Glycine Decarboxylase Is an Unusual Amino Acid Decarboxylase Involved in Tumorigenesis

ARTICLE *in* BIOCHEMISTRY · JANUARY 2014

Impact Factor: 3.02 · DOI: 10.1021/bi4014227 · Source: PubMed

4 AUTHORS, INCLUDING:

Maybelle Go
National University of Singapore
9 PUBLICATIONS **127** CITATIONS
SEE PROFILE

Wen Cai Zhang
Agency for Science, Technology and Research …
7 PUBLICATIONS **174** CITATIONS
SEE PROFILE

Bing Ru Lim
National University of Singapore
93 PUBLICATIONS **10,387** CITATIONS
SEE PROFILE

BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

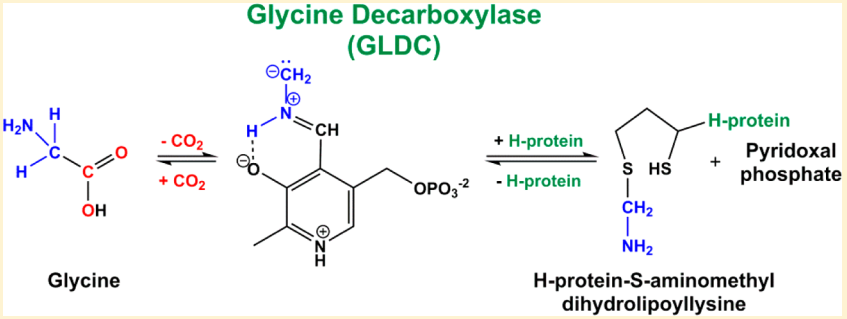
Glycine Decarboxylase Is an Unusual Amino Acid Decarboxylase Involved in Tumorigenesis

Maybelle Kho Go,[†] Wen Cai Zhang,[‡] Bing Lim,[‡] and Wen Shan Yew*,[†]

[†]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 8 Medical Drive, Singapore 117597

[‡]Genome Institute of Singapore, 60 Biopolis Street, Singapore 138672

Supporting Information



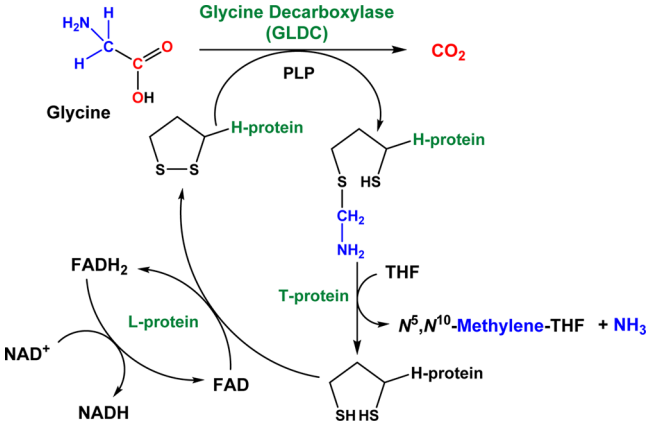
ABSTRACT: Glycine decarboxylase (GLDC) is a metabolic oncogene that links glycine metabolism with tumorigenesis. In humans, GLDC is part of a multienzyme complex (which includes the lipoyl-containing H-protein) that couples the decarboxylation of glycine to the biosynthesis of serine. Details of the GLDC-catalyzed glycine decarboxylation reaction are critical to drug development but remain elusive. This is the first report on the mechanism of the GLDC-catalyzed reaction and shows that GLDC is an unusual PLP-containing α-amino acid decarboxylase that removes carbon dioxide from the glycine substrate without releasing the expected amine (methylamine, a metabolic precursor of toxic formaldehyde) as a product. In an unusual decarboxylation mechanism, the resulting aminomethyl moiety is instead transferred to an accessory H-protein. This study defines the role of H-protein in GLDC-catalyzed glycine decarboxylation. (1) H-Protein is not required for glycine decarboxylation but, instead, is required for the release of the aminomethyl moiety from the quinonoid adduct. (2) Glycine decarboxylation is reversible and presumably proceeds through a stable quinonoid intermediate. (3) The physiological product of glycine decarboxylation is H-protein-*S*-aminomethyl dihydrolipoyllysine and not methylamine (in the absence of H-protein, the aminomethyl moiety remains as a quinonoid adduct). Mechanistic insights obtained from this study will inform future efforts for targeted anticancer therapeutic development.

Recent studies on cancer cell metabolism have highlighted the unexpected link between glycine metabolism and tumorigenesis.[1,2] In particular, glycine decarboxylase (GLDC) has been identified as a metabolic oncogene. Overexpression of GLDC in primary cells was sufficient to cause transformation. Overexpression of mutant inactive GLDC did not result in transformation, and knockdown of the GLDC gene in tumor cells led to a dramatic decrease in the incidence of mouse xenograft tumors. GLDC is a pyridoxal phosphate (PLP)-dependent α-amino acid decarboxylase;[3] in humans, it is part of a multienzyme complex [the glycine cleavage system,[4] together with H-protein, T-protein, and L-protein (Scheme 1)] in which it couples the decarboxylation of glycine to the biosynthesis of serine via adducts with PLP and lipoylated H-protein to form N^5,N^{10}-methylene-tetrahydrofolate (the substrate for serine hydroxymethyltransferase). Previous studies of GLDC have reported that the enzyme alone was almost inactive and required an accessory protein, known as the H-protein, to catalyze the decarboxylation reaction;[5,6] unlike those of typical amino acid decarboxylases, the GLDC-catalyzed decarboxylation reaction did not proceed readily, and the activity (or lack thereof) of GLDC was apparent only when it was "activated" by the accessory H-protein.

The inability of PLP and the holoenzyme GLDC to mediate glycine decarboxylation remains a mechanistic mystery. This is in contrast to prototypical amino acid decarboxylases, such as ornithine decarboxylase, a well-studied PLP-dependent α-amino acid decarboxylase.[7−9] In ornithine decarboxylase, the decarboxylation reaction readily proceeds through an electron sink provided by the PLP cofactor β to the carboxyl group, without the need for any accessory protein. In GLDC, the physiological product of glycine decarboxylation is proposed to be the covalent adduct of H-protein-*S*-aminomethyl dihydrolipoyllysine (H_{AM}, species **8** in Scheme 2);[10] however, there is

Received: October 18, 2013
Revised: January 26, 2014
Published: January 27, 2014

Scheme 1



no experimental evidence to corroborate the existence of H_{AM}. In addition, the question of whether the theoretical methylamine product of glycine decarboxylation is formed during catalysis remains (incidentally, methylamine is a primary amine whose oxidatively deaminated metabolite[11] is the toxic formaldehyde); in the absence of H-protein, methylamine could be released from the quinonoid intermediate (species **4** in Scheme 2, obtained upon glycine decarboxylation), regenerating the GLDC−PLP internal aldimine complex.

The mechanism of GLDC-catalyzed glycine decarboxylation is proposed as illustrated in Scheme 2. The reaction is initiated by the formation of an internal aldimine Schiff base between the active site lysine (Lys754 in human GLDC[12]) and PLP (species **1** in Scheme 2). A ternary complex is subsequently formed upon addition of glycine to the GLDC−PLP internal aldimine complex (species **2** in Scheme 2); the complex collapses by a series of proton transfers, forming the glycine−PLP external aldimine (species **3** in Scheme 2). Decarboxylation of the external aldimine releases CO_2 and yields a quinonoid intermediate (species **4** in Scheme 2); this quinonoid intermediate is stabilized by the delocalization of the negative charge across the PLP backbone.[13] In principle, the quinonoid intermediate can proceed in one of two ways. (1) A ternary complex can be formed between the quinonoid intermediate and GLDC (forming an aminomethyl−PLP−GLDC ternary complex), and protonation and subsequent hydrolysis of this complex will yield methylamine and the GLDC−PLP internal aldimine. (2) The quinonoid intermediate can nucleophilically attack the oxidized lipoic acid moiety of H-protein, forming an H-protein−*S*-aminomethyl−GLDC−PLP quaternary complex (species **6** in Scheme 2). Resolution of this complex regenerates the GLDC−PLP internal aldimine complex (species **7** in Scheme 2) and yields the second product of glycine decarboxylation, H_{AM} (species **8** in Scheme 2). Corroborating previous suggestions, we observed that the aminomethyl group is transferred to the lipoic acid moiety of H-protein to form H_{AM}, regenerating the GLDC−PLP internal aldimine complex. In addition, we found no evidence of the expected release of methylamine as the product, making GLDC an unusual amino acid decarboxylase; instead, in the absence of H-protein, PLP becomes a cosubstrate (rather than a cofactor) and glycine decarboxylation yields an aminomethyl−quinonoid "product". This study aims to understand the mechanistic contribution of H-protein to GLDC catalysis and gain insights into the mechanism of the GLDC-catalyzed reaction. Ultimately, the new mechanistic insights gained could inform rational approaches for targeted drug design against GLDC, providing a novel avenue for anticancer therapeutic development.

