Substitution of Ser for Arg-443 in the Regulatory Domain of Human Housekeeping (GLUD1) Glutamate Dehydrogenase Virtually Abolishes Basal Activity and Markedly Alters the Activation of the Enzyme by ADP and L-Leucine*

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Human glutamate dehydrogenase (GDH) exists in GLUD1 (housekeeping) and in GLUD2-specified (brain-specific) isoforms, which differ markedly in their basal activity and allosteric regulation. To determine the structural basis of these functional differences, we mutagenized the GLUD1 GDH at four residues that differ from those of the GLUD2 isoenzyme. Functional analyses revealed that substitution of Ser for Arg-443 (but not substitution of Thr for Ser-331, Leu for Met-370, or Leu for Met-415) virtually abolished basal activity and totally abrogated the activation of the enzyme by L-leucine (1–10 mM) in the absence of other effectors. However, when ADP (0.025–0.1 mM) was present in the reaction mixture, L-leucine (0.5–6.0 mM) activated the mutant enzyme up to >2,000%. The R443S mutant was much less sensitive to ADP (SC50 = 383.9 ± 14.6 μM) than the GLUD1 GDH (SC50 = 31.7 ± 4.2 μM, p < 0.001); however, at 1 mM ADP the Vmax for the mutant (136.67 μmol min⁻¹ mg⁻¹) was comparable with that of the GLUD1 GDH (152.95 μmol min⁻¹ mg⁻¹). Varying the composition and the pH of the reaction buffer differentially affected the mutant and the wild-type GDH. Arg-443 lies in the “antenna” structure, in a helix that undergoes major conformational changes during catalysis and is involved in intersubunit communication. Its replacement by Ser is sufficient to impair both the catalytic and the allosteric function of human GDH.

Mammalian glutamate dehydrogenase (GDH) (E.C.1.4.1.3) catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate using NAD(H) or NADP(H) as co-factors (1). GDH is expressed at high levels in the liver, brain, kidney, pancreas, heart and lungs (2). The enzyme may play a key role in cellular metabolism and energy homeostasis (2). In the pancreatic beta cells, GDH is involved in insulin secretion mechanisms (3–6), whereas in the kidney the enzyme may play a role in ammonia homeostasis (7). In the nervous system, GDH is enriched in glial cells associated with glutamatergic nerve terminals and thought to be involved in the metabolism of neurotransmitter glutamate (8–10).

There is increasing evidence that GDH activity is regulated in vivo (1, 6, 8) and that this regulation is of particular biological importance (11, 12). In pancreatic beta cells, which express the GLUD1-derived GDH, enzyme activity is under tonic inhibition by GTP (6). Allosteric activators such as ADP and L-leucine can counteract this inhibition leading to an enhanced flux of glutamate through this pathway. This in turn is shown to boost the cellular energy charge (ATP levels) with resultant insulin release (4). This concept is supported by recent findings, which show that mutations rendering the enzyme resistant to GTP are associated with enhanced insulin release (hyperinsulinism) in children (6).

Human GDH exists in housekeeping and in nerve tissue-specific isoforms encoded by the GLUD1 and GLUD2 genes, respectively (13, 14). Although GTP potently inactivates the housekeeping GDH, the brain-specific isoform is resistant to this compound (15). This may represent an adaptation that permits the enzyme to function in the GTP-rich environment that prevails in the nerve tissue (16). Instead of being inhibited by GTP, the brain-specific GDH has evolved to remain largely inactive at its basal state and to be regulated via activation by ADP and L-leucine (12, 15). These properties may allow the nerve tissue-specific GDH to remain dormant under resting conditions and to be called into action when low energy charge (high ADP levels), associated with intense glutamatergic transmission, prevails. Under these conditions, GDH function may prove essential for enhancing glutamate detoxification and for restoring cellular energy stores (12, 16).

