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Histidine 454 plays an important role in polymerization of human glutamate dehydrogenase

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Abstract **Although previous chemical modification studies have suggested several residues to be involved in the maintenance of the quaternary structure of glutamate dehydrogenase (GDH), there are conflicting views for the polymerization process and no clear evidence has been reported yet. In the present study, cassette mutagenesis at seven putative positions (Lys333, Lys337, Lys344, Lys346, Ser445, Gly446, and His454) was performed using a synthetic human GDH gene to examine the polymerization process. Of the mutations at the seven different sites, only the mutagenesis at His454 results in depolymerization of the hexameric GDH into active trimers as determined by HPLC gel filtration analysis and native gradient polyacrylamide gel electrophoresis. The mutagenesis at His454 has no effects on expression or stability of the protein. The K_M values for NADH and 2-oxoglutarate were 1.5-fold and 2.5-fold greater, respectively, for the mutant GDH than for wild-type GDH, indicating that substitution at position 454 had appreciable effects on the affinity of the enzyme for both NADH and 2-oxoglutarate. The V_{max} values were similar for wild-type and mutant GDH. The k_{cat}/K_M value of the mutant GDH was reduced up to 2.8-fold. The decreased efficiency of the mutant, therefore, results from the increase in K_M values for NADH and 2-oxoglutarate. The results with cassette mutagenesis and HPLC gel filtration analysis suggest that His454 is involved in the polymerization process of human GDH.**

Key words: Glutamate dehydrogenase; Polymerization; Quaternary structure; Reactive histidine

1. Introduction

Glutamate dehydrogenase (GDH; EC 1.4.1.3) catalyzes the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+ or $NADP^+$ as coenzyme [1]. GDH falls into two oligomeric classes. The bacterial and fungal $NADP^+$-linked and vertebrate dual specificity GDHs have six identical subunits, with a subunit size between 48 kDa (bacterial, e.g. *Escherichia coli*) [2] and 55–57 kDa (vertebrate) [1,3], whereas the NAD^+-linked enzymes have either six identical subunits of around 48 kDa (e.g. *Clostridium symbiosum*) [4], or four identical subunits of 115 kDa (e.g. *Neurospora crassa*) [5].

The largest difference between mammalian and bacterial GDH is a long antenna domain formed by the 48 amino acid insertion starting at residue 395 and there is little identity between the 100 residues in the C-terminus [6]. In contrast to the extensive allosteric regulations such as activation by ADP and inhibition by GTP observed in mammalian GDH, bacterial forms of GDH are relatively unregulated [1,7–9]. The recently elucidated atomic structure of bovine liver GDH has suggested that the allosteric regulation and negative cooperativity observed in mammalian GDH may be facilitated by subunit interactions via intra-trimer contacts and performed by changing the energy required to open and close the catalytic cleft during enzymatic turnover [10–12]. Recently, it has been reported that mutations, such as S445L, G446D, and H454Y, identified in patients with hyperinsulinism–hyperammonemia syndrome are within exons 11 and 12 of GDH [13,14] and lie within a sequence of amino acids that has been identified as a GTP binding site that is very close to the antenna region [11,12,15,16]. Therefore, it seems likely that the antenna may play a major role in subunit communication and that subunit communication responsible for negative cooperativity occurs. However, previous knowledge from chemical modification studies has suggested that several well conserved Lys residues including K333, K337, K344, and K346 that are not close to the antenna region may play a role in the regulation or maintenance of the quaternary structure of GDH [17–26]. No direct experimental evidence has been reported to identify the specific amino acid residue(s) involved in polymerization of hexameric human GDH. It is clear that direct evidence such as site-directed mutagenesis would be necessary to identify the residue(s) required for the polymerization process of human GDH.

In the present work, we have expanded the identification of the amino acid residue(s) involved in the polymerization process of human GDH using cassette mutagenesis and high performance liquid chromatography (HPLC) gel filtration analysis. For the present study, mutant human GDHs at seven putative sites for polymerization of human GDH have been expressed in *E. coli* as soluble proteins, purified, and characterized. To our knowledge, this is the first study by site-directed mutagenesis showing that His454 plays an important role in the maintenance of the quaternary structure of human GDH.