MATERIALS AND METHODS

^{13}C nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AVANCE 500 MHz NMR spectrometer. All reagents were the highest-quality grade commercially available.

Recombinant Expression and Purification of GLDC and H-Protein. The genes encoding GLDC and H-protein from *Homo sapiens* were chemically synthesized (DNA 2.0) and separately cloned into a pJexpress-416 vector with a C-terminal six-histidine tag. The genes were codon optimized for expression in *Escherichia coli* BL21(DE3) cells. Briefly, for recombinant GLDC expression in *E. coli* strain BL21(DE3), transformed cells were grown at 37 °C in LB broth (supplemented with 100 μg/mL ampicillin) to an OD_{600} of 0.6, and isopropyl D-thiogalactopyranoside (IPTG) (0.1 mM) was added to induce protein expression for 16 h at 25 °C. The cells were harvested by centrifugation, resuspended in binding buffer [5 mM imidazole, 0.1 mM PLP, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9)], and lysed by sonication. The lysate was cleared by centrifugation, and the protein was purified using a column of chelating Sepharose Fast Flow (GE Healthcare Bio-Sciences Corp.) charged with Ni^{2+} ion. The cell lysate was applied to the column in binding buffer, washed with buffer containing 154 mM imidazole, 0.1 mM PLP, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9), and eluted with 100 mM L-histidine, 0.1 mM PLP, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9). Fractions containing the protein were pooled and dialyzed against 20 mM Tris-HCl (pH 7.0), 0.1 mM PLP, and 100 mM NaCl. The protein was lyophilized after purification.

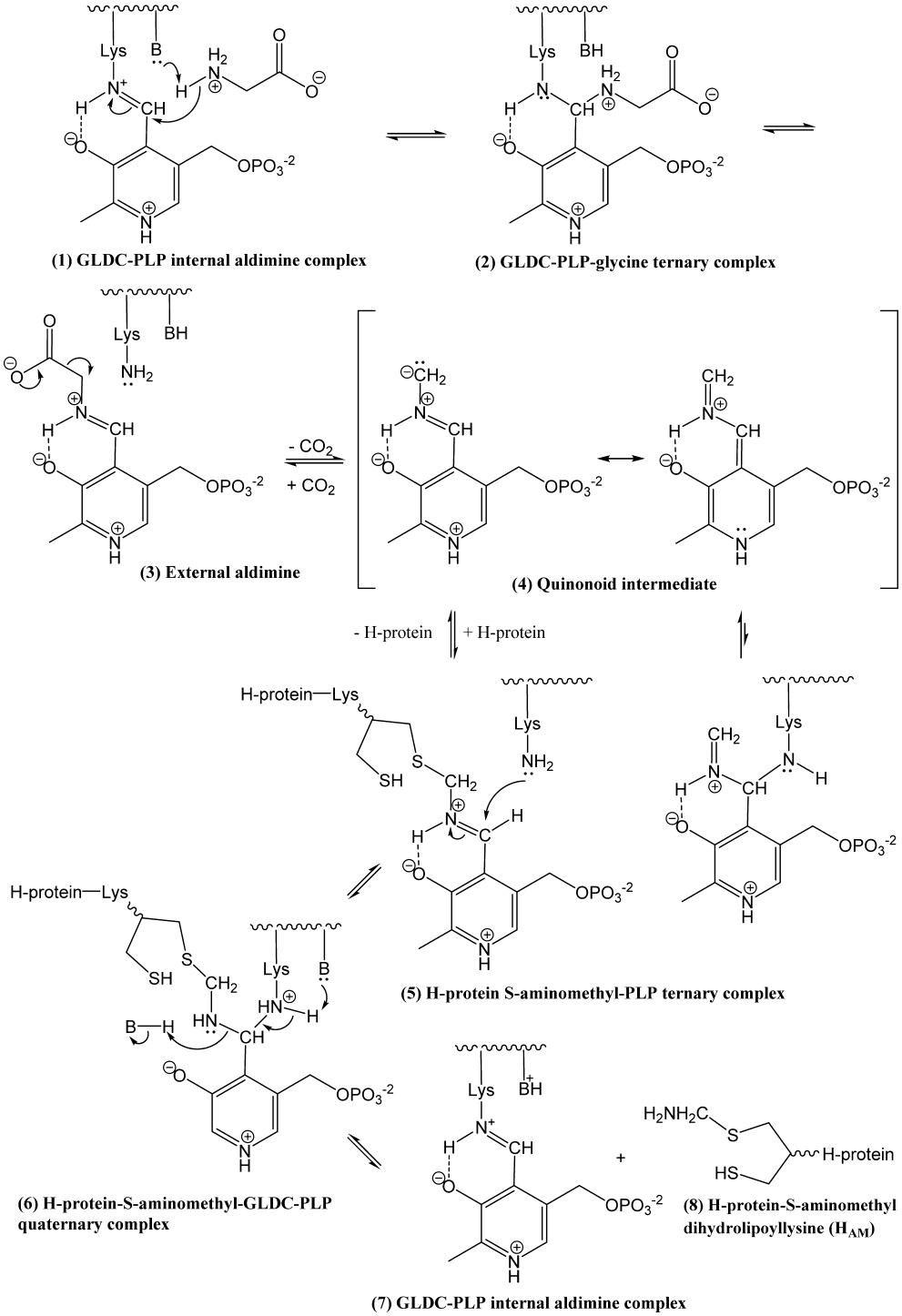
H-Protein was similarly expressed in *E. coli* strain BL21(DE3) and purified as described above with the following modifications. BL21(DE3) cells were grown and induced at 37 °C, and the buffers used for purification did not contain PLP.

Co-expression and Purification of GLDC and H-Protein. The genes encoding human GLDC and H-protein were subcloned from the respective pJexpress-416 vectors into a pRSFDuet-1 (Novagen) vector for co-expression. Briefly, the polymerase chain reaction (PCR) reaction mixture (100 μL) contained 1 ng of plasmid DNA, 10 μL of 10× *Pfx* amplification buffer, 1 mM $MgSO_4$, dNTPs (0.4 mM each), 40 pmol of each primer [for GLDC, forward primer (5′-GAGAGACATATGCAAAGCTGCGCACGCGCATGG-3′) and reverse primer (5′-CTCTCTGGTACCTTACTCGAGTTAATGGTGATGATG-3′); for H-protein, forward primer (5′-GAGAGACCATGGCAAGCGTACGTAAATTCACTGAG-3′) and reverse primer (5′-CTCTCTGGATCCTTACTCTTCGATGGACTTAATGTATTTC-3′)], and 5 units of Platinum *Pfx* polymerase. The gene was amplified using a PTC-0200G Thermal Cycler (Bio-Rad Laboratories), with the following parameters: 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 75 s, and 68 °C for 3 min, and a final extension of 68 °C for 10 min.

GLDC was first subcloned into the pRSFDuet-1 vector, followed by H-protein. GLDC was subcloned with a C-terminal six-histidine tag, while H-protein was subcloned without an affinity tag. The proteins were co-expressed in *E. coli* BL21(DE3). Transformed cells were grown at 25 °C in LB broth (supplemented with 100 μg/mL ampicillin) to an OD_{600} of 0.6, and IPTG (0.1 mM) was added to induce protein

dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947−956

Scheme 2



expression for 16 h. GLDC was purified from the harvested cells as previously described.

