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Roles of cysteine residues in the inhibition of human glutamate dehydrogenase by palmitoyl-CoA

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Human glutamate dehydrogenase isozymes (hGDH1 and hGDH2) have been known to be inhibited by palmitoyl-CoA with a high affinity. In this study, we have performed the cassette mutagenesis at six different Cys residues (Cys59, Cys93, Cys119, Cys201, Cys274, and Cys323) to identify palmitoyl-CoA binding sites within hGDH2. Four cysteine residues at positions of C59, C93, C201, or C274 may be involved, at least in part, in the inhibition of hGDH2 by palmitoyl-CoA. There was a biphasic relationship, depending on the levels of palmitoyl-CoA, between the binding of palmitoyl-CoA and the loss of enzyme activity during the inactivation process. The inhibition of hGDH2 by palmitoyl-CoA was not affected by the allosteric inhibitor GTP. Multiple mutagenesis studies on the hGDH2 are in progress to identify the amino acid residues fully responsible for the inhibition by palmitoyl-CoA. [BMB Reports 2012; 45(12): 707-712]

INTRODUCTION

Glutamate dehydrogenase (GDH) is found in all organisms and catalyzes the oxidative deamination of glutamate to 2-oxoglutarate. While this enzyme does not exhibit allosteric regulation in plants, bacteria, or fungi, its activity is tightly controlled by a number of compounds in mammals (1-3). Unique to the animal structures, there is a 48-residue "antenna" that protrudes above this NAD^+-binding domain (4-6). In the human, GDH exists in a housekeeping isozyme encoded by the *GLUD1* gene and a neural and testicular tissue-specific isozyme encoded by the *GLUD2* gene (7-9). Although the two GDH isozymes (hGDH1 and hGDH2) are highly homologous (showing a 97% amino acid identity), they differ markedly in their regulatory properties such as heat stability and allosteric regulation by ADP, L-leucine, and GTP (10-12).

Glutamate dehydrogenase is inhibited by palmitoyl-CoA and the inhibition is further enhanced by α-ketoglutarate and malate (13-15). Palmitoyl-CoA inhibition is the most primitive form of allosteric inhibition and appears to also be dependent upon other allosteric regulators (14, 16). For instance, allosteric modifiers such as ATP, GTP, and leucine decrease inhibition of glutamate dehydrogenase by palmitoyl-CoA (14, 16). Thus, the palmitoyl-CoA binding site may be apparently in the vicinity of the site of these allosteric modifiers (14). The site-directed mutagenesis at R463 residue, known to be involved in the binding of ADP, dramatically reduces ADP activation as well as palmitoyl-CoA inhibition (16). Kawaguchi & Bloch (13) found that palmitoyl-CoA converts liver glutamate dehydrogenase to enzymatically inactive dimeric subunits and that the inhibitor binds tightly to these subunits. Removal of the inhibitor from the palmitoyl-CoA-dimer complex fails to regenerate enzyme activity. In contrast, palmitoyl-CoA does not alter the quaternary structure of any of the malate dehydrogenases and binds only weakly to these enzymes (14).

Previous studies have reported that palmitoylated proteins have no clear consensus sequence for the palmitoylation and palmitate is transferred onto variably located cysteine residues of proteins, either enzymatically by a variety of enzymes known as protein fatty acyl transferases or spontaneously from palmitoyl-CoA (17-23). Cysteine residues are obvious target for modification of several reasons; their relative rarity and the availability of reasonably specific reagents provide an opportunity for unambiguous modification, and cysteine side-chains are frequently involved in enzyme catalysis (24). In the human glutamate dehydrogenase, there are six Cys residues at the positions of 59, 93, 119, 201, 274, and 323. However, the palmitoyl-CoA-modified residues of GDH have not been reported in

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any species. In the present study, we have performed the cassette mutagenesis at all Cys residues (Cys59, Cys93, Cys119, Cys201, Cys274, and Cys323) to identify palmitoyl-CoA binding sites within hGDH2.

