), H_{apo} (0.5 -400 μM), and LplA (10 μM) The reaction was started with the addition of ATP. The amount of lipoylated H protein in the mixture was determined using HPLC as described in our previous study (Zhang et al., 2019).

2. **Activity assay of the lipoate adenylation reaction (Step 1).**The reaction activity was determined in a reaction mixture of 0.25 mL containing 10 μM LplA, 2 mM ATP, 2 mM $MgCl_2$, and 2 mM R-(+)-lipoic acid. The final volume was made up with 50 mM Tris-HCl (pH 7.5). The reaction was initiated by adding ATP, incubated at 30 ^{o}C for 15 min (unless stated otherwise), and terminated by heating for 90s in boiling water to completely denature and precipitate LplA. Lip-AMP was chromatographically separated and quantified as described previously (Fujiwara et al., 2010), using a gradient HPLC on a Shimadzu Shim pack GIST C18 column (5 μm, 4.5 × 150 mm), with the mobile phase A being acetonitrile containing 0.1% trifluoroacetic acid, and the mobile phase B being 50 mM PBS buffer (pH 6.6) containing 5 mM tetrabutylammonium hydrogen sulfate (TBAHS). The formation of Lip-AMP can be observed as a single, clearly resolved peak. Because no chemical standard of Lip-AMP is commercially available, Lip-AMP was quantified indirectly as follows: after ATP, ADP and AMP were quantitatively determined, Lip-AMP is calculated as the difference between the initial amount of ATP and the total amount of the ATP remained and the ADP and AMP formed.
3. **Activity assay of the lipoate transfer reaction (Step 2).** The determination of the second step activity was similar to that in the first step, in which lipoic acid, ATP and $MgCl_2$ were replaced with Lip-AMP and H_{apo}. Lip-AMP was obtained through an overnight reaction of the first step and after a heat treatment of the reaction mixture in boiling water for 1 minute to denature LplA completely. Then, the sample was centrifuged at 10,000 x g and 4 °C for 1 minute. The supernatant was collected and used as the substrate of the second step. The lipoate transfer reaction was initiated by adding LplA, incubated at 30 ^{o}C for 15 min, and terminated by heating 1 minute in boiling water. The method for the detection of H_{lip} was the same as that used for the overall reaction.

2.6 Determination of influences of different forms of H protein

Different forms of H proteins (H_{apo}, H_{lip}, H_{K64A}) were used to investigate their effects on the reactions catalyzed by LplA. The reaction mixture (200 μL) contained 2 mM lipoic acid, 2 mM ATP, 2 mM $MgCl_2$, 10 μM LplA, Tris-HCl (50 mM, pH 7.0) and different concentrations of H proteins (from 0.4 to 400 μM). The components except ATP were premixed and centrifuged before the reactions were initiated by adding ATP to the reaction mixture.

3. RESULTS AND DISCUSSION

3.1 Activation of H protein lipoylation

Previously, H protein lipoylation catalyzed by LplA was only studied in a low concentration ranges of H_{apo} (up to about 5 μM) due to the limitation of the isotope radioactive labeling method used for the activity assay (Fujiwara et al., 2005). Here, we used our newly developed HPLC methods (see Materials and Methods) to quantitatively analyze the rate of H protein lipoylation in a wide range of concentrations of H_{apo}. At the same time, we also examined the possible effects of H_{lip} as the reaction product on the lipoylation process. Previous *in vivo* study of the lipoylation process in *E. coli* revealed a limited lipoylation of H protein and implied a possible inhibition of H protein lipoylation by its final product H_{lip} at low expression of LplA (Zhang et al., 2019). Unexpectedly, in this study, both H_{apo} as the reaction substrate and H_{lip} as the reaction product showed positive effects on the lipoylation process (Fig. 2a-f).