Cloning, Expression, and Purification of Phosphoenolpyruvate Carboxylase. The gene encoding phosphoenolpyruvate carboxylase (PEPCase) (GI: 32141095) was amplified via PCR from genomic DNA isolated from *Streptomyces coelicolor* A3(2) (ATCC) using Platinum *Pfx* DNA polymerase (Invitrogen). The PCR mixture (100 μL) contained 1 ng of plasmid DNA, 10 μL of 10× *Pfx* amplification buffer, 1 mM $MgSO_4$, dNTPs (0.4 mM each), 40 pmol of each primer [forward primer (5′-CAAGGAGCCGCACATATGAGCAGTGCCGACGACCAGACCACC-3′) and reverse primer (5′-GTACGGGGGGATCCTCAGCCGGTGTTGCGCAGGCCCGCTG-3′)], and 5 units of Platinum *Pfx* polymerase. The gene was amplified using a PTC-0200G Thermal Cycler (Bio-Rad Laboratories), with the following parameters: 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 75 s, and 68 °C for 3 min, and a final extension of 68 °C for 10 min. The amplified gene was cloned into a modified pET-15b vector (Novagen) in which the N-terminus contained 10 His residues (kindly provided by J. Gerlt, University of Illinois at Urbana-Champaign, Urbana,

dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947−956

IL).[14] The protein was expressed in *E. coli* BL21(DE3). Transformed cells were grown at 25 °C in LB broth (supplemented with 100 μg/mL ampicillin) to an OD_{600} of 0.6, and IPTG (0.1 mM) was added to induce protein expression for 16 h. PEPCase was purified from the harvested cells as previously described for H-protein.

Cloning, Expression, and Purification of Malate Dehydrogenase. The gene encoding malate dehydrogenase (MDH) (GI: 32141095) was amplified via PCR from genomic DNA isolated from *S. coelicolor* A3(2) (ATCC) using Platinum *Pfx* DNA polymerase (Invitrogen). The PCR mixture (100 μL) contained 1 ng of plasmid DNA, 10 μL of 10× *Pfx* amplification buffer, 1 mM $MgSO_4$, dNTPs (0.4 mM each), 40 pmol of each primer [forward primer (5′-CATACGGAGCATATGATGACTCGCACTCCCGTGAACGTCA-3′) and reverse primer (5′-GGGCGGACTCAGATGAGGCCGAGGGATCCCACCGCC-3′)], and 5 units of Platinum *Pfx* polymerase. The gene was amplified using a PTC-0200G Thermal Cycler (Bio-Rad Laboratories), with the following parameters: 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 75 s, and 68 °C for 3 min, and a final extension of 68 °C for 10 min. The amplified gene was cloned into a modified pET-15b vector (Novagen) in which the N-terminus contained 10 His residues, as previously described. The protein was expressed in *E. coli* BL21(DE3). Transformed cells were grown at 25 °C in LB broth (supplemented with 100 μg/mL ampicillin) to an OD_{600} of 0.6, and IPTG (0.1 mM) was added to induce protein expression for 16 h. MDH was purified from the harvested cells as previously described for H-protein.

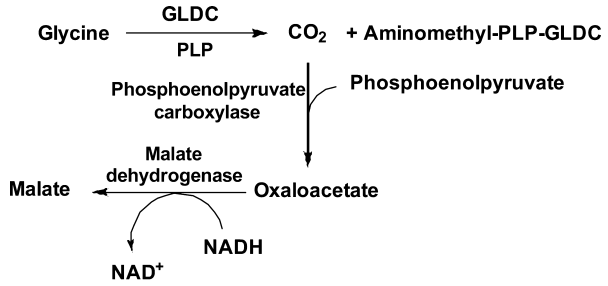
Cloning, Expression, and Purification of Lipoate-Protein Ligase A. The gene encoding lipoate-protein ligase A (LplA) (GI: 49175990) was amplified via PCR from genomic DNA isolated from *E. coli* K12 using Platinum *Pfx* DNA polymerase (Invitrogen). The PCR mixture (100 μL) contained 1 ng of plasmid DNA, 10 μL of 10× *Pfx* amplification buffer, 1 mM $MgSO_4$, dNTPs (0.4 mM each), 40 pmol of each primer [forward primer (5′-GGGCGGGTAAGGATCCCTACCTTACAGCCCCCGCCATC-3′) and reverse primer (5′-GAAATCGTTCATATGTCCACATTACGCCTGCTCATCTCTGAC-3′)], and 5 units of Platinum *Pfx* polymerase. The gene was amplified using a PTC-0200G Thermal Cycler (Bio-Rad Laboratories), with the following parameters: 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 75 s, and 68 °C for 3 min, and a final extension of 68 °C for 10 min. The amplified gene was cloned into a modified pET-15b vector (Novagen) in which the N-terminus contained 10 His residues, as previously described. The protein was expressed in *E. coli* BL21(DE3). Transformed cells were grown at 37 °C in LB broth (supplemented with 100 μg/mL ampicillin) to an OD_{600} of 0.6, and IPTG (0.1 mM) was added to induce protein expression for 16 h. LplA was purified from the harvested cells as previously described for H-protein.

Analysis of the GLDC−H-Protein Complex by Gel Filtration Chromatography. The amount of H-protein that associated with GLDC was determined by size-exclusion chromatography using a Bio-Rad Bio-Sil SEC 250-5 column (300 mm × 7.8 mm). The following samples were analyzed: (a) GLDC expressed in pJexpress-416 vector (GLDC-pJexp), (b) $GLDC_{coexp}$ expressed in pRSFDuet-1 vector (GLDC-pRSF), and (c) H-protein expressed in pJexpress-416 vector.

Lipoylation of H-Protein Catalyzed by Lipoyltransferase. Purified apo-H protein was lipoylated *in vitro* using lipoate-protein ligase A (LplA) from *E. coli* K12. The reaction mixture contained 100 mM Tris (pH 7.0), 12 mM adenosine 5′-triphosphate (ATP), 12 mM $MgCl_2$, 4.2 mM lipoic acid, 9 mM dithiothreitol (DTT), 0.30 mM LplA, and 0.35 mM H-protein. The mixture was incubated at 37 °C for 10 min while being shaken. The formation of lipoylated H-protein (H_{LA}) was verified by mass spectrometry (theoretical mass of H_{LA}, 14870.9 Da; observed mass of H_{LA}, 14870.4 Da).

Coupled Enzyme Assay for the GLDC-Catalyzed Glycine Decarboxylation Reaction. The kinetic parameters of GLDC were determined using a coupled enzyme continuous spectrophotometric assay. The rate of CO_2 formation was coupled to the oxidation of NADH measured at 340 nm (Scheme 3). Briefly, the assay (50 μL at 25 °C) contained 1 μM

Scheme 3



Monitor reaction rate *via* consumption of NADH

GLDC, 100 mM Tris-HCl buffer (pH 7.0), 20 mM $MgCl_2$, 0.3 mM PLP, 1.0 mM phosphoenolpyruvate, 0.3 mM NADH (340 nm; $\varepsilon = 6220\ M^{-1}\ cm^{-1}$), 2 units of PEPCase, 2 units of MDH, and 0.1 μM to 10 mM glycine substrate. To minimize the amount of contaminating atmospheric CO_2, enzymes were lyophilized and taken up in CO_2-free buffer (prepared by boiling water and cooling sealed aliquots of water under vacuum); stock solutions of the various reagents were also similarly prepared using CO_2-free water. Initial rates (ν_o) were corrected for the background rate of NADH oxidation in the absence of enzyme. Kinetic parameters were determined by fitting the initial rates to the Michaelis−Menten equation using Enzfitter (Biosoft).

