Single Amino Acid Substitution (G456A) in the Vicinity of the GTP Binding Domain of Human Housekeeping Glutamate Dehydrogenase Markedly Attenuates GTP Inhibition and Abolishes the Cooperative Behavior of the Enzyme*

Ioannis Zaganas and Andreas Plaitakis‡

From the Department of Neurology, University of Crete, School of Health Sciences, Section of Medicine, Heraklion, 71500 Crete, Greece

Human glutamate dehydrogenase (GDH) exists in two isoforms encoded by the GLUD1 and GLUD2 genes, respectively. Although the two enzymes share in their mature form all but 15 of their 505 amino acids, they differ markedly in their allosteric regulation. To identify the structural basis for these allosteric characteristics, we performed site-directed mutagenesis on the human GLUD1 gene at sites that differ from the GLUD2 gene using a cloned GLUD1 cDNA. Results showed that substitution of Ala for Gly-456, but not substitution of His for Arg-470 or Ser for Asn-498, renders the enzyme markedly resistant to GTP inhibition (IC50 = 2.80 μM) as compared with the wild type GLUD1-derived GDH (IC50 = 0.19 μM). The G456A mutation abolished the cooperative behavior of the enzyme, as revealed by the GTP inhibition curves. The catalytic and kinetic properties of the G456A mutant and its activation by ADP were comparable with those of the wild type GDH. Gly-456 lies in a very tightly packed region of the GDH molecule, and its replacement by Ala may lead to steric clashes with neighboring amino acids. These, in turn, may affect the conformational state of the protein that is essential for the allosteric regulation of the enzyme by GTP.

Glutamate dehydrogenase (GDH)1 (E.C.1.4.1.3) catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate using NAD(H) or NADP(H) as cofactors (1). The mature GDH protein is composed of six identical subunits consisting of 505 amino acids each. The enzyme is thought to play a key role in cellular metabolism and energy homeostasis (2). In the pancreatic β cells, GDH is thought to be involved in insulin secretion mechanisms, whereas in the nervous system the enzyme may play a role in the metabolism of neurotransmitter glutamate and in neurodegenerative processes (3, 4).

GDH in humans exists in two different isoforms: a housekeeping and a nerve tissue-specific isoenzyme encoded by the GLUD1 and the GLUD2 gene, respectively (5–8). GLUD1 contains 13 exons and is located on the 10th chromosome, whereas the GLUD2 gene lacks introns and is X-linked. Mammalian GDH is shown to be allosterically regulated by diverse compounds, such as purine nucleotides, steroid hormones, etc (1). GDH regulation is of particular biological importance as exemplified by observations showing that regulatory mutations of the GLUD1 GDH are associated with clinical manifestations in children (9).

Although the two GDH isoenzymes are highly homologous (showing a 97% amino acid identity), they differ markedly in their regulatory properties (8, 10). Thus, while the GLUD1-derived GDH is sensitive to GTP inhibition, the GLUD2 GDH is resistant to this compound. In contrast, the GLUD2 GDH is much more sensitive to allosteric activation by ADP or L-leucine than the GLUD1-derived enzyme (10). In addition, there are significant differences between the two isoforms with respect to the Km values for the substrates of the enzyme. Because the GLUD1- and GLUD2-derived polypeptides share in their mature form all but 15 of their 505 amino acids, these functional differences must arise from amino acid residues that are not common between the two isoenzymes. Our objective is to identify by mutagenesis of the GLUD1 gene these critical residues. In this study, we selected three such residues (Gly-456, Arg-470, and Asn-498) located in the C-terminal region, which is thought to be part of the regulatory domain of mammalian GDH (11). Using site-directed mutagenesis, we created three GLUD1 mutants, each containing one of these amino substitutions. 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 to homogeneity and studied with respect to their kinetic and regulatory characteristics. Results showed that substitution of Ala for Gly at position 456 (but not substitution of His for Arg-470 or Ser for Asn-498) of the GLUD1 GDH markedly attenuated GTP inhibition and abolished the cooperative behavior of the enzyme. The G456A substitution did not affect the allosteric activation of the mutant GDH by ADP or its kinetic properties as determined in the absence of allosteric inhibitors. The structural, functional, and evolutionary implications of these findings are discussed.

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 Invitrogen. Modified baculovirus (BaculoGold) was obtained from BD Pharmingen (San Diego, CA). NADPH, ADP, GTP (lithium salt), and bovine liver glutamate dehydrogenase were from Roche Molecular Biochemicals (Mannheim, Germany). Glutamic acid (monosodium salt) was from Sigma. Phenyl-Sepharose high

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† To whom correspondence should be addressed: Dept. of Neurology, University of Crete, School of Health Sciences, Section of Medicine, 71500 Heraklion, Crete, Greece. Tel.: 30-810-394648; Fax: 30-810-394639; E-mail: plaitakis@med.uoc.gr.