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Abbreviations: GDH, glutamate dehydrogenase; HPLC, high performance liquid chromatography

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2. Materials and methods

2.1. Materials

ADP, NADH, 2-oxoglutarate, and isopropyl-β-D-thiogalactose (IPTG) were purchased from Sigma. Human GDH gene (pHGDH) has been chemically synthesized and expressed in *E. coli* as a soluble protein in our laboratory as described elsewhere [27]. ADP-Sepharose and Resource-Q were purchased from Amersham Pharmacia Biotech. Protein Pak DEAE-5PW column was purchased from Waters. Restriction enzymes were purchased from New England Biolabs. All other chemicals and solvents were reagent grade or better.

2.2. Bacterial strains

E. coli DH5α [28] was used as the host strain for cassette mutagenesis. *E. coli* PA340 (*thr-1 fhuA2 leuB6 lacY1 supE44 gal-6 gdh-1 hisG1 rfbD1 galP63 Δ(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 [20] and was used to test plasmids for GDH activity. *E. coli* BL21(DE3) [29] was used for high level expression of the recombinant GDH.

2.3. Construction of mutants

A series of single amino acid substitutions was constructed by cassette mutagenesis of plasmid pHGDH. Plasmid DNA (5 μg) was digested with restriction enzymes to remove the flanking fragment that encodes target amino acids and the flanking fragment was replaced with seven synthetic DNA duplexes containing a substitution on both DNA strands at different positions to make K333L, K337L, K344L, K346L, S445L, G446D, and H454Y mutant proteins. Mutagenic oligonucleotides were annealed, ligated, and transformed into DH5α and the resultant mutant plasmids were identified by DNA sequencing using plasmid DNA as a template. Each of these mutants has been expressed in *E. coli* strain DE3 and purified to homogeneity, and has had its steady-state kinetic parameters determined as described below. The gene expression levels of the mutant proteins were examined by Western blot using monoclonal antibodies previously produced in our laboratory against bovine brain GDH [30].

2.4. Protein purification and kinetic studies

Fresh overnight cultures of DE3/pHGDH were used to inoculate 1 l of LB containing 100 μg/ml ampicillin. DE3/pHGDH 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/pHGDH 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 crude extracts were loaded onto an ADP-Sepharose column (1.5×5 cm) that was equilibrated with buffer A (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 5 mM dithiothreitol). The column was washed with buffer A until the breakthrough peak of protein had been eluted. The enzyme was then eluted by a gradient up to 500 mM NaCl in buffer A. The fractions containing GDH activity were pooled, concentrated, and applied to a FPLC Resource-Q anion exchange column equilibrated with buffer A. The enzyme was then eluted using a linear gradient made with buffer A in increasing concentrations of NaCl up to 100 mM at 0.5 ml/min. The purified GDH was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentration was determined by the method of Bradford [31] using bovine serum albumin as a standard.

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 [3]. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADH per minute at 25°C. Since *E. coli* only has an NADPH-dependent GDH [20], the enzyme assay was performed with NADH as a coenzyme. For determination of K_M and V_{max} values, the assays were carried out by varying the substrate under investigation while keeping the other substrate and reagents at the optimal concentrations. The K_M and V_{max} values were calculated by linear regression analysis of double-reciprocal plots and are given together with standard errors. Catalytic efficiency was estimated by use of the equation $v/[E_0] = (k_{cat}/K_M)[S]$ [32].

2.5. HPLC gel filtration analysis

Molecular size of the wild-type and mutant proteins was determined by a HPLC Protein-Pak 300SW gel filtration column with a Waters HPLC system. The column equilibrated with 100 mM Tris–HCl, pH 7.5 containing 0.05 M NaCl. The wild-type and mutant GDHs were separately loaded onto a Protein-Pak 300SW column (Waters) and eluted with the same buffer at a flow rate of 1 ml/min. The protein was monitored at 280 nm and the GDH activity was monitored by the standard assay method as described above. The molecular size of the wild-type and mutant GDHs was analyzed from the standard curve performed with high molecular weight standard markers (Amersham Pharmacia Biotech) under the same experimental conditions.