At present, the molecular basis for this adaptation has not been fully understood. Because the functional differences between the housekeeping and the nerve tissue-specific GDH arise from amino acid residues not common between the two isoenzymes, we have sought to identify these critical residues by mutagenizing the GLUD1 gene at sites that differ from the corresponding sites of the GLUD2 gene. Initial studies that explored three such amino acid residues, located in the C-terminal region of this protein (a section of which forms part of the regulatory domain of GDH), revealed that substitution of Ala for Gly-456 rendered the enzyme resistant to GTP inhibition without affecting its base-line activity or its activation by ADP (17).

Here we sought to study further structure-function relationships in this enzyme by performing site-directed mutagenesis on the GLUD1 gene at four amino acid residues (Fig. 1) located...
Allosteric regulation of mutant and wild-type GLUD1-derived GDHs by ADP and GTP in crude extracts

| Activator SC50 (μM ADP) | R443S | M415L | M370L | S331T |
|-------------------------|-------|-------|-------|-------|
| GC50                    | 27.4 ± 2.2 | 405.6 ± 14.8 | 28.1 ± 2.1 | 21.0 ± 3.0 |
| Inhibitor GC50 (μM GTP) | 12.2 ± 0.5 | 16.2 ± 1.1 | 14.7 ± 0.8 | 11.3 ± 1.1 |
| Hill coefficient (GTP Inhibition) | 2.5 ± 0.1 | 2.0 ± 0.1 | 2.3 ± 0.2 | 2.1 ± 0.2 |

*SC50 value for the R443S mutant is significantly different (p < 0.001) as compared with that of the wild type GLUD1-derived GDH, the M415L mutant, the M370L mutant, and the S331T mutant.*

just upstream from the C-terminal region we studied previously (17). In each of these sites the amino acid residue present in the GLUD2 GDH replaced the corresponding amino acid of the GLUD1 enzyme. The mutated cDNAs were expressed in Sf21 cells, and the obtained mutant GDH isoproteins were purified and studied with respect to their kinetic and regulatory characteristics. Results showed that substitution of Ser for Arg at position 443 of the GLUD1 GDH virtually abolished basal enzyme activity (determined in the absence of allosteric activators) and markedly altered the activation of this GDH isoform by ADP and l-leucine. These data provide further insight into the molecular mechanisms underlying enzyme function and the evolutionary processes that resulted in human GDH multiplicity.

EXPERIMENTAL PROCEDURES

Materials—Sf21 cells and the baculovirus expression vectors were obtained from Invitrogen. The media for the Sf21 insect cells and fetal calf serum were obtained from Life Technologies. The Modified baculovirus (BaculoGold) was obtained from BD Pharmingen. NADPH, ADP, and GTP (liithium salt) were from Roche Molecular Biochemicals. L-Leucine was from Amersham Biosciences. Phenyl-Serylharose High Performance from Amersham Biosciences, and Hydroxyapatite Bio-Gel HT was from Bio-Rad.

Site-directed Mutagenesis of the GLUD1 cDNA—A GLUD1 cDNA, cloned in pBSKII+ vector, was mutagenized at specific sites (Fig. 1) using the GeneEditor Mutageneis system according to the manufacturer’s protocol (Promega, Madison, WI). Mutagenic oligonucleotides (25–30 bp in length) were phosphorylated and annealed to the GLUD1 template by heating the reaction mixture at 75 °C and then slowly cooling it (1.5 °C/min) to 37 °C. The annealing reaction also contained primers (provided by the manufacturer) designed to mutate the beta lactamase gene of the pBSKII+ vector. The plasmid was then amplified by T4 DNA polymerase (nicks were ligated by T4 DNA ligase) and used to transform the BHN 71-18 mutS strain of Escherichia coli (to prevent repair of the newly synthesized strand by the microorganism). The cells were grown in the presence of an appropriate antibiotic selection mix; plasmid DNA was isolated and used to transform the JM109 strain of E. coli. Clones containing plasmids with the desired mutations were selected by restriction digestion analysis and by DNA sequencing.