Determining the Reversibility of Glycine Decarboxylation. ^{13}C NMR spectroscopy was used to determine the reversibility of glycine decarboxylation. Briefly, the reaction mixture (1 mL) contained 100 mM Tris (pH 7.0), 20 mM glycine, 20 mM $NaH^{13}CO_3$, and 3 μM GLDC. $NaH^{13}CO_3$ was used as the $^{13}CO_2$ source. The reaction mixture was incubated for 1 week at 25 °C, and ^{13}C NMR spectra were recorded. The acquisition time was 1 s, with a delay time of 2 s, and 400 spectra were recorded.

Determining the Products of Glycine Decarboxylation. Mass spectrometry and ^{13}C NMR spectroscopy were used to determine the products of glycine decarboxylation. The formation of H_{AM} was first assessed by mass spectrometry. The reaction mixture (1 mL) contained 100 mM Tris (pH 7.0), 20 mM glycine (unlabeled glycine or [2-^{13}C]glycine), 85 μM lipoylated H-protein (H_{LA}), and 3 μM GLDC. The reaction mixtures were incubated for 1 week at 25 °C, after which GLDC was removed by centrifugation through an Amicon (50000 Da) centrifugal filter device. The filtrate was desalted using a ZipTip-C4 (Millipore) before mass determination.

Formation of H_{AM} was also confirmed using ^{13}C NMR spectroscopy. Briefly, the reaction mixture (1 mL) contained

dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947−956

100 mM Tris (pH 7.0), 20 mM [2-^{13}C]glycine, and 3 μM GLDC. In the reaction with H-protein, 15 μM lipoylated H-protein (H_{LA}) was added to the reaction mixture. The reaction mixture was incubated for 1 week at 25 °C, and ^{13}C NMR spectra were recorded.

Determining the Reversibility of the Glycine Cleavage System. ^{13}C NMR spectroscopy was used to determine the reversibility of the glycine cleavage system. Briefly, the reaction mixture (1 mL) contained 100 mM Tris (pH 7.0), 10 mM $^{13}CH_3NH_2$, 10 mM $NaHCO_3$, and 3 μM GLDC. A parallel reaction mixture contained 10 mM CH_3NH_2 and 10 mM $NaH^{13}CO_3$. $NaHCO_3$ and $NaH^{13}CO_3$ were used as the CO_2 and $^{13}CO_2$ sources, respectively. The reaction mixtures were incubated for 1 week at 25 °C, and ^{13}C NMR spectra were recorded.

H_{AM} and carbon dioxide products were also used to probe the reversibility of the glycine cleavage system. H_{LA} was incubated with [2-^{13}C]glycine and GLDC to form [^{13}C]H_{AM}; purified [^{13}C]H_{AM} was subsequently incubated with $^{13}CO_2$ and GLDC to form [1,2-^{13}C]glycine. Briefly, the reaction mixture (1 mL) contained 100 mM Tris (pH 7.0), 20 mM [2-^{13}C]glycine, 85 μM H_{LA}, and 3 μM GLDC. The reaction mixture was incubated for 1 week at 25 °C, after which GLDC was removed by centrifugation through an Amicon (50000 Da) centrifugal filter device. The filtrate was then incubated with 50 mM $H^{13}CO_3^-$ and an additional 3 μM GLDC. The reaction mixture was incubated for 1 week at 25 °C, and ^{13}C NMR spectra were recorded.

RESULTS AND DISCUSSION

H-Protein Is Not Required for GLDC-Catalyzed Glycine Decarboxylation. We sought to understand the role of H-protein in the GLDC-catalyzed reaction. To rationalize the previously observed lack of activity of GLDC in the absence of H-protein, we wondered if H-protein is required for functional expression of GLDC because protein co-expression has been shown to be required for functional expression in multienzyme complexes.[15] GLDC was expressed either separately (without H-protein) or simultaneously with H-protein (here termed GLDC or $GLDC_{coexp}$, respectively) in the *E. coli* expression system and purified to homogeneity. There were no detectable differences in mass between GLDC and $GLDC_{coexp}$ as assessed by mass spectrometry (observed mass of GLDC, 112730 Da; observed mass of $GLDC_{coexp}$, 112730 Da). Size-exclusion chromatograms of purified GLDC, $GLDC_{coexp}$, and H-protein (Figure S1 if the Supporting Information) indicated that untagged H-protein is readily separated from six-histidine-tagged $GLDC_{coexp}$ during purification by affinity chromatography and suggested that $GLDC_{coexp}$ did not associate with H-protein; $GLDC_{coexp}$ was purified to homogeneity by affinity chromatography with a C-terminal six-histidine tag, while H-protein in the co-expression system was expressed without an affinity tag. Unlike GLDC orthologues in chicken and *Synechocystis*[16,17] where the enzyme exists as a homodimer, recombinant human GLDC is active in the monomeric form. Recombinant H-protein used for exogenous addition during decarboxylation assays (*vide infra*) was expressed in the *E. coli* host but was not lipoylated;[18] lipoylated H-protein (H_{LA}) was obtained through an *in vitro* lipoylation reaction using lipoate-protein ligase A[19] (LplA).

Release of CO_2 from glycine was assayed using a continuous coupled enzyme spectrophotometric assay, in which the production of CO_2 was coupled to the oxidation of NADH. Importantly, this assay was performed in the presence of excess PLP to facilitate the displacement of the quinonoid intermediate in the absence of an acceptor for the methylamine moiety (of decarboxylated glycine) to allow multiple enzyme turnovers. The kinetic parameters for GLDC-catalyzed glycine decarboxylation are listed in Table 1. GLDC expressed in the absence of H-protein exhibited barely detectable activity ($k_{cat} \leq 1.0 \times 10^{-4}$ s^{-1}); when lipoic acid was added to the reaction mixture, no significant increase in the rate of release of CO_2 was observed ($k_{cat} \leq 1.4 \times 10^{-4}$ s^{-1}), contrary to reports of lipoic acid-mediated activation of GLDC.[6] Upon addition of exogenous unlipoylated H-protein, k_{cat} increased 190-fold to 1.9×10^{-2} s^{-1}; in the absence of the lipoic acid moiety on H-protein, the methylamine adduct with PLP (aminomethyl–quinonoid intermediate) is released as the product of the GLDC-catalyzed decarboxylation of glycine. Despite the increase in the rate of release of CO_2 observed in the presence of unlipoylated H-protein, this rate still falls far short of rates typical for most PLP decarboxylases.[9,13] Surprisingly, addition of exogenous H_{LA} had the same effect on the rate of release of CO_2 as unlipoylated H-protein ($k_{cat} = 1.6 \times 10^{-2}$ s^{-1}), suggesting that either (1) the release of the aminomethyl–quinonoid intermediate is not rate-limiting or (2) GLDC is not expressed in an active form.

Table 1. Kinetic Parameters of Glycine Decarboxylation Catalyzed by GLDC

enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
GLDC	$\leq 1.0 \times 10^{-4}$		
GLDC and H-protein	$(1.9 \pm 0.03) \times 10^{-2}$	$(2.1 \pm 0.12) \times 10^{3}$	9.0
GLDC and H_{LA}	$(1.6 \pm 0.07) \times 10^{-2}$	$(1.3 \pm 0.29) \times 10^{3}$	12
GLDC and lipoic acid	$\leq 1.4 \times 10^{-4}$		
$GLDC_{coexp}$	$(3.1 \pm 0.10) \times 10^{-2}$	0.30 ± 0.08	1.0×10^{5}
$GLDC_{coexp}$ and H-protein	$(3.3 \pm 0.02) \times 10^{-2}$	0.37 ± 0.04	8.9×10^{4}
$GLDC_{coexp}$ and H_{LA}	$(4.7 \pm 0.20) \times 10^{-2}$	0.31 ± 0.10	1.5×10^{5}

In contrast, $GLDC_{coexp}$ exhibited significant activity for the release of CO_2 from glycine. The catalytic efficiency (k_{cat}/K_M) of $GLDC_{coexp}$-catalyzed glycine decarboxylation was 1.0×10^5 M^{-1} s^{-1}, an increase of >10000-fold compared to that of the GLDC-catalyzed reaction in the presence of exogenously added H-protein or H_{LA} (PLP is in excess in all kinetic assays). However, addition of unlipoylated H-protein or H_{LA} to the $GLDC_{coexp}$-containing reaction mixture did not significantly increase the rates of release of CO_2, suggesting that the role of H-protein in the GLDC-catalyzed reaction is not rate enhancement. Furthermore, we conclude that despite co-expression with H-protein, the robust decarboxylase activity observed is due solely to the $GLDC_{coexp}$ enzyme because we found no evidence of contaminating H-protein in the $GLDC_{coexp}$ preparation during sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (Figure S2 of the Supporting Information).