Fig. 2a shows that the effect of H_{apo} concentration on H_{lip} formation is significant even in a short reaction time of 15 min. A reaction time of 5-15 min and a H_{apo}concentration in the range of 0.19-4.5 μM were previously used in literature for the activity assay of LplA and the determination of the k_m value of H_{apo} (Fujiwara et al., 2005). In this range we also observed very low lipoylation of H_{apo}. In our study, when higher initial concentration of H_{apo} was used, the concentration of the formed H_{lip} significantly increased. In fact, it increased with the increase of the initial H_{apo} concentration up to 200 μM (Fig. 2a). As shown in Fig. 2b for two starting concentrations (10 μM and 100 μM, respectively) of H_{apo}, the concentration of H_{lip} reached maximum with a nearly 100% of lipoylation only after about 5 to 15 hours under the experimental conditions. From the reaction curves as shown in Fig. 2a we can calculate the initial reaction rate (v_{lip}) of H_{lip} as a function of initial H_{apo} concentration (Fig. 2c). It turned out that v_{lip} does not follow a

typical Michaelis-Menten kinetics as implied in previous studies (Fujiwara et al., 2005). Instead, it shows first a pseudo-saturation up to an initial H_{apo} concentration of about 10-50 μM, but increases again when the initial H_{apo} concentration was higher than 50 μM. Because the lipoylation product H_{lip} also significantly affect v_{lip} (Fig. 2d-f), an exact estimation of the k_m value even for this pseudo-saturation is not possible. Neglecting the effects of both H_{apo} and H_{lip} as activators resulted in an apparent k_m value of less than 3 μM (assuming a saturation at about 10 μM H_{apo}). This is in the same range of 1.2 μM as determined by Fujiwara *et al.* (Fujiwara et al., 2005). These authors also determined a v_{max} of 0.26 μmol/min/mg Protein for *E. coli* LplA. This is comparable to a v_{lip} value at a H_{apo} concentration of about 8 μM in Fig. 2c. In our assay v_{lip} increased significantly when the initial H_{apo} was beyond 50 μM. Additional measurements with LplA collected from a new batch of protein expression and purification showed that v_{lip} increased nearly linearly in a H_{apo} concentration range of 50 - 400 μM (data not shown). These results indicated that H_{apo} may act not only as a substrate, but also as an activator for its own lipoylation.

Surprisingly, H_{lip} also acts as an activator of H protein lipoylation (Fig. 2d and 2e). The relationship between the externally added initial H_{lip} concentration and the initial reaction rate v_{lip} showed a pattern similar to that between H_{apo} and v_{lip} (Fig. 2f). H_{lip} can active the lipoylation process, rather than cause a product inhibition. In general, it can be stated that H_{lip} causes a positive feedback activation of the lipoylation process.

To further examine the effects of different forms of H protein on the lipoylation of H_{apo}, we constructed a mutant protein of H_{apo} with mutation of the key residue lysine64 into alanine (H_{K64A}). The lysine residue at the position 64 in H_{apo} is the active site for the binding of lipoic acid. When it is mutated to alanine, the derived H_{apo} mutant should not participate in the lipoylation as a substrate. As shown in Fig. 3a, H_{K64A} did slightly promoted the lipoylation of H protein, but the activation was much less significant compared to those of H_{apo} and H_{lip}. It is noted that H_{apo} has the most significant effect on the formation of H_{lip}. This might be due to the fact that H_{apo} is both a substrate and an activator.

3.2 Hydrolysis of LplA·Lip-AMP complex and effectors

During the quantitative analysis of the lipoylation process, we noticed that the ratio of AMP/H_{lip} (Fig. 3b) is often much higher than the theoretical value which would be expected to be 1.0 from the stoichiometry of the lipoylation reaction (Fig. 1), indicating that a part of Lip-AMP bound on LplA might be hydrolyzed during the transfer of Lip-AMP to H_{apo}. This has not been reported before and is therefore examined in this work in more detail.

Fig. 4a depicts the kinetics of the formation of H_{lip}, AMP and Lip-AMP in a typical assay of the lipoylation process with 10 μM initial H_{apo}. It is noticed that AMP increased faster than H_{lip}, especially at the beginning of the assay. It is also interesting to note that there is a rapid accumulation of Lip-AMP, indicating that the first reaction step is much faster than the second reaction step. This was first thought to be the main reason for the hydrolysis of Lip-AMP, but turned out to be not true.