RESULTS AND DISCUSSION

Construction and analysis of Cys mutants

Mammalian GDHs are inhibited by palmitoyl-CoA (13-18). Previous studies have showed that cysteine residues of proteins spontaneously can be palmitoylated by palmitoyl-CoA (17-23) and that cysteine residues may be present at the active site of the mammalian GDHs (24-26). Previously, we reported that chemical modification or site-directed mutagenesis of Cys323 residue causes a loss of hGDH activity (26) and that Cys119 played an important role in the regulation of hGDH isozymes by ADP-ribosylation (30). However, the palmitoyl-CoA-modified residues of GDH have not been identified in any species. In the present study, we performed the cassette mutagenesis at six different Cys residues (Cys59, Cys93, Cys119, Cys201, Cys274, and Cys323) to identify palmitoyl-CoA binding site within hGDH2. All six cysteine mutant proteins constructed in the present study were efficiently expressed in *E. coli* as soluble proteins (Fig. 1). Analysis of crude cell extracts by Western blotting showed that the plasmids encoding Ala substitution of the six Cys residues directed the synthesis of proteins that interacted with monoclonal antibodies against GDH at almost identical levels to the wild type hGDH2 (Fig. 1A). The mutant proteins could also be purified to homogeneity by the same method used to purify of wild type hGDH2 (Fig. 1B).

Palmitoyl-CoA inhibition of wild-type hGDH2 and Cys mutants

Incubation of the wild-type hGDH2 with increasing concentrations of palmitoyl-CoA resulted in a progressive decrease in enzyme activity (Fig. 2). Among the Cys mutants, the efficiency (k_{cat}/K_m) of the C323A mutant was only ~10% of that of the wild-type hGDH2 and no further inhibition studies by palmitoyl-CoA was performed with this mutant. Our results showed that the C119A mutant showed almost identical inhibitory pattern with the wild-type hGDH2 by palmitoyl-CoA, suggesting that Cya119 is not involved in the inhibition of hGDH2 by palmitoyl-CoA (Fig. 2). In contrast, the C59A, C93A, C201A, or C274A change reduced some, but not all, of the inhibitory properties of hGDH2 by palmitoyl-CoA (Fig. 2). These results suggest that C59, C93, C201, or C274 residue may be involved, at least in part, in the inhibition of hGDH2 by palmitoyl-CoA. However, it also seems likely that none of the residues at C59, C93, C201, or C274 in hGDH2 can be solely responsible for the binding of palmitoyl-CoA. Therefore, additional amino acid residues should be responsible for the inhibition of hGDH2 by palmitoyl-CoA.

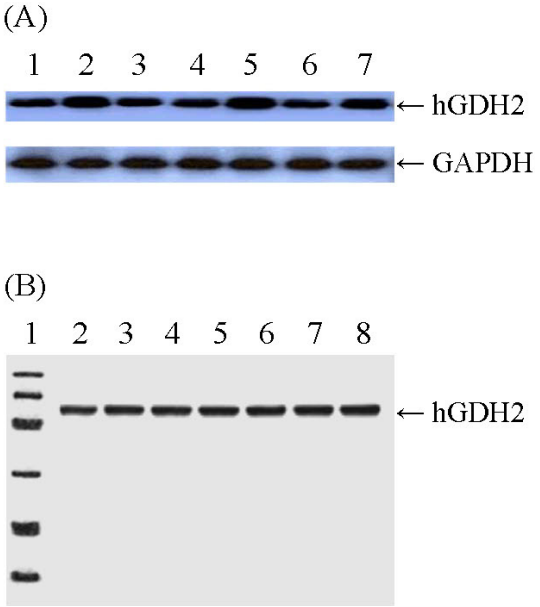

Fig. 1. Electrophoretic analysis of wild-type hGDH2 and Cys mutants. (A) Western blotting of wild-type hGDH2 and Cys mutants in crude extracts of *E. coli*. In each lane, 60 μg of protein from cell extracts were used. Lane 1, wild-type; lane 2, C59A; lane 3, C93A; lane 4, C119A; lane 5, C201A; lane 6, C274A; lane 7, C323A. (B) SDS-PAGE analysis of the purified wild-type hGDH2 and Cys mutants. Lane 1, low molecular weight marker proteins; lane 2, wild-type; lane 3, C59A; lane 4, C93A; lane 5, C119A; lane 6, C201A; lane 7, C274A; lane 8, C323A.