3. Results and discussion

To test the involvement of several putative amino acids in subunit polymerization of human GDH, we constructed a series of mutants at seven different sites by cassette mutagenesis. The flanking fragment in pHGDH was replaced with synthetic DNA duplexes containing a substitution on both DNA strands at positions encoding Lys333, Lys337, Lys344, Lys346, Ser445, Gly446, and His454 to make K333L, K337L, K344L, K346L, S445L, G446D, and H454Y mutant proteins. The mutant proteins were efficiently expressed in *E. coli* as soluble proteins and homogeneously purified using ADP-Sepharose column followed by FPLC Resource-Q column. Analysis of crude cell extracts by Western blot showed that each mutant plasmid encoding an amino acid substitution at seven different positions directed the synthesis of a 56.5 kDa protein that interacted with monoclonal antibodies raised against GDH at almost identical levels for all seven mutants and wild-type GDH (Fig. 1). Specific activities of these mutants were 1.02–1.18 U/mg, compared with a wild-type value of 1.12 U/mg, suggesting that the sites tested are not strictly required for catalytic activity. These results indicate that mutagenesis at the seven different sites has no effects on expression or stability of the mutant enzymes. The mutant enzymes also appeared to be as stable as wild-type human GDH, on the basis of their stability toward proteolysis and retention of activity upon prolonged storage at 4°C (data not shown).

Table 1
Kinetic parameters of the purified wild-type GDH and mutant GDHs

	k_{cat} (s^{-1})	K_M (mM)		k_{cat}/K_M (s^{-1} mM^{-1})	
		NADH	2-Oxoglutarate	NADH	2-Oxoglutarate
Wild-type	104	0.081	1.25	1284	83
K333L	93	0.079	1.29	1177	72
K337L	98	0.088	1.45	1113	68
K344L	105	0.090	1.31	1166	80
K346L	100	0.076	1.31	1315	76
S445L	111	0.098	1.47	1132	76
G446D	102	0.089	1.50	1146	68
H454Y	95	0.122	3.13	778	30

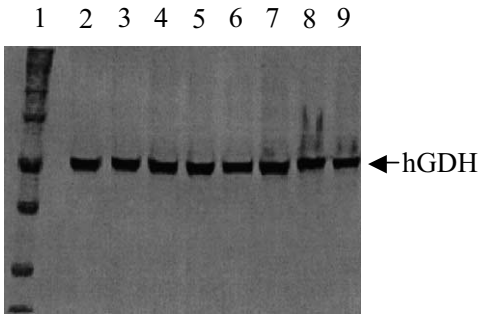

Fig. 1. Western blot analysis of the mutant GDHs in crude extracts. The supernatants of the crude extracts were loaded on a 12% sodium dodecyl sulfate–polyacrylamide gel, subjected to electrophoresis and immunoblotted with the monoclonal antibody directed against bovine brain GDH [30]. The final amount of total proteins loaded on each lane (lanes 2–9) was 0.8 mg. Lane 1, prestained marker proteins (Novex); lane 2, wild-type GDH; lanes 3–9, mutant GDHs (K333L, K337L, K344L, K346L, S445L, G446D, and H454Y).

A more detailed investigation of the catalytic activities of the mutant enzymes revealed essentially unchanged apparent K_M values for 2-oxoglutarate and NADH as well as the respective apparent V_{max} values in K333L, K337L, K344L, K346L, S445L, and G446D mutants (Table 1). The similarity of the K_M values for the wild-type and mutant enzymes indicates that mutagenesis at the six positions does not affect the affinity of the enzyme for these two substrates. However, the H454Y mutant GDH showed a different kinetic pattern from wild-type GDH. The K_M values for 2-oxoglutarate were 1.25 and 3.13 mM, respectively, for the wild-type and H454Y mutant GDH, and the K_M values for NADH were 0.081 and 0.122 mM, respectively, for the wild-type and H454Y mutant GDH (Table 1). The 2.5-fold and 1.5-fold greater K_M values for 2-oxoglutarate and NADH, respectively, for the K454Y mutant GDH than for wild-type GDH suggest that substitution at position 454 had appreciable effects on the affinity of the enzyme for both NADH and 2-oxoglutarate. The V_{max} values were similar for wild-type and H454Y mutant GDH, but the enzyme efficiency (k_{cat}/K_M) of the H454Y mutant GDH was reduced up to 2.8-fold. The decreased enzyme efficiency of the H454Y mutant, therefore, results from the increase in K_M values for NADH and 2-oxoglutarate.

