Important role of Ser443 in different thermal stability of human glutamate dehydrogenase isozymes[1]

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Abstract **Molecular biological studies confirmed that two glutamate dehydrogenase isozymes (hGDH1 and hGDH2) of distinct genetic origin are expressed in human tissues. hGDH1 is heat-stable and expressed widely, whereas hGDH2 is heat-labile and specific for neural and testicular tissues. A selective deficiency of hGDH2 has been reported in patients with spinocerebellar ataxia. We have identified an amino acid residue involved in the different thermal stability of human GDH isozymes. At 45°C (pH 7.0), heat inactivation proceeded faster for hGDH2 (half life = 45 min) than for hGDH1 (half-life = 310 min) in the absence of allosteric regulators. Both hGDH1 and hGDH2, however, showed much slower heat inactivation processes in the presence of 1 mM ADP or 3 mM L-Leu. Virtually most of the enzyme activity remained up to 100 min at 45°C after treatment with ADP and L-Leu in combination. In contrast to ADP and L-Leu, the thermal stabilities of the hGDH isozymes were not affected by addition of substrates or coenzymes. In human GDH isozymes, the 443 site is Arg in hGDH1 and Ser in hGDH2. Replacement of Ser by Arg at the 443 site by cassette mutagenesis abolished the heat lability of hGDH2 with a similar half-life of hGDH1. The mutagenesis at several other sites (L415M, A456G, and H470R) having differences in amino acid sequence between the two GDH isozymes did not show any change in the thermal stability. These results suggest that the Ser443 residue plays an important role in the different thermal stability of human GDH isozymes.**

Key words: Glutamate dehydrogenase; Thermal stability; Isozyme; Cassette mutagenesis; Reactive serine; Allosteric effector

1. Introduction

Glutamate dehydrogenase (GDH; EC 1.4.1.3) is a family of enzymes catalyzing a reversible deamination of L-glutamate to α-ketoglutarate directly connected to the Krebs cycle [1]. Therefore, GDH serves as the major link between carbohydrate and amino acid metabolism and various roles of GDH have been reported. For instance, partial deficiency of GDH has been reported in some patients with cerebellar degeneration, suggesting that the enzymes are important in brain function [2]. In addition, GDH has shown neuroprotective value in model systems where glutamate reuptake is inhibited [3]. It has also been reported that inhibition of GDH expression by antisense oligonucleotides was toxic to cultured mesencephalic neurons, with dopaminergic neurons being affected at the early stages of this inhibition [4]. Specific alteration in the expression of GDH was also observed in rats with genetic absence epilepsy [5]. Besides, a membrane-bound form of GDH possesses microtubule-binding activity [6] and GDH reacts as an RNA-binding protein and shows a possible role in regulation of transcription [7,8]. Cavallaro et al. [9] have identified GDH as one of the late memory-related genes in the hippocampus and Frattini et al. [10] have identified GDH as a new member of the ring finger gene family in Xq24–25. The existence of the hyperinsulinism–hyperammonemia syndrome further highlights the importance of GDH in the regulation of insulin secretion and indicates that GDH has an important role in regulating hepatic ureagenesis [11–14].

GDH has long been used as a marker for mitochondria in brain and other tissues. However, the activity of GDH is also enriched in the nuclear fraction as well as in the mitochondrial fraction. Recent molecular biological studies have confirmed that two human GDH isozymes (hGDH1 and hGDH2) of distinct genetic origin are expressed in human tissues and that the activities of the GDH isotypes differ in their relative resistance to thermal inactivation, detergent extractability, and allosteric regulation characteristics [2,15–17]. hGDH1 (housekeeping GDH) is expressed widely, whereas hGDH2 (nerve-specific GDH) is specific for neural and testicular tissues [18–22]. hGDH2 is the heat-labile form of human GDH. However, it is not known whether the distinct properties of the hGDH isozymes are essential for the regulation of glutamate metabolism. Although many studies show the subcellular localization of GDH, the origin of the GDH polymorphism is not fully understood and the functional significance and reaction mechanism of nerve-specific GDH isotype in nerve tissue remains to be studies. Therefore, further characterization of the structure and function of human GDH isozymes is needed to elucidate the physiological nature of the GDH-related disorders [2,5,11].