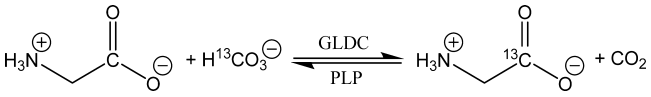
Because addition of H-protein or H_{LA} to $GLDC_{coexp}$ did not significantly change the observed rates of glycine decarboxylation, we speculate that H-protein functions as a molecular chaperone and/or activator during GLDC expression as well as

dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947–956

an acceptor for the methylamine moiety if the H-protein is lipoylated. This could account for the observed kinetic parameters for GLDC, where addition of exogenous H-protein [either unlipoylated or lipoylated (H_{LA})] slightly increased the rates of glycine decarboxylation because it suggests that the H-protein is able to facilitate the correct folding and/or activation of a fraction of the inactive GLDC, albeit much less efficiently than during co-expression. The exact nature of this enhancement of activity is being investigated. In addition, the fact that the presence of the lipoyl moiety on H-protein did not affect the rates of GLDC (or $GLDC_{coexp}$)-catalyzed glycine decarboxylation further corroborated the suggestion that H-protein (or H_{LA}) does not directly participate in and is not required for release of CO_2 from glycine in the formation of the PLP−quinonoid intermediate, an important distinction that is contrary to earlier findings. For the sake of clarity, $GLDC_{coexp}$ will hereafter be termed GLDC.

Exchange of C1 of Glycine: Probing the Stability of the Quinonoid Intermediate. Previous studies involving partially purified mitochondrial preparations from chicken and rat liver suggested that glycine decarboxylation is reversible.[3,6,20,21] If true, one would reason that the proposed quinonoid intermediate formed during glycine decarboxylation (species **4** in Scheme 2) would exist for a finite lifetime and have an associated stability to allow back incorporation of CO_2 to form the glycine substrate. To examine this, the mechanism of release of CO_2 was explored using ^{13}C NMR spectroscopy. We probed for the existence of the quinonoid intermediate and the reversibility of its formation from glycine by incubating GLDC with both unlabeled glycine and $NaH^{13}CO_3$ as an exogenous $^{13}CO_2$ source (Scheme 4). Figure 1 depicts the

Scheme 4



partial ^{13}C NMR spectrum of [1-^{13}C]glycine formed from the incubation of unlabeled glycine, $^{13}CO_2$, and GLDC and provides evidence of the exchange of C1 of glycine (reaction of the quinonoid intermediate with $^{13}CO_2$ to form glycine). The resonance for C1 is a singlet at 172.4 ppm; the resonance for $H^{13}CO_3^-$ is a singlet at 160.2 ppm. With the nonenzymatic control experiment (in which no resonance for C1 of glycine representing [1-^{13}C]glycine was observed), our observation provides evidence of the exchange of C1 of glycine vis-à-vis the quinonoid intermediate.

In contrast to previous reports about the reversibility of the GLDC-catalyzed glycine decarboxylation reaction in which both GLDC and H-protein were reportedly required for glycine decarboxylation, our study showed that recombinant GLDC alone was able to catalyze the release of CO_2 without "assistance" from H-protein. The incorporation of exogenous CO_2 requires the formation of the quinonoid intermediate that has a sufficient lifetime to allow incorporation of $^{13}CO_2$ to form [1-^{13}C]glycine aldimine (Scheme 5); subsequent hydrolysis of [1-^{13}C]glycine aldimine would form the observed [1-^{13}C]-glycine.

The observed reversibility for the release of CO_2 (vis-à-vis glycine decarboxylation) is unusual, as decarboxylation catalyzed by known PLP-dependent amino acid decarboxylases has been found to be irreversible; examples of irreversible decarboxylation catalyzed by PLP-dependent α-amino acid decarboxylases include ornithine decarboxylase from *Trypanosoma brucei*,[9] histidine decarboxylase from *Morganella morganii*,[22] tyrosine decarboxylase from *Streptococcus faecilis*,[23] and arginine decarboxylase,[24] glutamate decarboxylase,[25] lysine decarboxylase,[26] and diaminopimelate decarboxylase[27] from *E. coli*.

Determining the Products of Glycine Decarboxylation. With the demonstration of a quinonoid intermediate on the reaction coordinate, and the GLDC-catalyzed reaction in the absence of H-protein (H_{LA}), we investigated the identity of the products upon glycine decarboxylation. In the absence of H-protein (H_{LA}), one would reason that products of glycine decarboxylation are either (1) methylamine and carbon dioxide, after hydrolysis from PLP (a cofactor in this instance), or (2) the quinonoid intermediate (in the presence of excess PLP, a cosubstrate in this instance) and carbon dioxide (Scheme 6); the corollary extension would suggest H-protein-*S*-aminomethyl dihydrolipoyllysine (H_{AM}) and carbon dioxide would be the products formed in the presence of H-protein as part of the glycine cleavage system (Scheme 7). The formation of H_{AM} was assessed by mass spectrometric analysis; in the presence of H_{LA}, the GLDC-catalyzed glycine decarboxylation proceeded with the detection of H_{AM} (theoretical mass of H_{AM}, 14901.9 Da; observed mass of H_{AM}, 14901.6 Da). In the absence of H_{LA}, methylamine was not detected by mass spectrometry.

We investigated the possibility of methylamine formation as previously reported by using [2-^{13}C]glycine as the substrate to conduct ^{13}C NMR experiments for the detection of [^{13}C]-methylamine. However, we did not detect the formation of methylamine upon GLDC-catalyzed glycine decarboxylation in the presence or absence of H_{LA} (Figure 2 and Figure S3 of the Supporting Information, respectively). Importantly, unlike glycine decarboxylation in the absence of H_{LA} (Figure S3 of the Supporting Information), when [2-^{13}C]glycine was reacted with GLDC in the presence of H_{LA}, a new resonance peak was observed (Figure 2) with a chemical shift that could plausibly be attributed to [^{13}C]-H-protein-*S*-aminomethyl dihydrolipoyllysine ([^{13}C]H_{AM}, 45.0 ppm theoretical[28] vs 40.87 ppm observed). The presence of [^{13}C]H_{AM} was confirmed by mass spectrometry (theoretical mass of [^{13}C]H_{AM}, 14902.9 Da; observed mass of [^{13}C]H_{AM}, 14902.5 Da).

To exclude the possibility that the amount of methylamine formed was below the detection limit of ^{13}C NMR spectroscopy, and with the knowledge that the GLDC-catalyzed reaction is reversible, we attempted to synthesize [1-^{13}C]glycine using the products methylamine and $^{13}CO_2$ and [2-^{13}C]glycine using $^{13}CH_3NH_2$ and carbon dioxide, respectively (Figures S4 and S5 of the Supporting Information, respectively). In both instances, we did not detect the formation of glycine using methylamine and carbon dioxide as substrates. Taken together, these data strongly suggested that methylamine was not released as a product during the GLDC-catalyzed decarboxylation of glycine.