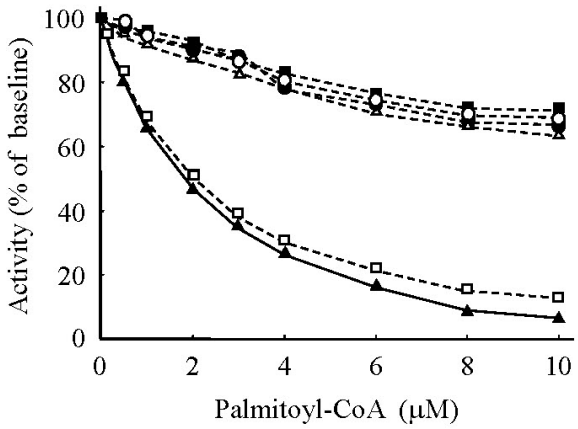

Fig. 2. Palmitoyl-CoA inhibition curves of wild-type hGDH2 and Cys mutants. Data points represent mean values from at least three experimental determinations and are expressed as percentage of baseline activity (no palmitoyl-CoA added). GDH activity was measured in the standard assay mixture in the presence of increasing concentrations of palmitoyl-CoA. Data points represent mean values from three experimental determinations and are expressed by a relative activity. Wild-type (▲-▲), C59A (■-■), C93A (Δ-Δ), C119A (□-□), C201A (○-○), and C274A (●-●).

Stoichiometry of palmitoyl-CoA inactivation for wild-type hGDH2 and Cys mutants

Correlation between palmitoyl-CoA binding and enzyme activity is shown in Fig. 3. There was a biphasic relationship, depending on the levels of palmitoyl-CoA, between palmitoyl-CoA binding and the loss of enzyme activity during the inactivation process. At lower levels, palmitoyl-CoA would linearly decrease hGDH2 activity, extrapolating to a stoichiometry of 1.2 mol of palmitoyl-CoA incorporation per mol of enzyme subunit. However, higher levels of palmitoyl-CoA did not produce additional linear inhibition of hGDH2 activity up to a stoichiometry of approximately 2 mol of palmitoyl-CoA binding per mol of enzyme subunit. The biphasic results may due to the formation of formation of micelles having a lower affinity for the enzyme at high concentration of palmitoyl-CoA as reported by other investigators (28). Further studies, therefore, are necessary to elucidate how many cysteine residues per molecule of enzyme subunit are required for the complete inactivation of hGDH2 by palmitoyl-CoA.

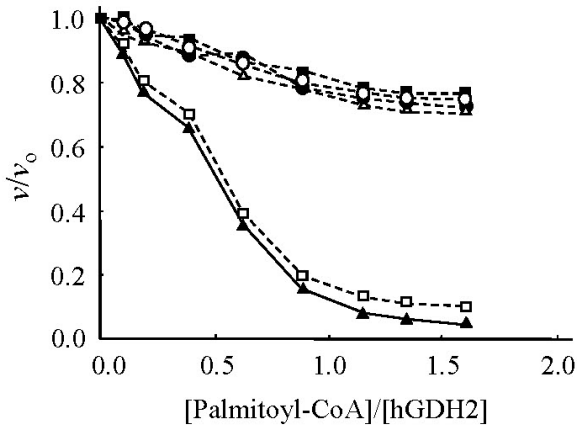

Fig. 3. Stoichiometry of palmitoyl-CoA inactivation for wild-type hGDH2 and Cys mutants. These results were obtained by incubating wild-type hGDH2 or Cys mutants with palmitoyl-CoA at various ratios for 3 min in the assay buffer at 25°C. After 3 min, aliquots were withdrawn and remaining activities were measured by adding the standard assay mixture. Data points represent mean values from three experimental determinations and are expressed by a plot of ratio of velocity in the presence of palmitoyl-CoA (v) to velocity in the absence of palmitoyl-CoA (v_o) versus molar ratio of palmitoyl-CoA to hGDH2. Wild-type (▲-▲), C59A (■-■), C93A (Δ-Δ), C119A (□-□), C201A (○-○), and C274A (●-●).