As summarized in Table 2, the formation rate of Lip-AMP is over 400 times higher than that of H_{lip} in the overall reaction assay. When the two reaction steps were separately assayed, the formation rate of Lip-AMP (Step 1) is still over 245 times faster than that of H_{lip} (Step 2) and the hydrolysis rate is about 1.24 times of that of the H_{lip} formation under the experimental conditions. A separate assay of Step 1 revealed a similar fast formation rate of Lip-AMP, but a neglectable hydrolysis of Lip-AMP in comparison to that in the overall reaction, suggesting that the accumulation of Lip-AMP is not the primary reason for its hydrolysis. A separate assay of Step 2 resulted in formation rates of AMP and H_{lip} both about 40% higher than those in the overall reaction. This might be because of the fact that Lip-AMP used for the assay of Step 2 was obtained as the reaction product of Step 1 and contained therefore also some residual lipoic acid and ATP. The residual ATP was between 127-173 μM in the different assays and lipoate in the range of 135-182 μM. Interestingly, the ratio of the formation rates of AMP and H_{lip} remained at 1.24. With the increase of H_{apo} concentration, the ratio of the formation rates of AMP and H_{lip} can be further increased (data not shown).

To better assess the effect of H protein on the hydrolysis of the Lip-AMP complex, we added H_{K64A} in the

overall reaction mixture. As shown in Fig. 4b and 4c, an enhanced hydrolysis (formation of AMP) was also observed with the addition of H_{K64A}. It is also clear from Fig. 4c and 4d that H_{K64A} increased the formation rates of AMP and H_{lip} as well, but not as significantly as it to the hydrolysis of Lip-AMP. The promotion of H_{K64A} on the hydrolysis of Lip-AMP complex is dose-dependent (Fig. 5a and 5b). Since no H_{apo} was added in these experiments, they represent a situation of reaction Step 1 under the influence of H_{K64A}. While the formation of Lip-AMP nearly finished after about 3 hours of assay, its hydrolysis continued as shown by the increased formation of AMP. After an assay time of 7 hours, the experiments with 10 μM and 100 μM initial H_{K64A} concentration produced 37% and 492% more AMP, respectively, compared to the control experiment.

The effect of H_{lip} on the kinetics of H_{lip} formation in the overall reaction was also studied. With the addition of 100 μM H_{lip} the formation rate of H_{lip} is increased from 0.066 to 0.234 μmol/min/mg (Fig. 4d). In a separate assay for reaction Step 1 (Fig. 6a), a much higher AMP formation rate was also observed under the influence of H_{lip}.

Taking the results together, it can be stated that the addition of various forms of H protein enhances the hydrolysis of Lip-AMP. It is noted that the hydrolysis is especially significant at low level of H_{apo}. As shown in Fig. 6b the amount of AMP formed at H_{apo} below 50 μM was much higher than H_{lip} formed. With the increase of $H_{apo}$to above 100 μM the ratio of AMP and H_{lip} is close to 1.0. This suggests that the hydrolysis of Lip-AMP competes with the lipoate transfer reaction.

3.3 A conceptual model of H protein lipoylation and competitive hydrolysis

Lipoylation of H protein plays a key role in the functionality of glycine cleavage system. Fujiwara *et al* . (Fujiwara et al., 2010) elaborated the crystal structures of LplA itself and its complexes with lipoate, Lip-AMP and octyl-AMP-H_{apo}, which contributed greatly to understanding the reaction mechanism of LplA-catalyzed H protein lipoylation as outlined in Fig.1. In general, the lipoylation process is considered to be composed of two steps: (1) the adenylation of lipoate catalyzed by LplA, forming thereby the complex Lip-AMP-LplA, and (2) the binding of H_{apo} to the complex, resulting in a transfer of the lipoate to H_{apo} which is subsequently released as H_{lip}. For the first time the kinetics of the overall lipoylation process and the two individual steps were studied in this work with the help of newly developed simple and efficient HPLC analytic methods to quantitatively determine the involved educts and products. The experimental results revealed unusual kinetic behavior of the overall and individual processes. Overall, no typical saturation kinetic behavior can be found with H_{apo} as a substrate of the lipoylation process (Fig.2). As summarized in Table 2, the specific rate of adenylation in the first step is over 420 times faster than that of lipoate transfer in the second step under the given experimental conditions. This leads to the accumulation of a high amount of the intermediate Lip-AMP during the lipoylation process *in vitro* . Furthermore, this study discovered two new phenomena of the H protein lipoylation process:

1. Whereas Lip-AMP itself is quite stable under the experimental conditions, the presence of protein H either in the forms of H_{apo}, H_{lip} or H_{K64A}can cause the hydrolysis of Lip-AMP bound to LplA. Enzymatic assays of the individual steps clearly showed that the hydrolysis proceeds in competition with lipoate transfer in the second step of the lipoylation process.
2. Both the rates of protein H lipoylation and Lip-AMP hydrolysis can be enhanced by H_{lip} and H_{K64A}. Obviously, H_{apo} acts not only as a substrate, but also as an activator for its lipoylation. For this reason, it was not possible to accurately determine the so-called k_m andv_{max} values of LplA towards H_{apo} as a substrate. In fact, the specific lipoylation rate of H_{apo} increased nearly linearly with H_{apo} concentration even up to 400 μM studied in this work.

We propose here an extended model of H protein lipoylation as presented in Fig.7 to account for these new experimental observations. We hypothesize that the presence of H protein can cause a conformational change of LplA (or LplA bound with Lip-AMP), which allows not only H_{apo} but also H_2O to attack the Lip-AMP complex easily, therefore, leading to an accelerated hydrolysis in accompany with lipoylation. As a result, the formation of AMP increased with the addition of various forms of H protein, and the production of H_{lip} linearly increased with increased concentration of H_{apo} and H_{lip}, because they can help to maintain the

easily attackable conformation of LplA, rather than causing a substrate inhibition or product inhibition.

When the mutant H protein H_{K64A} is present and the C-terminal domain of LplA is opened, no amine group could attack but only the hydroxyl group of water. Based on the structure of LplA, in the first step, the C-terminal domain of LplA adopts a binding conformation (Fig. 1) which can form a Lip-AMP intermediate bound to LplA. The intermediate Lip-AMP or H protein induces a LplA rearrangement from the β3 strand to the α2 helix, including a cleavage of the charge-charge interaction between the N-terminal domain (residue Arg47) and the C-terminal domain (residue Glu291). As a consequence, the free C-terminal domain rotates by 180°, adopting a stretched conformation with a "closed" C-terminal domain (Fujiwara et al., 1992). When the C-terminal domain is in the closed form, Lip-AMP cannot combine with LplA, further protecting Lip-AMP from the attack of H_2O, and yield less hydrolysis product AMP. And the open form of LplA will drive the amine group on the lysine residue of H_{apo} to attack the carbonyl group of Lip-AMP, yielding H_{lip}. At the same time, the hydroxyl group of H_2O would also attack the carbonyl group competitively, yielding the hydrolysis product of lipoic acid and AMP.

This hypothesis can explain our experimental results very well. First, few AMP was produced in Step 1, since no H protein was added in the reaction system and the C-terminal domain of LplA is closed. As a result, less H_2O could attack the Lip-AMP complex. Second, the hydrolysis by the attack of H_2O competes with the lipoylation by the attack of H_{apo}. When H_{apo} was increased from 1 μM to 50 μM, the ratio between AMP and H_{lip} amplified, since more H_2O could attack the carbonyl group of Lip-AMP rather than lipoylation of H_{apo}. However, with further increase of H_{apo} concentration, the attack from H_{apo}dominates the reaction, rather than the attack from H_2O. Thus, the ratio between AMP and H_{lip} returned to nearly 1, as H_{apo} increased from 50 μM to 200 μM (Fig. 6). The exact nature of the conformational change of LplA induced by protein H is not known at this stage. In view of the importance of protein lipoylation for several essential enzymes of cellular metabolism it is certainly worth further study, ideally with new experimental tools for studying protein interactions under dynamic conditions. It would be also interesting to examine if similar phenomena as reported here could be observed for relevant enzyme systems such as pyruvate dehydrogenase and 2-oxoglutarate complexes.