Subcloning in pVL1393—The mutated GLUD1 cDNA was cleaved from the pBSKII+ vector using BamHI and PstI restriction endonucleases and ligated to the baculovirus transfer vector pVL1393. The ligation products were isolated and used to transform the JM109 strain of E. coli. The proper orientation of the insert was verified by sequencing. The subcloned mutated GLUD1 gene was bidirectionally sequenced in its entire length in order to confirm the presence of the desired mutation and exclude incidental DNA alterations induced during the above mutagenesis steps. DNA sequencing was carried out using the LI-COR 4200 system (LI-COR, Lincoln, NE).

Expression of Recombinant Proteins—Mutated GLUD1 cDNAs, along with the wild-type GLUD1 cDNA (used here as a control for protein expression and enzymatic analysis), were expressed in Sf21 cells using the baculovirus expression system described previously (14, 17). Cells of the insect Spodoptera frugiperda (Sf21) were co-transfected with the plasmid DNA (pVL1393 vector containing the GLUD1 insert) and modified baculovirus DNA (BaculoGold, BD Pharmingen), and incubated at 27 °C for 4–5 days. The virus was amplified by two to three rounds of infection. The cultured cells were harvested 5 days post-infection and used for extracting the recombinant GDH proteins. For this purpose, the collected cells were homogenized in a buffer containing 0.05 M Tris-HCl, pH 7.4, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 M NaCl. The resulting whole homogenate was centrifuged at 7000 × g (4 °C) for 10 min, and the supernatant was used for studies employing crude extracts. Protein determination was done using the Lowry method (18).

Enzyme Purification—GDH was purified from Sf21 cell extracts essentially as described previously (17, 19). The cultured cells were subjected to two to three cycles of freeze-thawing and homogenized (5–10%
Human GDH Mutagenesis Affects Basal Activity and Regulation

RESULTS

Production of Recombinant Mutant and Wild-type GDHs—Expression of the wild-type GLUD1 gene and the four mutants in S21 cells produced recombinant GDH proteins capable of catalyzing the reversible interconversion of glutamate to α-ketoglutarate in the presence of 1 mM ADP. As described previously (14), the host insect cells (S21) used here to express human GDH contain endogenous GDH that shows an absolute specificity for NADPH. Because nontransfected insect cells showed zero GDH activity when NADP(H) was used as a cofactor, all enzyme assays of S21 cells infected with recombinant baculovirus were done in the presence of NADP(H). This
permitted the study of the recombinant human enzymes without the interference of the endogenous insect GDH.

Studies on Crude Tissue Extracts—Enzymatic assays carried out in crude tissue extracts showed that the S331T, M370L, and M415L mutants exhibited a basal catalytic activity similar to that of the wild-type GLUD1 GDH (data not shown). Additional functional analyses revealed that the S331T, M370L, and M415L mutants exhibited an allosteric regulation pattern by ADP and GTP that was comparable with that of the wild-type GLUD1-derived GDH (Table I). In contrast, the R443S mutant was essentially inactive under base-line conditions and required much higher levels of ADP for its activation (SC50 = 405.6 ± 14.8 μM) than the wild-type (SC50 = 27.4 ± 2.2 μM; p < 0.001) GDH (Table I). On the other hand, the GTP inhibition pattern of the R443S mutant was similar to that of the wild-type GDH (Table I).

Purification of Mutant and Wild-type GDH—The R443S mutant and the wild-type recombinant GLUD1-derived human GDH were studied further after they were purified from cell extracts. As an additional control, we used the endogenous human enzyme purified from human liver as previously described (17). About 40–50% of the GDH activity present in the crude tissue extracts was recovered in the purified fractions as previously described (17, 19). SDS-PAGE analysis of GDH-positive fractions eluted from the hydroxypapite column revealed that the enzyme was more than 95% pure (Fig. 2). As shown in Fig. 2, the molecular mass of the R443S mutant was identical to that of the recombinant wild-type GLUD1-derived GDH.