Effects of GTP on the inhibition of wild-type hGDH2 and Cys mutants by palmitoyl-CoA

In contrast to plants and fungi, animals perform α-oxidation of medium and long chain fatty acids mainly in the mitochondria, while the very long chain fatty acids are catabolized in the peroxisomes. Therefore, it has been proposed that the antenna evolved to link fatty acid and amino acid catabolism in the mitochondria. While we do not yet know the location of the palmitoyl-CoA-binding site, it is tempting to speculate that the binding of palmitoyl-CoA might be related to the antenna domain and inhibited by affecting the conformational changes associated with the opening and closing of the catalytic cleft (4, 5). According to the previous reports, allosteric modifirers such as ATP, GTP, and leucine decrease inhibition of bovine GDH by palmitoyl-CoA (14). Thus, the palmitoyl-CoA binding site might be apparently in the vicinity of the site of these allosteric modifiers. In this study, we further investigated the effects of GTP on the inhibition of the Cys mutants by palmitoyl-CoA. The data in Table 1 shows that inhibition of hGDH2 by palmitoyl-CoA was not affected by the allosteric inhibitor GTP. K_3 value, the affinity for palmitoyl-CoA binding, was not changed by the presence of GTP for both wild-type hGDH2 and Cys mutants (Table 1), indicating that inhibition by palmitoyl-CoA was complete in the absence or presence of GTP. These results are contrast to those obtained from bovine GDH, where GTP reduced inhibition of bovine GDH by palmitoyl-CoA (14). These discrepancies are not clearly explained, but probably may be due to the different sensitivity to GTP inhibition between GDH isotypes (2, 3, 10-12).

Palmitoyl-CoA is the acyl donor for protein palmitoylation and inhibits several enzymes including bovine liver glutamate dehydrogenase (29) and bovine liver methylmalonyl semialdehyde dehydrogenase (14). The results presented here show that palmitoyl-CoA inhibits hGDH2 with a high affinity suggesting that hGDH2 may be vulnerable to inhibition by palmitoyl-CoA at

Table 1. Effects of GTP on the inhibition of wild-type hGDH2 and Cys mutants by palmitoyl-CoA. K_3 values were determined by non-regression analysis of as described in Materials and Methods. The palmitoyl-CoA concentration was constant (5 μM) while the GTP concentration was held varied as indicated. The data represent mean values for three independent experiments

GTP (μM)	K_3 (μM)					
	WT	*C59A*	*C93A*	*C119A*	*C201A*	*C274A*
0.0	1.82	2.15	2.60	1.98	2.47	2.31
0.4	1.77	2.09	2.57	2.00	2.42	2.30
0.8	1.84	2.10	2.62	2.04	2.40	2.33
1.2	1.80	2.11	2.61	2.08	2.41	2.31
1.6	1.80	2.20	2.59	1.99	2.50	2.36
2.0	1.79	2.09	2.62	1.99	2.49	2.29

physiological condition. The cysteine residues at positions of C59, C93, C201, or C274 may be involved, at least in part, in the inhibition of hGDH2 by palmitoyl-CoA. The sulphydryl group of a cysteine has an ionization constant p*KA* of 8.5, which makes the formation of a thiolate under the cytosolic conditions of the cell (pH 7.2-7.4) unlikely. However, in the context of a peptide or a protein, proximal polar or charged side chains can notably modulate the pKa of a cysteine and, therefore, its potential to form a thiolate. Indeed, the pKa of a cysteine can be reduced by as much as six orders of magnitude to a value of 3 (30), which makes the formation of thiolates highly likely. The dependence of the pKa on the position of a cysteine in a protein could also explain the peculiar observation that autoacylation targets the same cysteines that are acylated *in vivo* (31, 32).

However, we still do not exactly know the binding site(s) of palmitoyl-CoA for the inhibition of hGDH2. Multiple mutagenesis studies on the hGDH2 are in progress in our laboratory to identify the amino acid residues fully responsible for the regulatory properties by palmitoyl-CoA. The fact that GDH is involved in amino acid catabolism, is fatty-acylated (33), and inhibited by palmitoyl-CoA shows the importance of GDH as a metabolic cross-talk between amino acid and fatty acid catabolic pathways.