4. CONCLUSIONS

The two-step lipoylation process of H protein catalyzed by *E. coli* LplA has been kinetically studied as a whole and in individual steps for the first time in this work. The experimental results revealed a highly asymmetric kinetic behavior and hitherto unreported phenomena of H protein lipoylation: the first adenylation step is faster than the second lipoate transfer step by 2-3 orders of magnitude, leading to a high accumulation of the intermediate Lip-AMP under *in vitro*assay conditions. Unexpectedly, the second step was activated not only by the substrate H_{apo} but also by the product H_{lip} and the mutant H_{K64A} lacking the binding site of the lipoate. Moreover, a competitive Lip-AMP hydrolysis side-reaction was found which is also activated by the different forms of H-proteins. These new experimental observations suggest that there may be local conformational changes in the Lip-AMP binding pocket of LplA induced by the interactions and binding of H protein and its variants. Lip-AMP is then activated and attacked by either the amino group of the lysine residue of H protein or by the hydroxyl group of H_2O competitively. Structural and molecular dynamic studies of interactions of LplA and H protein could shed more light into H protein lipoylation, an essential process of several multiple enzyme systems with high impacts in biomedicine and biotechnology.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ABBREVIATIONS BCKDH, branched-chain alpha-ketoacid dehydrogenase complex; C1, one-carbon; GCS, glycine cleavage system; Hapo, apo-H protein; H_{K64A}, a mutant protein of Hapo with mutation of the key residue lysine64 into alanine; H_{lip}, lipoylated form of H protein; KDHC, 2-oxoglutarate dehydrogenase complex; Lip-AMP, lipoyl-AMP; LplA, lipoate-protein ligase A; PDHC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid;

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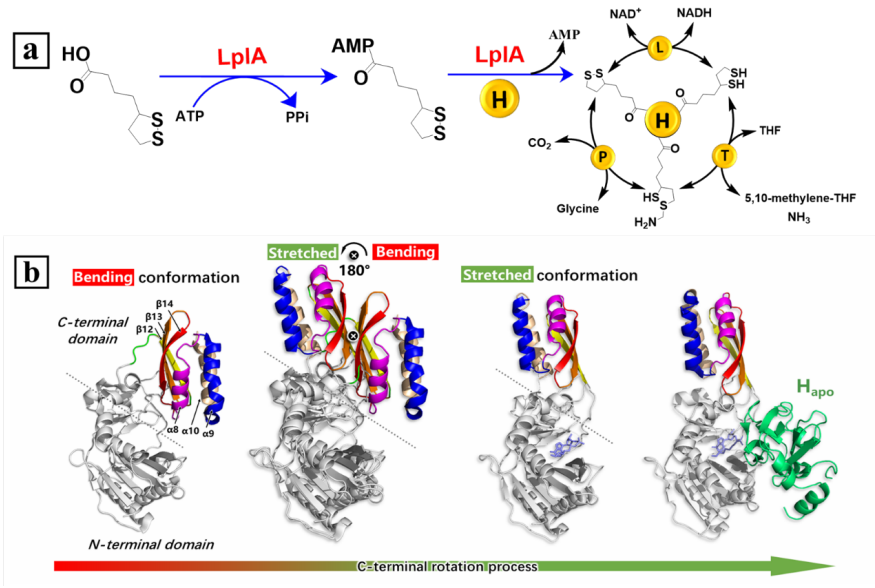

Figure 1 . (a) The lipoylation process of H protein in *E. coli* and the shuttling role of lipoylated H protein

in the glycine cleavage system. LplA catalyzes the lipoylation process in two steps: 1. Adenylation of lipoic acid, and 2. lipoate transfer to H protein(Morris et al. 1995). (b) Main conformational changes of LplA during the lipoylation process. LplA contains a N-terminal domain and a movable C-terminal domain. The latter rotates from a bending (closed) conformation to a stretched (open) conformation upon binding of lipoate and ATP and probably under the effect of H_{apo} (Fujiwara et al. 1991). Key secondary structures of the C-terminus are indicated by colors: β-12 in yellow, β-13 in orange, β-14 in red, α-8 in magenta, α-9 in blue, α-10 in beige.