Catalytic and Allosteric Properties of Purified Mutant and Normal Human GDHs—Functional analyses of the purified enzymes confirmed that the R443S mutation rendered the enzyme virtually inactive in the absence of allosteric effectors (basal state). In enzyme assays performed here in the presence of 0.3–0.5 μg of purified mutant enzyme/1.0 ml of TRA, pH 8.0, buffer (see "Experimental Procedures"), no measurable basal activity was detected. Increasing the mutant enzyme up to 5 μg of purified protein/1 ml of reaction mixture yielded a basal activity (4.1 ± 0.4 μmol mg−1 min−1) that was about 7% that of the wild-type GDH (54.0 ± 4.3 μmol mg−1 min−1) (Table II). The addition of ADP to 1 mM (final concentrations) resulted in full activation of the R443S mutant with the maximal specific activity of this mutant approaching that of the wild-type GDH (Fig. 3). This activation was observed even when no measurable basal activity was detectable (in assays involving 0.3–0.5 μg of purified mutant enzyme). However, study of the ADP stimulatory curves (performed in TRA buffer, pH 8.0) revealed that the mutant enzyme was much less sensitive to ADP than the wild-type GDH (Fig. 3). At 0.1 mM ADP (concentration capable of inducing a near maximal activation of the wild-type GDH), the specific activity of the mutant remained about 13% that of the wild-type GDH (Fig. 3). The SC50 values for the R443S mutant determined in TRA, pH 8.0 buffer were about 10-fold higher than those of the wild-type expressed GDH (Table II) or the endogenous human liver enzyme (data not shown).

The addition of L-leucine to the reaction mixture (1–10 mM final concentrations) failed to activate the R443S mutant (Fig. 4). Although L-leucine when used alone was totally ineffective in stimulating the activity of the Arg-443 mutant, the presence of low concentrations of ADP (25–100 μM) rendered the mutant enzyme amenable to activation by L-leucine. As shown in Fig. 4, L-leucine induced in the presence of ADP a concentration-dependent activation of the R443S mutant, which was proportionally greater than that of the wild-type GDH. Under these conditions, L-leucine was capable of maximally activating the mutant enzyme (enhancement of the mutant enzyme activity by >2000%).

Effect of Buffer Composition and pH—The basal activities of the R443S mutant remained markedly decreased when assayed at different pH values in either TRA or phosphate buffer. Although the basal activity of the wild-type enzyme in TRA was...
somewhat higher at pH 8.0 and 7.5 than at pH 7.0, the specific activity of the R443S mutant increased 2-fold when the pH was lowered from 8.0 to 7.0 (Table II). In phosphate buffer, the basal activity of the R443S mutant GDH also remained markedly decreased compared with that of the wild-type expressed enzyme. Again, the mutant enzyme was substantially more active in phosphate at pH 7.0 than at 8.0 (Table II).

Study of the ADP stimulatory curves revealed that the R443S mutant was more sensitive to ADP activation in phosphate than in TRA, particularly at pH 7.5 and 8.0 (Table II). In contrast, the wild-type GDH was more sensitive to ADP activation in TRA than in phosphate at all pH values studied (Table II). A comparison of the two enzymes revealed that the SC50 values for the R443S mutant were about 7–10-fold and 2–3-fold higher than those for the wild-type enzyme for analyses done in TRA and in phosphate, respectively.

Kinetic Properties and GTP Inhibition—Functional analyses carried out in the presence of 1 mM ADP revealed that the kinetic properties (Km and Vmax for α-ketoglutarate) of the R443S mutant were similar to those of the GLUD1-derived GDH and the endogenous enzyme purified from human liver (Table III). Study of GTP inhibitory curves in the presence of 1 mM ADP revealed that the purified R443S mutant enzyme behaved similarly to that the wild-type GDH (data not shown), thus confirming observations obtained in crude extracts (Table I).

Structural Analysis—A study of the structure of human GLUD1-derived GDH suggests that Arg-443 lies in the “antenna-like” structure of the enzyme (25, 26). As shown in Fig. 5, Arg-443 is located near the junction of the antenna with the pivot helix, which is shown to rotate during the movement of the NAD+ binding domain that occurs with substrate binding. Modeling of the introduced Ser-443 side chain suggests that this amino acid substitution may disrupt potential hydrogen bonds that exist between Arg-443 of one subunit with Ser-409 of a neighboring GDH subunit, as shown in Fig. 5.