MATERIALS AND METHODS

Materials

NADH, 2-oxoglutarate, ADP, and isopropyl b-D-thiogalactopyranoside (IPTG) were purchased from Sigma Chemical Co. Human GDH2 genes (pHGDH2) have been chemically synthesized and expressed in *E. coli* as soluble proteins in our laboratory as described elsewhere (34). ADP-Sepharose, and Resource-Q were purchased from Amersham Pharmacia Biotech. Restriction enzymes were purchased from New England Biolabs. Pre-stained marker proteins for Western blot were purchased from NOVEX. Low molecular weight protein marker for SDS/PAGE was purchased from Bio-Rad. All other chemicals and solvents were reagent grade or better.

Bacterial strains

Escherichia coli DH5a was purchased from Invitrogen and used as the host strain for plasmid-mediated transformations for cassette mutagenesis. *E. coli* PA340 (thr-1 fhuA2 leuB6 lacY1 supE44 gal-6 gdh-1 hisG1 rfbD1 galP63 D(gltB-F)500 rpsL19 malT1 xyl-7 mtl-2 argH1 thi-1; kindly provided by Dr. Mary K.B. Berlyn, *E. coli* Genetic Stock Center, Yale University) lacked both GDH and glutamate synthase activities (35) and was used to test plasmids for GDH activity. *E. coli* BL21 (DE3) (36) was used for high level expression of the recombinant GDH.

Construction of mutants

To make hGDH2 mutant proteins (C59A, C93A, C119A, C201A, C274A, and C323A), amino acid substitutions at six different Cys sites were constructed by cassette mutagenesis of synthetic hGDH2 gene (pHGDH2) as described elsewhere (15, 26, 34). Plasmid DNA was digested with restriction enzymes to remove the flanking fragment that encodes target amino acid. The flanking fragment was replaced with synthetic DNA duplex containing a substitution on both DNA strand at six different Cys sites. Mutagenic oligonucleotides were annealed, ligated, and transformed into DH5α and resultant mutant plasmids were identified by DNA sequencing using plasmid DNA as a template.

Expression and purification of Cys mutant proteins

Fresh overnight cultures of DE3/pHGDH2 mutant was used to inoculate 1 L of LB containing 100 μg of ampicillin per ml. DE3/pHGDH2 mutant was grown at 37°C until the A_{600} reached 1.0 and then IPTG was added to a final concentration of 1 mM. After IPTG induction, DE3/pHGDH2 mutant was grown for an additional 3 h at 37°C and then harvested by centrifugation. Cell pellets were suspended in 100 ml of 100 mM Tris-HCl, pH 7.4/1 mM EDTA/5 mM dithiothreitol and lysed with a sonicator. Cellular debris was removed by centrifugation and the mutant proteins were purified by ADP-Sepharose column followed by FPLC Resource-Q column as described elsewhere (15, 26, 34). The purified mutant proteins were analyzed by SDS-PAGE and the western blot analysis as reported elsewhere (37, 38).

Inhibition of hGDH2 by palmitoyl-CoA

Inhibition studies were determined with the purified proteins unless otherwise indicated. GDH activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 nm as described before (34). All assays were performed in triplicate and initial velocity data were related to a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, and 2.6 mM EDTA at 25°C. One unit of enzyme is defined as the amount required to oxidize 1 μmol of NADH per min at 25°C.

Effects of mutagenesis on different palmitoyl-CoA inhibitions of hGDH2 were examined by incubating the wild type and mutant enzymes with palmitoyl-CoA at various concentrations in the assay buffer at 25°C. At intervals after the initiation with palmitoyl-CoA, aliquots were withdrawn for the assay of hGDH activity.

The K_3 value was determined with the following equation described by Shemisa and Fahien (39), which is appropriate for both full and partial inhibitors:

$$1/(V_1 - v) = 1/(V_1 - V_2) + K_3/(V_1 - V_2)[M]$$

where V_1 is the velocity in the absence of effector, v is the velocity in the presence of effector, V_2 is the velocity in the presence of saturating concentrations of effector, [M] is the concentration of effector, and K_3 is the concentration of effector necessary to obtain $V = (V_1 + V_2)/2$. Data were analyzed by fitting the $(V_1 - v)$ vs [M] curves by non-linear regression to obtain K_3.

Statistical analysis

The significance of the effect of palmitoyl-CoA on the activities

of hGDH2 was analyzed by an unpaired two-tailed *t* test. Unless otherwise mentioned, each experimental point represents the mean of triplicate determinations from different preparations. At some points, error bars were omitted in the figures for the purpose of clarity.

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