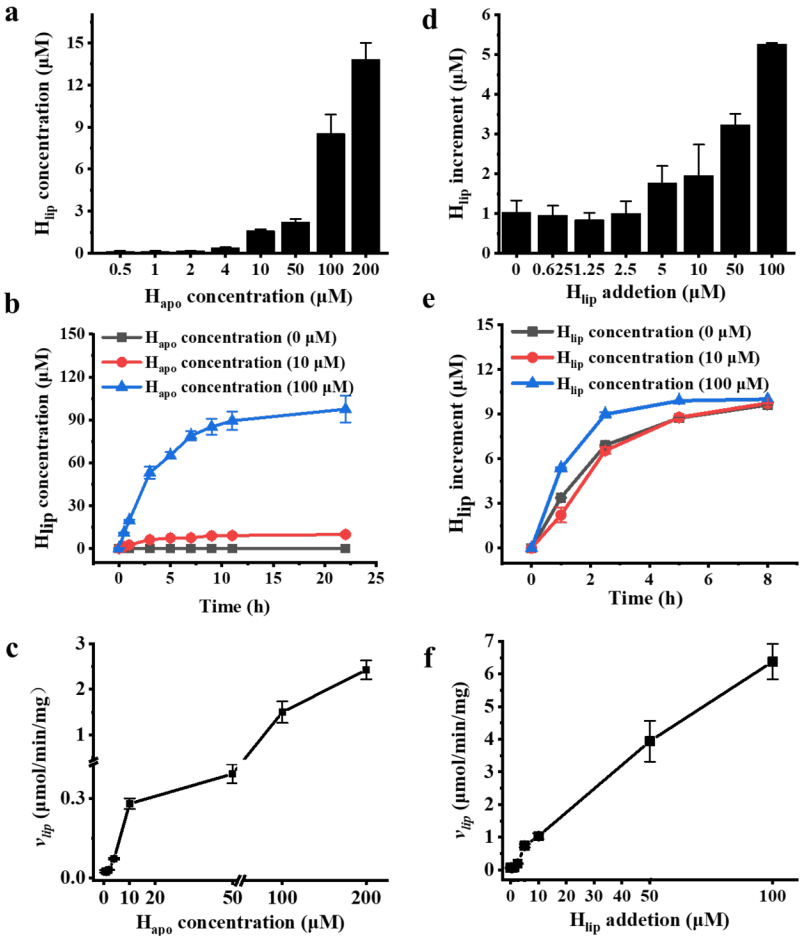

Figure 2. (a) Effect of H_{apo} concentration on the concentration of H_{lip} formed after a reaction time of 15 min; (b) Time course of H_{lip} formation at different concentrations of H_{apo}; (c) Relationship between the initial H_{apo} concentration and the specific lipoylation rate catalyzed by LplA; (d) Effect of H_{lip}concentration on its own production (increment) after a reaction time of 40 min; (e) Time course of H_{lip} formation (increment) at different concentrations of H_{lip} externally added; (f) Relationship between the initial H_{lip} concentration and the specific lipoylation rate catalyzed by LplA.

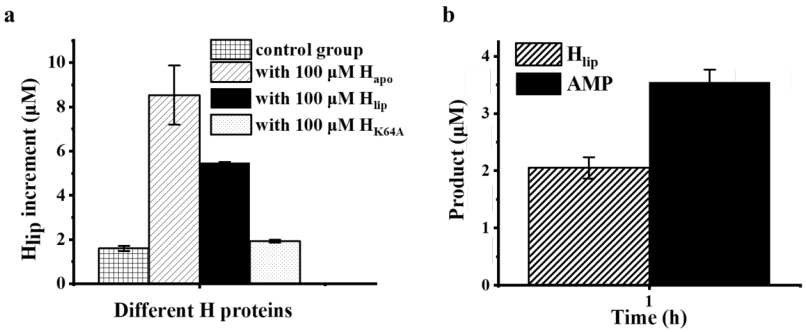

Figure 3. (a) Effects of H_{apo}, H_{lip} and H_{K64A} (all added at 100 μM) on the formation (or increment) of H_{lip} in the overall reaction of H protein lipoylation compared with the control assay with only 10 μM H_{apo}; (b) The concentrations of H_{lip} and AMP after a reaction time of 1 h in the control assay with 10 μM H_{apo}.