Reversibility of the Glycine Cleavage System. In an attempt to understand the energetics of glycine decarboxylation vis-à-vis product release, we prepared [^{13}C]H_{AM} by incubating H_{LA} with [2-^{13}C]glycine and GLDC (Scheme 8); purified [^{13}C]H_{AM} was then incubated with $^{13}CO_2$ and GLDC to form [1,2-^{13}C]glycine (Figures 3 and 4). The resonance associated with C1 of [1,2-^{13}C]glycine was represented as a doublet at 172.42 ppm [J_{CC} = 212 Hz (Figure 3)]; the corresponding resonance associated with C2 of [1,2-^{13}C]glycine was also

dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947−956

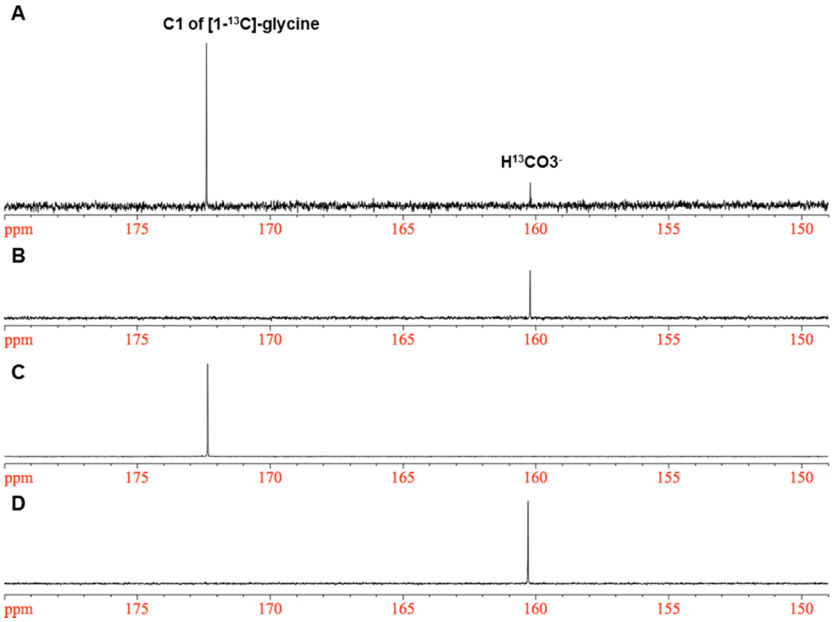
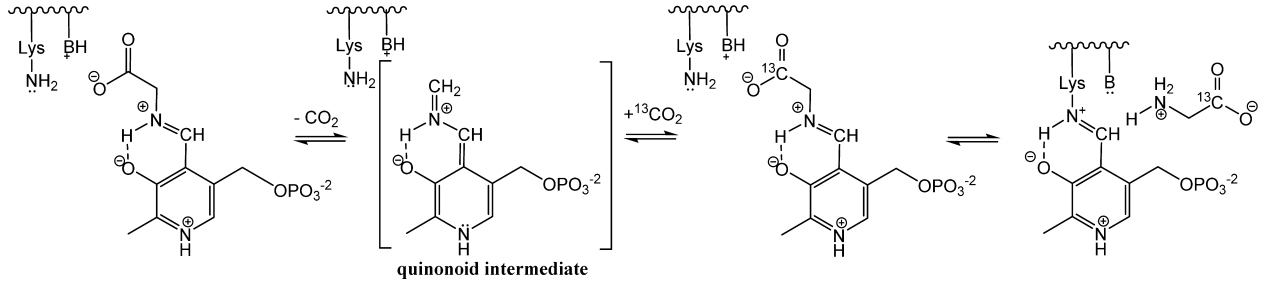
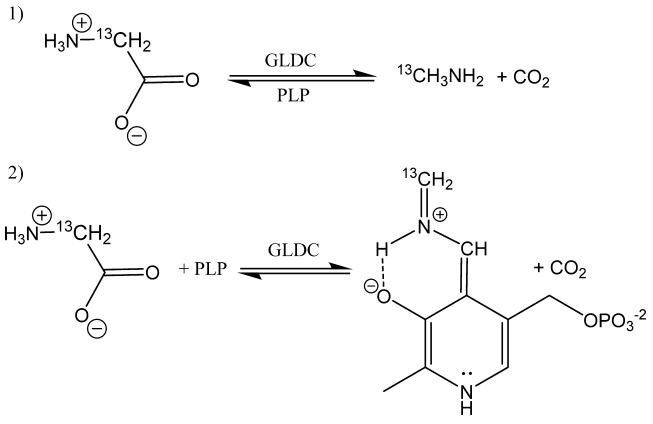


Figure 1. Partial ^{13}C NMR spectrum of [1-^{13}C]glycine formed by the incorporation of $^{13}CO_2$ into glycine. (A) The resonance for C1 of [1-^{13}C]glycine was observed as a singlet at 172.4 ppm; the resonance for $H^{13}CO_3^-$ was observed as a singlet at 160.2 ppm. (B) Nonenzymatic control, in which unlabeled glycine was incubated with $NaH^{13}CO_3$. (C) Partial ^{13}C NMR spectrum of 10 mM [1-^{13}C]glycine standard. (D) Partial ^{13}C NMR spectrum of 10 mM $NaH^{13}CO_3$ standard.

Scheme 5



Scheme 6

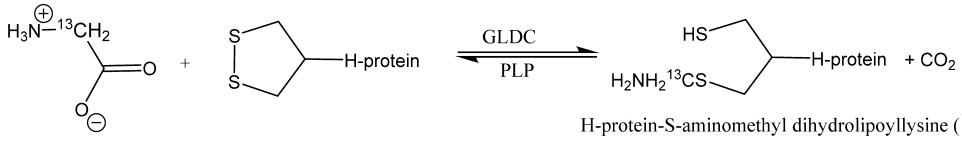


observed as a doublet at 41.45 ppm [J_{CC} = 212 Hz (Figure 4)]. After incubation for 1 week, the reaction mixture contained [1,2-^{13}C]glycine (substrate for the forward decarboxylation reaction) and [2-^{13}C]glycine (formed via incorporation of unlabeled CO_2 present), while the resonance associated with [^{13}C]H_{AM} (product for the forward decarboxylation reaction) was not detected, suggesting that the glycine cleavage system is reversible.

Summary Thoughts for Glycine Decarboxylation by Human GLDC. On the basis of this study, we propose the following for glycine decarboxylation by human GLDC.

(1) Release of CO_2 from glycine is the rate-determining step. The presence of lipoylated H-protein (H_{LA}) does not affect the rate of decarboxylation.

Scheme 7



H-protein-S-aminomethyl dihydrolipoyllysine (^{13}C-H_{AM})

dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947−956

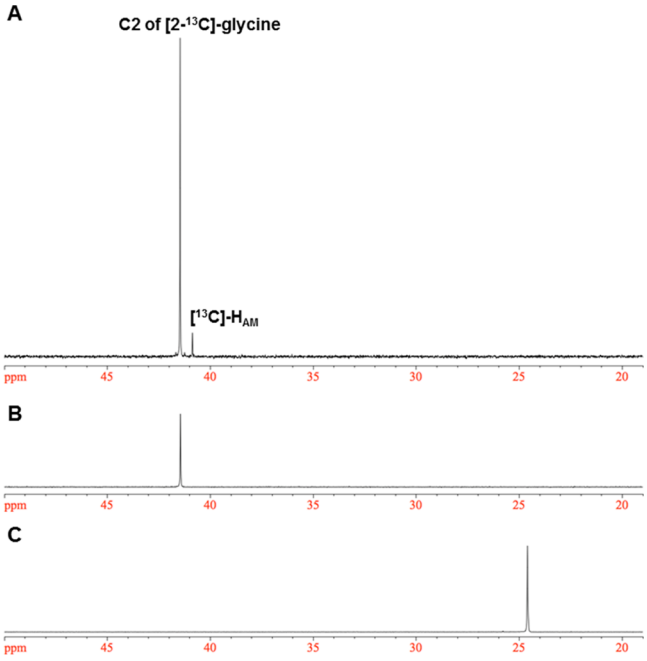


Figure 2. Partial ^{13}C NMR spectrum of [2-^{13}C]glycine incubated with GLDC and H_{LA}. (A) The resonance for C2 of glycine was observed as a singlet at 41.47 ppm. The resonance associated with [^{13}C]-H-protein-*S*-aminomethyl dihydrolipoyllysine ([^{13}C]H_{AM}) was observed as a singlet at 40.87 ppm. (B) Partial ^{13}C NMR spectrum of 10 mM [2-^{13}C]glycine standard. (C) Partial ^{13}C NMR spectrum of 10 mM $^{13}CH_3NH_2$ standard.