Recently, the human GDH1 gene has been chemically synthesized and expressed in *Escherichia coli* in our laboratory

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[1] The sequence reported in this paper has been deposited in the GenBank data base (accession number: AY177294).

Abbreviations: GDH, glutamate dehydrogenase

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[23]. Using this synthetic human GDH1 gene, reactive amino acid residues for catalysis [23], for GTP base binding [24], for NAD^+ base binding [25], and for ADP base binding [26] have been identified by cassette mutagenesis. One of the important differences between the two GDH isozymes is their thermal stability. In the present work, therefore, we have investigated the amino acid residue involved in different thermal stability between the two human GDH isozymes. For these studies, a 1557-bp gene that encodes human GDH2 has been synthesized based on the amino acid sequence of the mature human GDH2 [22]. Since the functional differences between hGDH1 and hGDH2 arise from amino acid residues not common between the two GDH isozymes, we have examined these critical residues by cassette mutagenesis of the synthetic hGDH2 gene at sites differing from the corresponding sites of the synthetic hGDH1 gene. Similar approaches have also been performed by other investigators [16,27] to identify the reactive residues involved in the functional differences between the GDH isozymes. The objective of this study was to identify accessible regions in the enzyme's structure with a view to the design of reactive amino acid residues of hGDH isozymes showing different thermal stabilities. Some of these residues in the C-terminal region have been selected because previous studies have showed the importance of the C-terminal region in the regulation of hGDH1 [16,24,27]. In each of these sites, the amino acid residue of the hGDH2 gene was replaced by the corresponding amino acid of the hGDH1 gene. To our knowledge, this is the first report of site-directed mutagenesis showing a critical role of the Ser443 residue in the different thermal stability of the human GDH isozymes.

2. Materials and methods

2.1. Materials

NADH, NAD^+, α-ketoglutarate, ADP, and factor Xa were purchased from Sigma Chemical Co. Synthetic human GDH1 gene was previously constructed in our laboratory [23]. Pre-cast gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and pre-stained marker proteins for Western blot were purchased from Novex. Low molecular weight marker proteins for SDS–PAGE were purchased from Bio-Rad. All other chemicals and solvents were reagent grade or better.

2.2. Bacterial strains

E. coli DH5α [28] 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* 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, New Haven, CT, USA) lacked both GDH and glutamate synthase activities [29] and was used to test plasmids for GDH activity. *E. coli* BL21 (DE3) [30] was used for high-level expression of the recombinant human GDH2.

2.3. Construction and expression of synthetic hGDH2 and mutants

The design of the synthetic human GDH2 gene was based on the amino acid sequence of the mature human GDH2 [22]. The synthetic hGDH2 gene was constructed from the hGDH1 gene previously synthesized in our laboratory [23] by cassette mutagenesis at different amino acid residues not common between the GDH1 and GDH2 isozymes [22]. General methods for design of the synthetic hGDH2 gene, cassette mutagenesis, subcloning, and bacterial transformation were as described [23]. The final synthetic hGDH2 gene, designated pHGDH2, was used for gene expression and mutagenesis studies. To make hGDH2 mutant proteins, a single amino acid substitution at Lys415, Ser443, Ala456, or His470 was separately constructed by cassette mutagenesis of the synthetic hGDH2 gene, pHGDH2. Plasmid DNA was digested with restriction enzymes at flanking sites to remove a fragment that encodes the target amino acid and replaced with a synthetic DNA duplex containing a substitution on both DNA strands at positions encoding Lys415, Ser443, Ala456, or His470 to make L415M, S443R, A456G, or H470R. The mutants were identified by DNA sequencing using plasmid DNA as a template.

The wild type and mutant proteins were expressed in *E. coli* strain DE3. The gene expression level in the crude extract was examined by Western blot using monoclonal antibodies against bovine brain GDH [31]. The expressed proteins were purified to homogeneity as described before [23] and analyzed by SDS–PAGE. To remove five additional N-terminal residues (M-I-E-G-R), the purified hGDH2 was treated with factor Xa (10 μg/mg GDH) in a reaction mixture of 20 mM Tris–HCl/100 mM NaCl/2 mM $CaCl_2$. After incubation at room temperature for 3 h, the reaction mixtures were purified by a high performance liquid chromatography (HPLC) Protein-Pak 300SW gel filtration column as described before [23]. HPLC-purified hGDH2 was subjected to automated amino acid sequencing. Steady-state kinetic parameters were determined with the purified proteins unless otherwise indicated.