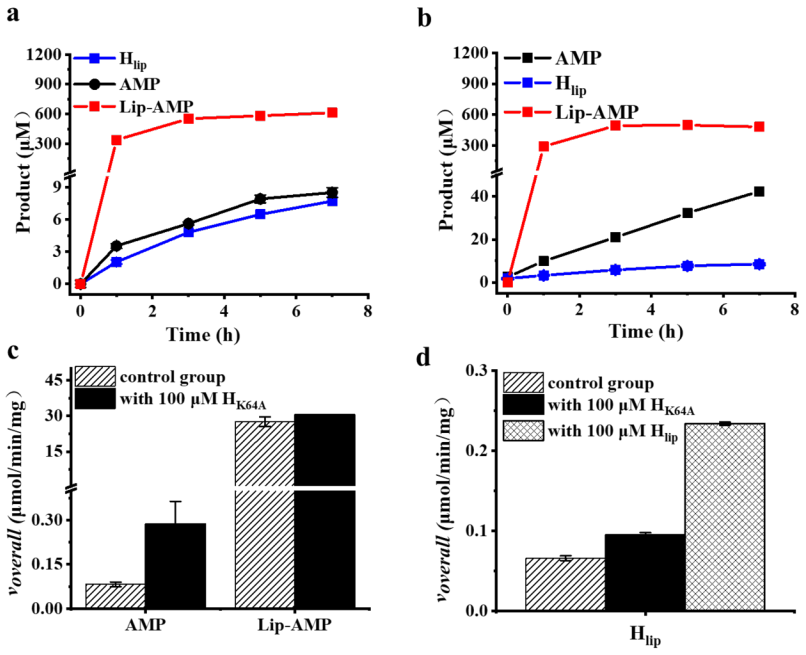

Figure 4. Time course of the formation and the specific formation rates of Lip-AMP, AMP and H_{lip} in the overall reaction: (a) in a normal assay with 10 μM H_{apo} without addition of H_{K64A}; (b) with the addition of 100 μM H_{K64A}. (c) the specific formation rates of Lip-AMP and AMP; (d) the specific formation rates of H_{lip}.

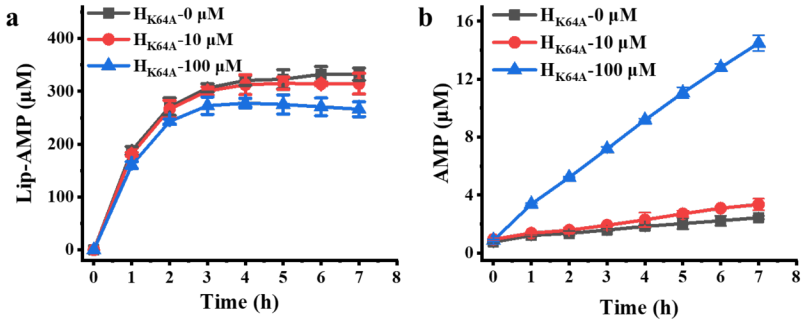

Figure 5. Time courses of Lip-AMP formation (a) and AMP formation (b) with H_{K64A}added at different concentrations.

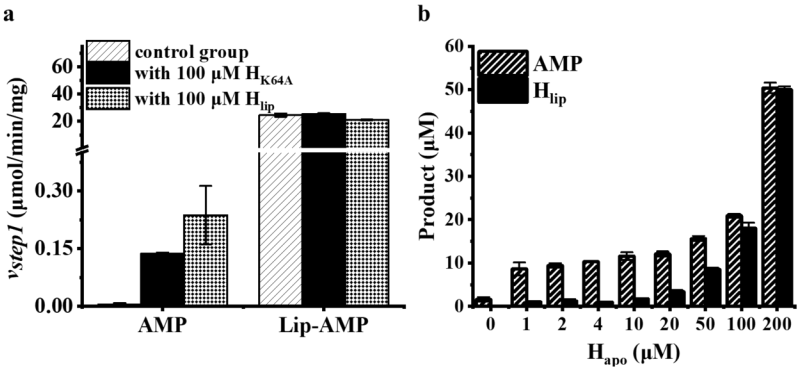

Figure 6. (a) The specific formation rates of AMP and Lip-AMP in the lipoate adenylation reaction (Step 1); (b) AMP and H_{lip} formations as function of the H_{apo} concentration determined for the lipoate transfer reaction step (Step 2).