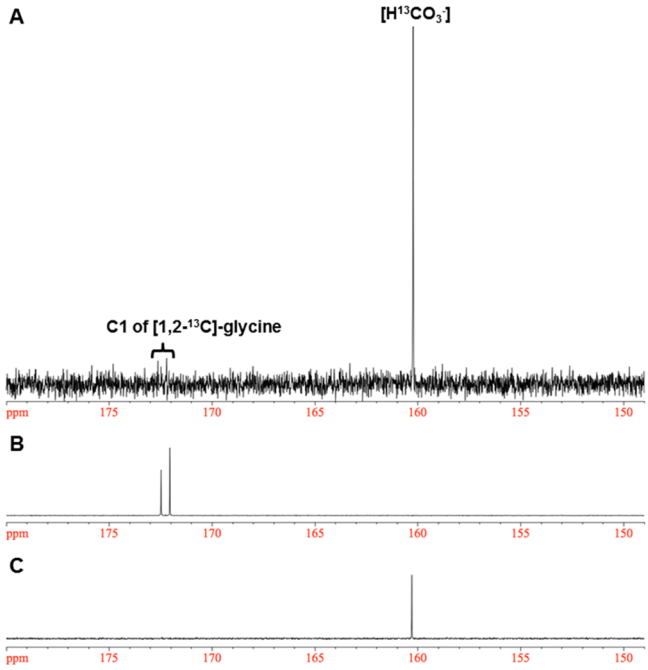


Figure 3. Partial ^{13}C NMR spectra showing the formation of [1,2-^{13}C]glycine from [^{13}C]H_{AM} and $^{13}CO_2$. (A) The resonance for C1 of [1,2-^{13}C]glycine was observed as a doublet at 172.42 ppm (J_{CC} = 212 Hz); the resonance for $H^{13}CO_3^-$ was observed as a singlet at 160.21 ppm (peak b). (B) Partial ^{13}C NMR spectrum for C1 of 10 mM [1,2-^{13}C]glycine standard. (C) Partial ^{13}C NMR spectrum of 10 mM $NaH^{13}CO_3$ standard.

(2) There is a lifetime associated with the quinonoid intermediate. In the absence of H_{LA}, CO_2 can react with the intermediate to form glycine.

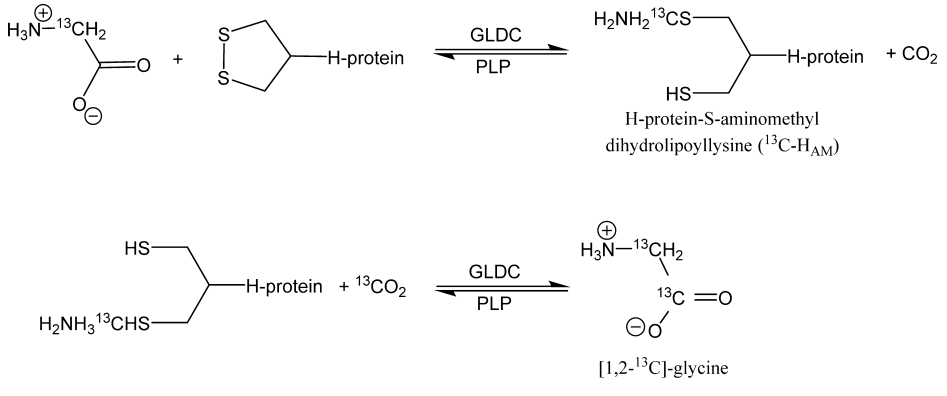
(3) Methylamine is not a product of GLDC-catalyzed glycine decarboxylation. It is possible that this is an evolutionary measure to prevent the release of methylamine to the cellular environment. Although methylamine itself is not toxic, it is oxidatively deaminated by semicarbazide-sensitive amine oxidases (SSAO) to the toxic formaldehyde.

(4) In the absence of H-protein, GLDC can release CO_2 from glycine to form the quinonoid intermediate; however, stoichiometric amounts of H_{LA} are required for the release of CO_2 from glycine when PLP is limiting (in catalytic amounts).

(5) In the presence of H_{LA}, the energy barrier for the release of the aminomethyl group from the quinonoid intermediate is lowered significantly. H_{LA} functions to "safely" channel the supposed methylamine "product" to T-protein (yielding N^5,N^{10}-methylene tetrahydrofolate) without releasing methylamine into the cellular environment.

Concluding Remarks. GLDC is an unusual PLP-dependent α-amino acid decarboxylase. It catalyzes glycine decarboxylation without releasing methylamine as a product; instead, the resulting aminomethyl moiety is transferred to lipoylated H-protein (H_{LA}) to form H-protein-*S*-aminomethyl dihydrolipoyllysine (H_{AM}). H-Protein (either lipoylated or unlipoylated) must be present during expression for the production of fully active GLDC; this study highlights the importance of co-expression of GLDC with H-protein for drug discovery and development. H_{LA} is not required for the release of CO_2 from glycine but, instead, is required for the transfer of the aminomethyl moiety from the quinonoid intermediate to form H_{AM}. The GLDC-catalyzed release of CO_2 from glycine

Scheme 8



dx.doi.org/10.1021/bi4014227 | *Biochemistry* 2014, 53, 947−956

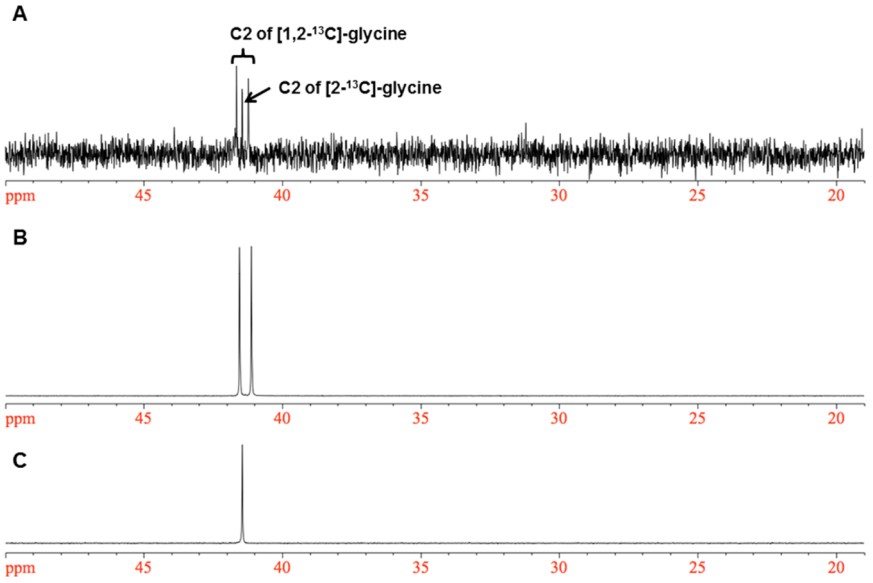


Figure 4. Partial ^{13}C NMR spectra showing the formation of [1,2-^{13}C]glycine from [^{13}C]H_{AM} and $^{13}CO_2$ and the formation of [2-^{13}C]glycine from [^{13}C]H_{AM} and CO_2. (A) The resonance for C2 of [1,2-^{13}C]glycine was observed as a doublet at 41.45 ppm (J_{CC} = 212 Hz); the resonance for C2 of [2-^{13}C]glycine (formed from CO_2 and [^{13}C]H_{AM}) was observed at 41.45 ppm. (B) Partial ^{13}C NMR spectrum for C2 of 10 mM [1,2-^{13}C]glycine standard. (C) Partial ^{13}C NMR spectrum for C2 of 10 mM [2-^{13}C]glycine standard.

(to form the quinonoid intermediate) is reversible. Contrary to reports, the physiological product of glycine decarboxylation is H_{AM} and not methylamine. A spectrophotometric coupled enzyme assay (for the GLDC-catalyzed reaction) that can be adapted for high-throughput inhibitor screens was established. We are currently using mechanistic insights from our study to inform rational approaches for targeted drug design against the oncogenic human GLDC.