DISCUSSION
Here we showed that single amino acid substitution (R443S) in the regulatory domain of the human housekeeping GDH is capable of virtually abolishing the basal catalytic activity of the enzyme. In addition, the R443S mutation markedly altered the regulation of the enzyme by ADP and l-leucine without affecting its allosteric inhibition by GTP. Although the R443S mutant was amenable to activation by ADP, this activation occurred at substantially higher ADP concentrations than required for the wild-type GLUD1-derived GDH.

In addition, the R443S mutation totally abrogated the activation of the enzyme by l-leucine used in the absence of other effectors. In the presence of low concentrations of ADP, however, l-leucine induced a concentration-dependent activation of the R443S mutant, which was proportionally greater than for the wild-type GDH. The synergistic effect of ADP and l-leucine, capable of producing a full activation of the mutant enzyme activity, is similar to that observed with the use of the GLUD2-derived GDH (15).

How can the present observations be reconciled with existing data on the structure and function of mammalian GDH? GDH is a hexameric molecule composed of six identical subunits (25). Its activity can be allosterically regulated by structurally diverse compounds (1, 14–17, 26–28). However, activators are not necessary for the catalytic function of the GLUD1 GDH, as the enzyme is active in the absence of these compounds (basal state). Based on these observations, it has been suggested (27) that GLUD1 GDH assumes in its basal state a configuration (open catalytic cleft) that permits catalytic activity regardless of whether the allosteric sites are functional.
X-ray crystallographic studies of the bovine liver enzyme (25) and more recently of the human GLUD1-derived GDH (26) revealed the presence of an antenna-like region in this enzyme. This antenna is a protruding domain of the protein that consists of an ascending helix and a descending random coil strand that contains a small α-helix toward the C-terminal end of the strand (Fig. 5). In the GDH trimer, the antenna of one subunit is intertwined with the antenna of the adjacent subunits; this interaction is thought to mediate intersubunit communication responsible for allosteric regulation of the enzyme (25, 26).

Original observations on bacterial GDH have shown that during substrate binding, the NAD⁺ binding domain of this dehydrogenase moves significantly (29). Crystallographic studies on bovine and human GDH have confirmed this movement of the NAD⁺ domain, which pivots about a long α-helix (pivot helix) connected to the descending strand of the antenna. It has been suggested further that opening of the catalytic cleft is stimulated by rotating the NAD⁺ domain along the long axis of the pivot helix (25). More recent studies in human GDH (26) have shown that, in addition to this movement, the NAD⁺ binding domain twists about the antenna in a clockwise fashion.

A comparison of the open and closed conformations of the human GDH revealed striking changes in the small helix of the descending strand of the antenna, which seems to recoil as the catalytic cleft opens (26). Moreover, closure of one subunit is associated with distortion of the small helix of the descending strand that is pushed into the antenna of the adjacent subunit (26). Residue Arg-443 is located on this small helix (Fig. 5). As shown in Fig. 5, the side chain of Arg-443 of one GDH subunit forms one or two H-bonds with the side chain of Ser-409 (part of the ascending strand of the antenna) from the adjacent subunit. This interaction is observed in both the open and closed conformations. Substitution of Ser for Arg-443 will result in the loss of this interaction thus affecting intersubunit communication.

As noted above, Arg-443 is located in the small helix of the descending strand of the antenna, which undergoes the greatest conformational changes upon the opening or closing of the active site. For the enzyme to function it needs to cycle between the open and closed states. It is thus likely that substitution of Ser for Arg-443 favors the closed enzyme conformation associated with the severely decreased basal activity of the mutant protein as observed here.

ADP can bind to both the open and closed states of the mammalian GDH (25) and is thought to facilitate the opening of the active site or to prevent hyper closure (26). Our findings suggest that relatively high concentrations of ADP are needed to allow the mutant enzyme to shift often enough to the open state for catalysis to occur. Also, our data showing that substitution of Ser for Arg-443 totally abrogated the ability of L-leucine to activate the enzyme further suggest that this mutation favors the closed enzyme conformation, as L-leucine is thought to bind at the active site (30). Closure of the catalytic cleft will prevent L-leucine from entering this site. Also, the fact that some minimal concentration of ADP (sufficient to double or triple the extremely low base-line activity of the mutant) is required to allow L-leucine to exert its effect is consistent with the possibility that opening of the catalytic site by ADP is necessary for L-leucine to act.