2.4. Enzyme assay, thermal stability, and kinetic studies

GDH activity was measured spectrophotometrically in the direction of reductive amination of α-ketoglutarate by following the decrease in absorbance at 340 nm as described before [32]. All assays were performed in duplicate and initial velocity data were correlated with 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. Enzyme assay was performed in the presence of 1 mM ADP unless otherwise indicated. The reaction started with the addition of α-ketoglutarate to 10 mM final concentration. Since *E. coli* only has an NADP(H)-dependent GDH [28], the enzyme assay was performed with NADH as a coenzyme as described elsewhere [32]. One unit of enzyme was defined as the amount of enzyme required for oxidizing 1 μmol of NADH/min at 25°C. For the thermal stability studies, the hGDH isozymes were incubated at 45°C in the presence or absence of various regulators. At various times, aliquots were withdrawn and the remaining activities were assayed by the addition of the standard assay mixture. The K_m and V_{max} values were calculated by linear regression analysis of double-reciprocal plots and given along with standard errors. Catalytic efficiency was estimated by use of the equation $v/[E_0] = (k_{cat}/K_m)[S]$. For the heat stability studies, the hGDH isozymes were incubated at 45°C in the presence or absence of various regulators. At various times, aliquots were withdrawn and the remaining activities were assayed by the addition of the standard assay mixture.

3. Results and discussion

The synthetic hGDH2 gene was constructed from the hGDH1 gene previously synthesized in our laboratory [23] by cassette mutagenesis at different amino acid residues not common between hGDH1 and hGDH2 [22]. *E. coli* strain PA340 lacks both GDH and glutamate synthase activities [29]. Activity measurements and SDS–PAGE of the crude extracts from *E. coli* strain PA340 transformed with pHGDH2 showed that the plasmid was directing the synthesis of catalytically active GDH (0.15 U/mg) to a level of ~1% of the total soluble protein. However, higher level expression of the synthetic hGDH2 gene was achieved by transformation of pHGDH2 into *E. coli* strain DE3. Upon induction with 1 mM of isopropyl-β-D-thiogalactose at 37°C for 3 h, expression of hGDH2 in soluble extracts increased to 1.49 U/mg. SDS–PAGE analysis of crude cell extracts and measurement of specific activities indicated that pHGDH2 directed GDH expression to a level of ~12% of total cellular protein upon induction with IPTG. The wild type and mutant enzymes were expressed in *E. coli* strain DE3. The gene expression of hGDH2 in the crude extract was also examined by Western blot using monoclonal antibodies against bovine brain GDH [31] as shown in Fig. 1A.

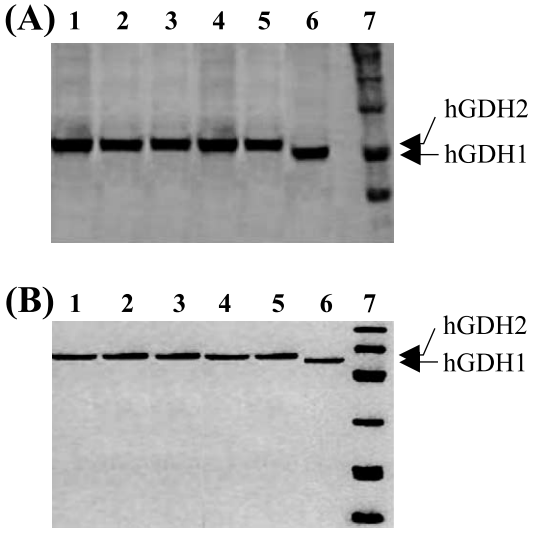

Fig. 1. Electrophoretic analysis of wild type hGDH isozymes and hGDH2 S443R mutant. A: Western blot analysis of hGDHs in crude extracts of *E. coli* expressing hGDH1 or hGDH2. In each lane of this immunoblot, 60 μg of protein from cell extracts were used. B: SDS–PAGE of purified hGDHs. Lane 1, wild type hGDH2; lanes 2–5, hGDH2 mutants (L415M, A456G, S443R, H470R); lane 6, wild type hGDH1; lane 7, molecular weight marker proteins.