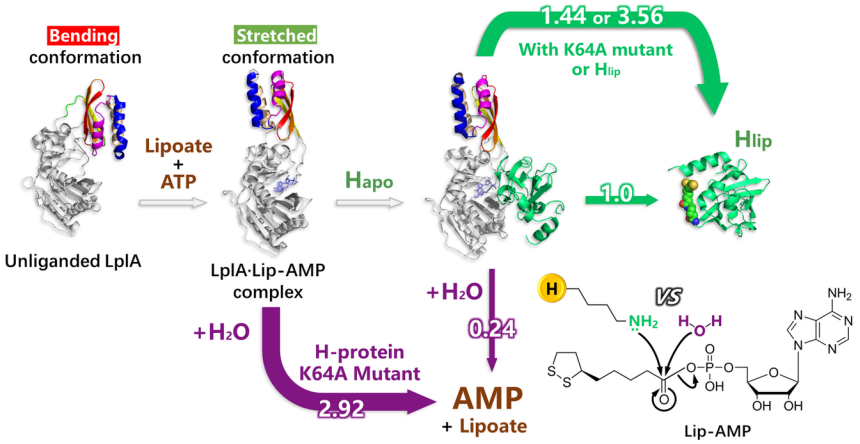

Figure 7. Extended reaction model of H protein lipoylation and its hydrolysis (modified from Fujiwara et al. (2010) (Fujiwara et al. 2010). Green arrow represents the generation of H_{lip} and purple arrow indicates

the hydrolysis of Lip-AMP. Numbers on the arrows represent the relative values of reaction rates obtained from assays for the overall reaction. The H_{lip} generation rate is set to 1.0. H_{apo} and water compete for the reaction with Lip-AMP and the black arrow represents electron transfer.

Table 1 . The forward and reverse primers used for the cloning of *ecH* , *ecLplA* and H_{k64A} genes into the pET28a$^+$ vector to obtain the plasmids pET28a-H, pET28a-LplA, and pET28a-H_{K64A}.

Primer	**Oligonucleotide Sequence**	**Restriction Site**	**NCBI NO.**
ecH-fwd	CATGCCATGGGCAGCAACGTACCAGCAGAACTGAAATAC	NcoI	WP_001295377.1
ecH-rev	CCGCTCGAGCTCGTCTTCTAACAATGCTTCGTATGC	XhoI	
ecLplA-fwd	CCATGGGCTCCACATTACGCCTGCTCATCTCT	NcoI	WP_000105885.1
ecLplA-rev	CTCGAGCTACCTTACAGCCCCCGCCAT	XhoI	
H_{K64A}-fwd	GCCGAATCGGTAGCAGCGGCGTCAGACATTTATG		
H_{K64A}-rev	GCTGCTACCGATTCGGCAACCGC		

Table 2 . Specific formation rates of H_{lip}, AMP and Lip-AMP in different assays. $v_{overall}$ represents values from assay of the overall lipoylation reaction (Fig.1); v_{Step1}gives rates from assay of the first reaction step without addition of H_{apo}; v_{Step2} gives rates from assay of the second reaction step with the use of Lip-AMP (236 μM) formed from the first reaction as substrate. The initial concentration of H_{apo} was 10 μM for the overall reaction and the Step 2 reaction.

Assy with 10 μM H_{apo} (null for v_{step1})	Specific rate (μmol/min/mg)	Lip-AMP	AMP	H_{lip}
	$v_{overall}$	28±2	0.082±0.008	0.066±0.003
	v_{step1}	25±1	0.005±0.003	-
	v_{step2}	-	0.124±0.005	0.100±0.021