Mammalian GDH is known to polymerize (31). A loss of subunit interaction in the antenna region, resulting from the introduced R443S mutation, may affect enzyme polymerization, as the antenna is involved in this hexamer-hexamer association (25). The ability of ADP to restore the activity of the mutant is consistent with this possibility, because this nucleotide promotes the aggregated state of the enzyme (31). Also, the improved function of R443S mutant in phosphate (observed here in the presence of ADP) may relate to the stabilizing effect.
of phosphate on GDH (32), as also reported for the S448P mutant described by Fang et al. (27). Ser-448 is also located in the descending strand of the antenna and its replacement by Pro is associated with decreased basal activity (27). However, the S448F mutation is associated with substantially higher basal activity in phosphate, pH 8.0 (19 ± 1.6 μmol min⁻¹ mg⁻¹), than the R443S mutation (3.8 ± 0.9 μmol min⁻¹ mg⁻¹) studied here. Also, the S448P mutation does not alter the activation of the mutant by l-leucine and ADP. In addition, it affects the GTP regulation significantly (27). Hence, the functional properties of the R443S mutant, as reported here, are substantially different that those of S448P mutant (27).

Analysis of the GDH structure also indicates that the residues involved in the mutations S331T and M415L studied here are surface residues and that their side chains are not involved in contacts with neighboring residues or co-factors. This observation is consistent with the fact that the mutations do not affect the activity of the enzyme. Met-370, involved in the mutation M370L, is buried and is not involved in any contact with co-factors or substrates. Its substitution with leucine is conservative and likely does not disturb the local packing of the molecule, which is consistent with the absence of any change in activity.

There is evidence that in the human the GLUD1 gene (located on the 10th chromosome) has been retro-posed to the X chromosome, where it gave rise to the intronless GLUD2 gene through random mutations and natural selection. As noted above, the GLUD2 gene might have adapted to the particular needs of the nervous system where it is specifically expressed (14). The present data, showing that replacement of Arg-443 by Ser renders the enzyme inactive (in the absence of GTP) and totally dependent on ADP for its function, provides a novel molecular mechanism for the regulation of GDH. Dependence on ADP should permit the recruitment of the enzyme under conditions of low energy charge (a high ADP:ATP ratio) such as those occurring under intense glutamatergic neurotransmission (16).

In addition, the present findings showing that replacement of Arg-443 by Ser renders GDH more active at pH 7.0 than at 8.0 may be of importance for glutamate metabolism in the nervous system. It is well known that the glutamate neurotransmitter action is terminated by uptake into glial cells (10). In the salamander Müller cells (a specialized type of glial cells found in the retina), glutamate uptake generated significant intracellular acidification (the pH dropped from approximately 7.2 to approximately 7.0) due to countertransport of OH⁻ (33, 34). This glutamate-induced acidification occurred in parallel with a rise in NAD+/PH⁺ that is thought to result from glutamate oxidation via GDH (34).

The results of the present investigations further our understanding of this important protein, particularly with respect to its proposed structural and functional models. Although, the introduced R443S mutation abolished the basal activity of the enzyme, this mutation did not reproduce other functional properties of the GLUD2 GDH. Previous studies (15) have shown that the GLUD2 GDH can be stimulated by l-leucine (in the absence of ADP) and by lower concentrations of ADP than required for activation of the R443S mutant. Also, resistance of the GLUD2 GDH to GTP inhibition (15) represents another difference between the functional properties of this nerve tissue-specific isoenzyme and those of the R443S GLUD1 mutant. In this regard, we have recently shown (17) that this GTP resistance relates to the presence of Ala rather than Gly at position 456 of the GLUD2 GDH. Additional studies are needed to clarify the structural basis of the functional diversity of human GDH and to provide insights into the role of this enzyme in the biology of mammalian systems.

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