The expressed hGDH2 was purified by ADP-Sepharose column and FPLC Resource-Q column (see Section 2). Since the recombinant hGDH2 was readily solubilized, no detergents were required throughout the entire purification steps. To remove five additional N-terminal residues (M-I-E-G-R) that were introduced to create an initiation codon and a factor Xa recognition site, the purified hGDH2 was treated with factor Xa, purified by a HPLC Protein-Pak 300SW gel filtration column, and subjected to automated Edman degradation. N-terminal sequence analysis of the first eight amino acids of S-E-L-V-A-D-R-E was identical with the published sequence of the mature human GDH2 [22]. Indeed, the five additional residues (M-I-E-G-R) did not show any significant changes in specific activities, thermal stability, and solubility of the recombinant human GDH isozymes. The purified wild type hGDH2 was estimated to be >98% pure by SDS–PAGE (Fig. 1B). The subunit size (~56.5 kDa) and the native size (~320 kDa) of the hexameric recombinant hGDH2 as determined by SDS–PAGE (Fig. 1B) and HPLC gel filtration chromatography (data not shown), respectively, were almost identical to those of the mature human brain GDH2 [22].

Compared to hGDH1, the hGDH2 protein is more basic and shows a slightly lower electrophoretic mobility (Fig. 1). These differences are consistent with the electrophoretic characteristics of GDH isozymes purified from human brain [22]. Previous studies showed that GDH activities were differentially altered in neurologic patients [33–38], thus suggesting that these activities are under different genetic control. Since electroretinographic abnormalities have been detected in patients with spinocerebellar ataxia who have a selective deficiency of the heat-labile GDH [2,38], studies on the molecular basis for the different thermal stability of the GDH isozymes may thus be of importance for understanding the biology of the human nervous systems and the genetic analysis of X-linked neurodegeneration. Expression of the hGDH2 gene in human brain and retina is of particular interest in view of data showing that glutamate is an important excitatory transmitter in brain and retina [39]. These results are similar to those originally described in human leukocytes [33] and suggest that the heat-resistant GDH activity represents the housekeeping enzyme and the heat-sensitive GDH activity represents the tissue-specific GDH.

There was a big difference in thermal stability between the two types of human GDH isozymes. Activities after 100 min incubation at 45°C (pH 7.0) in the absence of ADP or L-Leu were 80% and 20% for hGDH1 and hGDH2, respectively. Therefore, heat inactivation proceeded faster for hGDH2 (half life = 45 min) than for hGDH1 (half-life = 310 min) in the absence of ADP or L-Leu (Fig. 2A,B), suggesting that hGDH1 is the heat-stable form and hGDH2 is the heat-labile form of human GDH. When the enzymes were incubated at 45°C (pH 7.0) in the presence of 1 mM ADP and/or 3 mM L-Leu, the patterns of the thermal stabilities were dramatically changed. Both hGDH1 and hGDH2 showed slow heat inac-