■ ASSOCIATED CONTENT

Supporting Information

Size-exclusion chromatograms of purified GLDC, $GLDC_{coexp}$, and H-protein (Figure S1) and additional figures and acquired and measured NMR spectra (Figures S2−S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: 65-6516-8624. Fax: 65-6779-1453. E-mail: bchyws@nus.edu.sg.

Funding

This work was supported by grants from the National University Health System and the Academic Research Fund of the Ministry of Education, Singapore, to W.S.Y. and grants from the Biomedical Research Council of A*STAR to B.L.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Professor John Gerlt and Dr. Heidi Imker for insightful discussions.

■ ABBREVIATIONS

GLDC, glycine decarboxylase; PLP, pyridoxal phosphate; H_{AM}, H-protein-*S*-aminomethyl dihydrolipoyllysine; IPTG, isopropyl D-thiogalactopyranoside; PEPCase, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase; LplA, lipoate-protein ligase A; H_{LA}, lipoylated H-protein; SSAO, semicarbazide-sensitive amine oxidase.

■ REFERENCES

(1) Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A. L., Kafri, R., Kirschner, M. W., Clish, C. B., and Mootha, V. K. (2012) Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science 336*, 1040−1044.

(2) Zhang, W. C., Shyh-Chang, N., Yang, H., Rai, A., Umashankar, S., Ma, S., Soh, B. S., Sun, L. L., Tai, B. C., Nga, M. E., Bhakoo, K. K., Jayapal, S. R., Nichane, M., Yu, Q., Ahmed, D. A., Tan, C., Sing, W. P., Tam, J., Thirugananam, A., Noghabi, M. S., Huei Pang, Y., Ang, H. S., Mitchell, W., Robson, P., Kaldis, P., Soo, R. A., Swarup, S., Lim, E. H., and Lim, B. (2012) Glycine Decarboxylase Activity Drives Non-Small Cell Lung Cancer Tumor-Initiating Cells and Tumorigenesis. *Cell 148*, 259−272.

(3) Hiraga, K., Kochi, H., Motokawa, Y., and Kikuchi, G. (1972) Enzyme complex nature of the reversible glycine cleavage system of cock liver mitochondria. *J. Biochem. 72*, 1285−1289.

(4) Kikuchi, G., and Hiraga, K. (1982) The mitochondrial glycine cleavage system. Unique features of the glycine decarboxylation. *Mol. Cell. Biochem. 45*, 137−149.

(5) Hiraga, K., and Kikuchi, G. (1980) The mitochondrial glycine cleavage system. Functional association of glycine decarboxylase and aminomethyl carrier protein. *J. Biol. Chem. 255*, 11671−11676.

(6) Hiraga, K., and Kikuchi, G. (1980) The mitochondrial glycine cleavage system. Purification and properties of glycine decarboxylase from chicken liver mitochondria. *J. Biol. Chem. 255*, 11664−11670.

(7) Brooks, H. B., and Phillips, M. A. (1997) Characterization of the reaction mechanism for *Trypanosoma brucei* ornithine decarboxylase by multiwavelength stopped-flow spectroscopy. *Biochemistry 36*, 15147−15155.

(8) Jackson, L. K., Brooks, H. B., Myers, D. P., and Phillips, M. A. (2003) Ornithine decarboxylase promotes catalysis by binding the carboxylate in a buried pocket containing phenylalanine 397. *Biochemistry 42*, 2933−2940.

(9) Osterman, A. L., Brooks, H. B., Jackson, L., Abbott, J. J., and Phillips, M. A. (1999) Lysine-69 plays a key role in catalysis by ornithine decarboxylase through acceleration of the Schiff base formation, decarboxylation, and product release steps. *Biochemistry 38*, 11814−11826.

(10) Fujiwara, K., and Motokawa, Y. (1983) Mechanism of the glycine cleavage reaction. Steady state kinetic studies of the P-protein-catalyzed reaction. *J. Biol. Chem. 258*, 8156−8162.
(11) Yu, P. H., and Zuo, D. M. (1993) Oxidative deamination of methylamine by semicarbazide-sensitive amine oxidase leads to cytotoxic damage in endothelial cells. Possible consequences for diabetes. *Diabetes 42*, 594−603.
(12) Kume, A., Koyata, H., Sakakibara, T., Ishiguro, Y., Kure, S., and Hiraga, K. (1991) The glycine cleavage system. Molecular cloning of the chicken and human glycine decarboxylase cDNAs and some characteristics involved in the deduced protein structures. *J. Biol. Chem. 266*, 3323−3329.
(13) Eliot, A. C., and Kirsch, J. F. (2004) Pyridoxal phosphate enzymes: Mechanistic, structural, and evolutionary considerations. *Annu. Rev. Biochem. 73*, 383−415.
(14) Yew, W. S., and Gerlt, J. A. (2002) Utilization of L-ascorbate by *Escherichia coli* K-12: Assignments of functions to products of the yjf-sga and yia-sgb operons. *J. Bacteriol. 184*, 302−306.
(15) Kerrigan, J. J., Xie, Q., Ames, R. S., and Lu, Q. (2011) Production of protein complexes via co-expression. *Protein Expression Purif. 75*, 1−14.
(16) Hasse, D., Andersson, E., Carlsson, G., Masloboy, A., Hagemann, M., Bauwe, H., and Andersson, I. (2013) Structure of the homodimeric glycine decarboxylase P-protein from *Synechocystis* sp. PCC 6803 suggests a mechanism for redox regulation. *J. Biol. Chem. 288*, 35333−35345.
(17) Kikuchi, G., Hiraga, K., and Yoshida, T. (1980) Role of the glycine-cleavage system in glycine and serine metabolism in various organs. *Biochem. Soc. Trans. 8*, 504−506.
(18) Fujiwara, K., Okamura-Ikeda, K., and Motokawa, Y. (1992) Expression of mature bovine H-protein of the glycine cleavage system in *Escherichia coli* and in vitro lipoylation of the apoform. *J. Biol. Chem. 267*, 20011−20016.
(19) Green, D. E., Morris, T. W., Green, J., Cronan, J. E., Jr., and Guest, J. R. (1995) Purification and properties of the lipoate protein ligase of *Escherichia coli*. *Biochem. J. 309* (Part 3), 853−862.
(20) Motokawa, Y., and Kikuchi, G. (1971) Glycine metabolism in rat liver mitochondria: V. Intramitochondrial localization of the reversible glycine cleavage system and serine hydroxymethyltransferase. *Arch. Biochem. Biophys. 146*, 461−466.
(21) Motokawa, Y., and Kikuchi, G. (1975) Glycine metabolism by rat liver mitochondria: Reconstitution of the reversible glycine cleavage system with partially purified protein components. *Arch. Biochem. Biophys. 164*, 624−633.
(22) Vaaler, G., and Snell, E. (1989) Pyridoxal 5′-phosphate dependent histidine decarboxylase: Overproduction, purification, biosynthesis of soluble site-directed mutant proteins, and replacement of conserved residues. *Biochemistry 28*, 7306−7313.
(23) Orlacchio, A., Borri-Voltattorni, C., and Turano, C. (1980) The Binding of the Coenzyme Pyridoxal 5′-Phosphate and Analogues of the Substrate-Coenzyme Complex to Tyrosine Decarboxylase. *Biochem. J. 185*, 41−46.
(24) Wu, W., and Morris, D. (1973) Biosynthetic arginine decarboxylase from *Escherichia coli*. Purification and properties. *J. Biol. Chem. 248*, 1687−1695.
(25) Capitani, G., De Biase, D., Aurizi, C., Gut, H., Bossa, F., and Grütter, M. (2003) Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. *EMBO J. 22*, 4027−4037.
(26) Sabo, D., Boeker, E., Byers, B., Waron, H., and Fischer, E. (1974) Purification and physical properties of inducible *Escherichia coli* lysine decarboxylase. *Biochemistry 13*, 662−670.
(27) White, P. (1971) Diaminopimelate decarboxylase (*Escherichia coli*). *Methods Enzymol. 17*, 140−145.
(28) Shoulders, H., and Welch, S. C. (1987) A very brief, rapid, simple, and unified method for estimating carbon-13 NMR chemical shifts: The BS method. *J. Chem. Educ. 64*, 915.