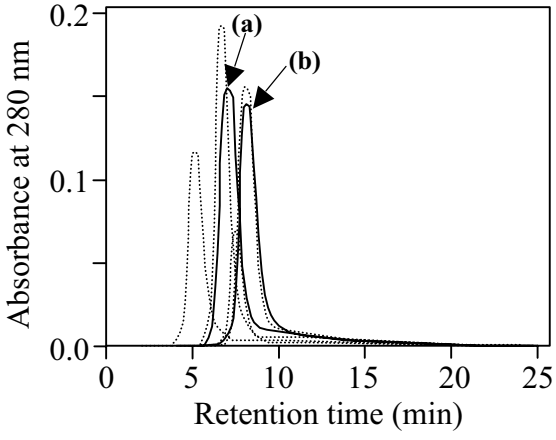

Fig. 2. HPLC gel filtration elution profiles. The purified wild-type and mutant GDHs were loaded onto a Protein-Pak 300SW gel filtration column equilibrated with 100 mM Tris–HCl, pH 7.5 containing 0.05 M NaCl. Proteins were eluted by the same buffer at a flow rate of 1.0 ml/min, monitored for protein at 280 nm, and assayed as described in the text. One-minute fractions were collected. Calibration was performed with the gel filtration high molecular weight marker proteins (dashed lines) from Amersham Pharmacia Biotech: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). The solid lines represent wild-type (a) and H454Y mutant GDH (b). Elution profiles of K333L, K337L, K344L, K346L, S445L, and G446D mutants were identical with those of wild-type and are not shown for clarity.

The role of the seven residues in polymerization of human GDH was further investigated by using HPLC gel filtration analysis. Calibration was performed with the high molecular marker proteins: thyroglobulin (670 kDa), ferritin (440 kDa), catalase (230 kDa), and aldolase (160 kDa). The retention times of the marker proteins were 5.1, 6.4, 7.3, and 7.7 min, respectively (Fig. 2 and Table 2). The result of gel filtration on HPLC Protein Pak 300SW column shows the identical retention time with the calculated molecular size of approximately 330 kDa for both wild-type GDH and mutant GDHs at positions Lys333, Lys337, Lys344, Lys346, Ser445, and Gly446 (Table 2). Since GDH is composed of identical subunits with a molecular size of ~56 kDa, these results indicate that the wild-type GDH and these mutant GDHs are composed of six identical subunits. The present work shows that kinetic parameters and molecular size of the mutant GDHs at positions Lys333, Lys337, Lys344, Lys346, Ser445, and Gly446 are almost identical with those of wild-type (Table 1, Fig. 2), suggesting that subunit composition and polymerization process of the mutant GDHs are not affected by the mutagenesis at each target amino acid. Although previous studies have suggested a possibility for the involvement of the residues at positions Lys333, Lys337, Lys344, Lys346, Ser445, and Gly446 in the maintenance of quaternary structure or subunit communication of mammalian GDH [11–14,17–23], the results from the present work show that those residues do not have a direct role for the polymerization process of hexameric human GDH.