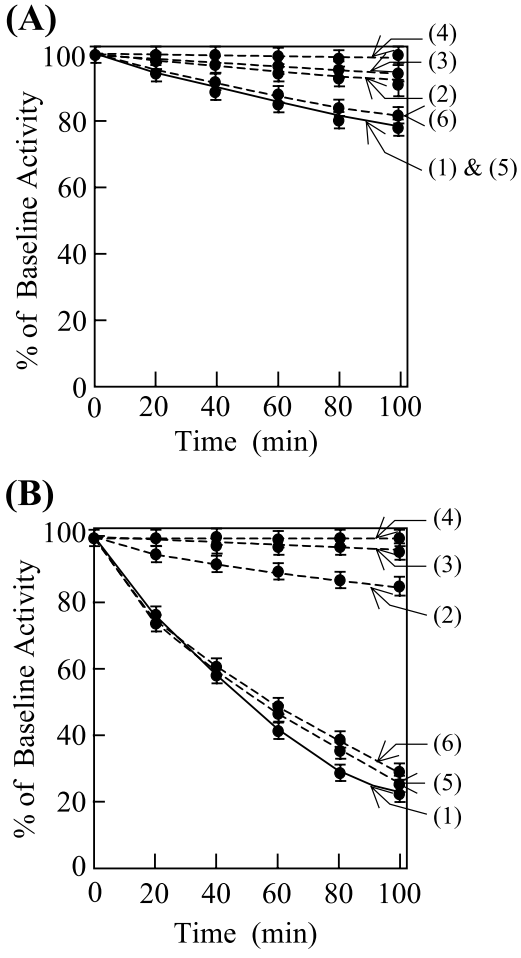

Fig. 2. Thermal stability of wild type hGDH isozymes. hGDH1 (A) and hGDH2 (B) were separately incubated at 45°C in 100 mM sodium phosphate buffer, pH 7.0 in the absence and presence of ADP and/or L-Leu. At various times, aliquots were withdrawn and the activity was assayed by the addition of the standard assay mixture at pH 8.0 in the presence of 1 mM ADP. GDH activity was measured in the direction of reductive amination of α-ketoglutarate. The data represent the mean of two independent experiments and are expressed as a percentage of baseline activity determined in the absence of regulators. (1) No effectors; (2) ADP (1 mM); (3) L-Leu (3 mM); (4) ADP (1 mM)+L-Leu (3 mM); (5) NADH (0.1 mM); (6) α-ketoglutarate (10 mM).

tivation in the presence of 1 mM ADP (Fig. 2A,B). ADP levels are known to vary from 0.05 mM to higher than 1.0 mM depending on the rate of oxidative phosphorylation [40]. The results of the present work, therefore, revealed that the nerve tissue-specific hGDH2 has evolved into a highly heat-sensitive enzyme that is markedly protected by ADP at physiological concentrations that can occur when cellular energy charge declines. Similar protective effects were also observed after treatment with 3 mM L-Leu (Fig. 2), although L-Leu protected hGDH1 and hGDH2 from the heat inactivation slightly better than ADP did. Virtually most of the enzyme activity remained after treatment with 3 mM L-Leu. In contrast to ADP or L-Leu, α-ketoglutarate or NADH did not affect the thermal stabilities of the hGDH isozymes (Fig. 2). Similar results were also obtained with L-glutamate and NAD^+ singly and in combination (data not shown).

The different heat stability between hGDH1 and hGDH2 was further examined by cassette mutagenesis of the synthetic hGDH2 gene at sites differing from the corresponding sites of the synthetic hGDH1 gene. Mutagenesis at site Lys415, Ser443, Ala456, or His470 having difference in amino acid sequence between the C-terminal regions of two human GDH isozymes was performed to make L415M, S443R, A456G, or H470R mutants. In each of these sites, the amino acid residue of the hGDH2 gene was replaced by the corresponding amino acid of the hGDH1 gene, expressed in *E. coli* DE3, and homogeneously purified as described above. The hGDH2 S443R mutant showed a dramatic change in thermal lability of hGDH2. In human GDH isozymes, the 443 site is Arg in hGDH1 and Ser in hGDH2. Analysis of crude cell extracts by Western blot showed that the mutant plasmid encoding an amino acid substitution at position 443 of hGDH2 directed the synthesis of a protein at almost identical levels for hGDH2 (Fig. 1A). The molecular size of the purified hGDH2 S443R protein was also identical to that of the wild type hGDH2 (Fig. 1B). Replacement of Ser by Arg at hGDH2 position 443 abolished the heat lability of hGDH2 and changed the half-life of hGDH2 from 45 min to approximately 300 min at 45°C, which is almost the same as that of hGDH1 (Fig. 3). These results suggest that the Ser443 residue plays an important role in the different thermal stability of human GDH isozymes.

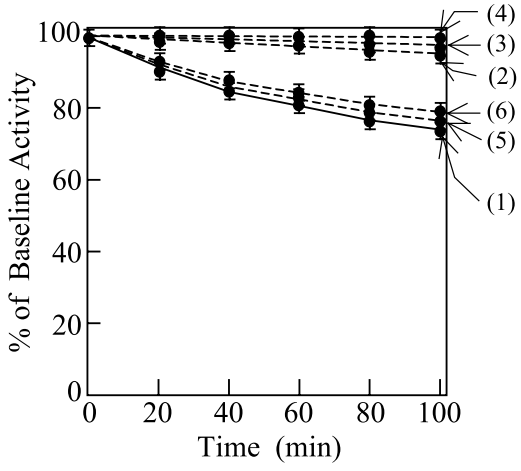

Fig. 3. Thermal stability of hGDH2 S443R mutant. The hGDH2 S443R mutant was incubated at 45°C in 100 mM sodium phosphate buffer, pH 7.0 and aliquots were withdrawn at various times to measure remaining activity. The data represent the mean of two independent experiments and are expressed as a percentage of baseline activity determined in the absence of regulators. (1) No effectors; (2) ADP (1 mM); (3) L-Leu (3 mM); (4) ADP (1 mM)+L-Leu (3 mM); (5) NADH (0.1 mM); (6) α-ketoglutarate (10 mM).

The effects of ADP, L-Leu, or substrates on thermal stability of the hGDH2 S443R mutant were similar to those observed with hGDH1 (Fig. 3). When the enzyme was incubated in the presence of ADP and L-Leu, heat inactivation was almost completely abolished and full enzyme activity remained for all enzymes tested. The synergistic effect of ADP and L-Leu, however, was not considerable because separate treatment of ADP or L-Leu already showed a good protection from the heat inactivation with a residual GDH activity of more than 85% after 100 min incubation at 45°C. Results of the SDS–PAGE showed no changes in the structure of wild type and S443R mutant proteins after 100 min incubation at 45°C (pH 7.0) in the absence or presence of 1 mM ADP and/or 3 mM L-Leu, (data not shown). Once again, α-ketoglutarate or NADH did not affect the thermal stabilities of the hGDH2 S443R mutant (Fig. 3).

Unlike the hGDH2 S443R mutant, none of the hGDH2 mutants L415M, A456G, or H470R showed any change in the heat inactivation process compared to wild type hGDH2. Protein expression level and molecular size of the hGDH2 mutants of L415M, A456G, or H470R were identical with those of wild type and S443R mutant of hGDH2 (Fig. 1). Activities after 100 min incubation at 45°C (pH 7.0) were 20–28% for hGDH2 L415M, A456G, or H470R mutants in the absence of ADP or L-Leu (Fig. 4A). Although the heat inactivation process was similarly protected by ADP as in the case of the S443R mutant, there were no differences in thermal stability between hGDH2 wild type and L415M, A456G, or H470R mutant enzymes (Fig. 4B), suggesting that none of the mutants at site Leu415, Arg456, or His470 is directly involved in the different thermal stability between the two hGDH isozymes.

Recently, Zaganas et al. [27] have shown the importance of the Ser443 residue in the allosteric activation of hGDH isozymes by ADP and L-Leu. In human GDH isozymes, the 443 site is Arg in hGDH1 and Ser in hGDH2. Replacement of Ser by Arg at the 443 site by cassette mutagenesis abolished the heat lability of hGDH2 with a similar half-life of hGDH1 (Fig. 3), whereas mutagenesis at several other sites (L415M, A456G, and H470R) having difference in amino acid sequence between the two GDH isozymes did not show any change in thermal stability (Fig. 4). These results suggest that the Ser443 residue plays an important role in the different heat stability between hGDH1 and hGDH2. Our results are also consistent with the recent report of Plaitakis et al. [2] demonstrating that substitution of Ser for Arg443 and Ala for Gly456 are the main evolutionary changes that led to the adaptation of hGDH2 to the unique metabolic needs of the nerve tissue.

In order to evaluate more detailed catalytic properties for the wild type hGDH isozymes and the hGDH2 S443R mutant enzyme, the enzyme efficiency (k_{cat}/K_m) for the individual substrates were determined (Table 1). The apparent K_m values for α-ketoglutarate and NADH were essentially similar for all wild type and mutant proteins at 81–88 μM and 1.25–1.41 mM for NADH and mutant GDHs, respectively (Table 1). These results suggest that substitution at position 443 might have no appreciable effect on the affinity of enzyme for substrate or coenzyme. However, there was an approximately

Table 1
Kinetic properties of hGDH1, hGDH2, and hGDH2 S443R mutant

	k_{cat} (s^{-1})	K_{m-NADH} (μM)	$K_{m-\alpha-KG}$ (mM)	k_{cat}/K_{m-NADH} (s^{-1} μM^{-1})	$k_{cat}/K_{m-\alpha-KG}$ (s^{-1} mM^{-1})
hGDH1 (wild type)	104	81	1.25	1.28	81.3
hGDH2 (wild type)	130	86	1.39	1.51	93.5
hGDH2 (S443R mutant)	134	88	1.41	1.52	95.0

K_m and k_{cat}/K_m values of wild type hGDH isozymes and hGDH2 S443R mutant were obtained from the initial velocity data and linear regression analysis of double-reciprocal plots as described in Section 2. GDH activity was measured in the presence of 1 mM ADP. The data represent the mean of two independent experiments. Duplicates of the kinetic values differed less than 5%.