Unlike the mutant GDHs at positions Lys333, Lys337, Lys344, Lys346, Ser445, and Gly446, there were differences in the biochemical properties between wild-type and His454 mutant GDH. The H454L mutant GDH shows a retention time of 7.7 min with a calculated molecular size of 160 kDa suggesting a composition of three subunits instead of a hexameric form (Fig. 2 and Table 2). Of the mutations at the seven different sites tested, therefore, only the mutagenesis at position His454 specifically results in depolymerization of the hexameric GDH into trimers. The K_M values for NADH and 2-oxoglutarate increased 1.5-fold and 2.5-fold, respectively, in

Table 2
Molecular size of the wild-type and mutant GDHs

	Retention time (min)	Molecular size (kDa)
Wild-type	7.0	330
K333L	7.0	330
K337L	7.0	330
K344L	7.0	330
K346L	7.0	330
S445L	7.0	330
G446D	7.0	330
H454Y	7.7	160
Thyroglobulin	5.1	670
Ferritin	6.4	440
Catalase	7.3	230
Aldolase	7.7	160

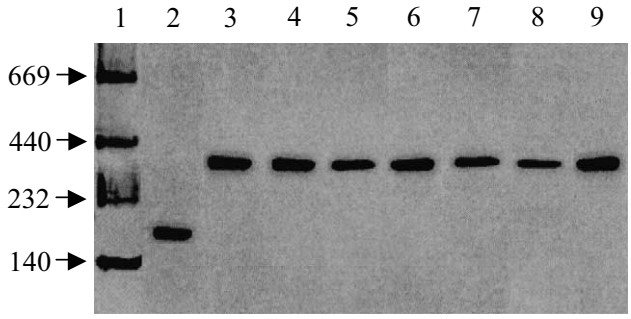

Fig. 3. Native gradient polyacrylamide gel electrophoresis of wild-type and mutant GDHs. The purified wild-type and mutant GDHs were separated on 5–12.5% polyacrylamide native gradient gel at 4°C and stained with Coomassie blue R-350. Lane 1, high molecular weight native gel electrophoresis markers (Amersham Pharmacia Biotech); lane 2, H454Y mutant; lanes 3–8, mutant GDHs (K333L, K337L, K344L, K346L, S445L, and G446D); lane 9, wild-type. Numbers beside arrows represent molecular weight in kDa.

the His454 mutant compared to those of the wild-type GDH (Table 1). Although the mutation at position 454 produces changes in the apparent K_M values for NADH and 2-oxoglutarate, H454Y mutant GDH and wild-type GDH show similar specific activities and k_{cat} values of the same order of magnitude (Table 1). The results of Western blot analysis (Fig. 1) show that the mutagenesis at His454 has no effects on expression or stability of the H454Y mutant GDH. Therefore, the dramatic reduction in the catalytic efficiency (k_{cat}/K_M) of the H454Y mutant reflects primarily changes in the K_M values for substrates, especially for 2-oxoglutarate.

Next we performed native gradient acrylamide gel electrophoresis to further confirm the native molecular size of the mutant GDHs. The results in Fig. 3 show that the molecular sizes of the mutant GDHs at positions Lys333, Lys337, Lys344, Lys346, Ser445, and Gly446 are almost identical with those of wild-type, whereas the H454L mutant GDH shows a different migration with a calculated molecular size of 170 kDa suggesting a composition of three subunits instead of a hexameric form. These results are consistent with those observed in HPLC gel filtration analysis (Fig. 2), demonstrating that mutagenesis at the His454 site results in the depolymerization of hexameric human GDH into trimers.

The His454 site identified as a functional residue in the polymerization of GDH in the present work is exactly located within the GTP binding domain that is very close to the antenna region. It has been reported that the six glutamate binding domains form the core of the GDH hexamer [11,12]. Since the six glutamate binding domains form an apparently rigid core structure as the catalytic cleft opens and closes, it seems likely that the subunit communication responsible for negative cooperativity occurs via intra-trimer contacts. Unlike mammalian GDHs, bacterial GDH does not exhibit negative cooperativity and does not have the antenna domain. It should, therefore, be mentioned that the antenna might play a major role in the subunit communication. Our results with cassette mutagenesis and HPLC gel filtration analysis suggest the possibility that His454 plays an important role in the polymerization process of hexameric human GDH, although the detailed kinetics of the H454Y mutant, in particular by rapid reaction kinetics and subunit complementation analysis, remains to be studied in order to elucidate the role of this residue more fully.

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