30% increase in k_{cat} values of wild type hGDH2 and hGDH2 S443R mutant compared to that of the wild type hGDH1. The increased enzyme efficiency (k_{cat}/K_m) of wild type hGDH2 and hGDH2 S443R mutant, therefore, results from the increase in k_{cat} values. Analysis of crude cell extracts by Western blot showed that the mutant plasmid encoding an amino acid substitution at position 443 of hGDH2 directed the synthesis of a protein at almost identical levels for hGDH2 (Fig. 1A). In addition, the hGDH2 S443R mutant protein was purified by the same methods as was the wild type enzyme, indicating that no gross conformational change in the hGDH2 S443R mutant protein had occurred. Therefore, the difference in the k_{cat} values between hGDH1 and hGDH2 is probably not due to the Ser443 residue but to the rest of the amino acids not common between hGDH1 and hGDH2.

To our knowledge, this is the first report identifying a reactive residue critically involved in the different thermal stability of the human GDH isozymes. The construction of a synthetic gene encoding hGDH isozymes will enable us to generate a large number of site-directed mutations at several positions in the coding region. The high level expression of hGDH1 and hGDH2 as a catalytically active soluble protein in *E. coli* will facilitate the purification of large quantities of mutant proteins to address a broad range of questions relating to the structure and function of human GDH isozymes.

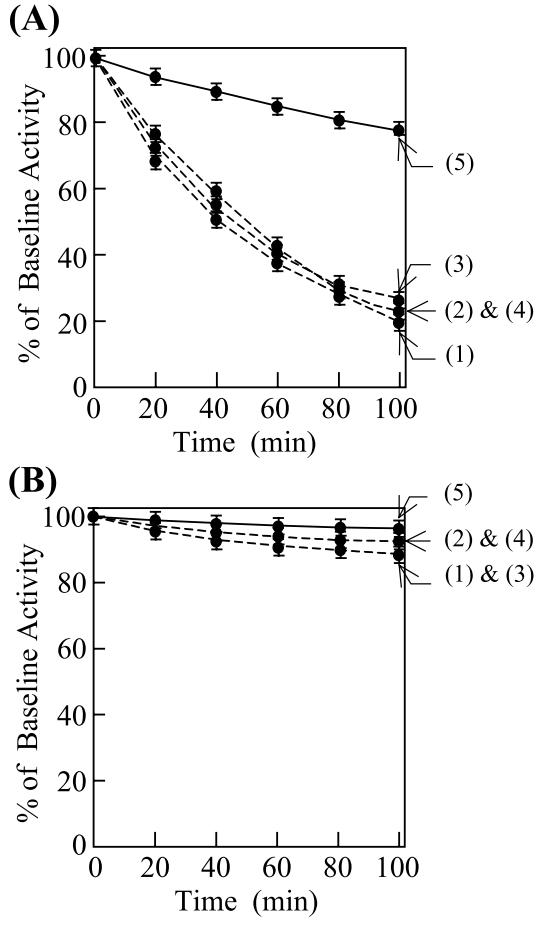

Fig. 4. Thermal stability of hGDH2 L415M, A456G, and H470R mutants. The mutant proteins were separately incubated at 45°C in 100 mM sodium phosphate buffer, pH 7.0 in the absence of ADP or L-Leu. At various times, aliquots were withdrawn and the activity was assayed by the addition of the standard assay mixture at pH 8.0 in the presence of 1 mM ADP. GDH activity was measured in the direction of reductive amination of α-ketoglutarate. The data represent the mean of two independent experiments and are expressed as a percentage of baseline activity determined in the absence (A) and presence (B) of ADP (1 mM) and L-Leu (3 mM). (1) Wild type hGDH2; (2) L415M; (3) A456G; (4) H470R; (5) wild type hGDH1.

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