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Human GDH Mutagenesis Affects GTP Inhibition

Site-directed Mutagenesis of the GLUD1 cDNA—A GLUD1 cDNA, cloned in pBSKII+ vector, was mutagenized at specific sites (Fig. 1) using the Gene Editor Mutagenesis 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 by slowly cooling it (1.5 °C/min) to 37 °C. The annealing reaction also contained primers (provided by the manufacturer) designed to mutate the β-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 BMH 71–15 mut5 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 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 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, Nebraska).

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 as previously described (7, 10). Cells of the insect Spodoptera frugiperda (SF21) were co-infected 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 postinfection and used for extracting the recombinant GDH proteins. For this, the cultured 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 for 10 min, and the supernatant was used for studies employing hydrophobic interaction chromatography as previously described (14). For this, fractions of the hydrophobic interaction column containing GDH activity were dialyzed (at 4 °C) against several changes of 100 mM Tris-HCl, 200 mM KCl, pH 7.15, buffer and loaded into the hydroxyapatite column (14). The column was eluted with a gradient of 10–400 mM sodium phosphate buffer, pH 7.4. Purified GDH was used for enzyme assays and for studying its electrophoretic mobility by SDS-PAGE. The latter was done using the Laemmli procedure (15).

Enzyme Purification—GDH was purified from SF21 cell extracts and from human liver obtained at autopsy. About 1 g of liver tissue and 200 × 10⁶ infected SF21 cells were used for enzyme purification, which was carried out by modification of our previously published method (13). The tissues were subjected to two to three cycles of freeze thaw and homogenized (5–10% w/v) in 10 mM Tris-HCl, pH 7.4, buffer containing 0.1 mM EDTA, 0.5 mM NaCl, 1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride. 50–55% ammonium sulfate (AS) cut obtained from these extracts was resuspended in 50 mM Tris-HCl, pH 6.0, buffer containing 15% AS, loaded on a hydrophobic interaction column packed with phenyl-Sepharose high performance (Amersham Biosciences), and equilibrated with the same buffer. The column was eluted with a double gradient of decreasing concentration of AS (15–0%) and increasing concentration of ethylene glycol (0–90%). Eluted GDH was precipitated with 60% AS and passed again through the same phenyl-Sepharose column. Because the G456A mutant has decreased affinity for GTP, we did not employ a GTP binding column (13). Instead, we used hydroxypatite chromatography as previously described (14). For this, fractions from the hydrophobic interaction column containing GDH activity were dialyzed (at 4 °C) against several changes of 100 mM Tris-HCl, 200 mM KCl, pH 7.15, buffer and loaded into the hydroxyapatite column (14). The column was eluted with a gradient of 10–400 mM sodium phosphate buffer, pH 7.4. Purified GDH was used for enzyme assays and for studying its electrophoretic mobility by SDS-PAGE. The latter was done using the Laemmli procedure (15).

Enzyme Assays, Kinetic, and Allosteric Regulation Studies—Enzyme activity was assayed spectrophotometrically at (340 nm) in the direction of reductive amination of α-ketoglutarate (10). The reaction mixture of 1 ml contained 50 mM triethanolamine buffer, pH 8.0, 100 mM ammonium acetate, 100 µM NADPH, and 2.6 mM EDTA. NADPH was used instead of NADPH only to test the activity of the endogenous insect cell GDH. Enzyme reaction was initiated by adding α-ketoglutarate to 8 mM (except as indicated). Initial rates (enzyme velocity during the first 30 s after initiation of the reaction) were recorded. Wild type and mutant GLUD1 proteins were studied in parallel.

Kinetic analyses were performed to determine the Michaelis-Menten constant (Km) for α-ketoglutarate and NADPH. Several sets of experiments were performed for each purified enzyme. In each of these experiments, α-ketoglutarate varied from 0.4 to 8.0 mM, while ADP concentration was kept constant at 0 (base line), 25, or 250 µM. NADPH varied from 10 to 100 µM in the absence of ADP.

Fig. 1. Schematic representation of the C-terminal region of GLUD1 and GLUD2 GDHs and of the three mutants (G456A, R470H, N498S). Dots indicate amino acid residues of the GLUD2 and the mutant GDHs that are identical to those of the wild type GLUD1-derived enzyme.

Fig. 2. GTP inhibition of the recombinant G456A mutant GDH and the effect of ADP. Enzyme assays were performed in the direction of reductive amination of α-ketoglutarate in the presence of increasing concentrations of GTP. ADP concentration was kept constant at 0 mM (No Additions), 0.1, and 1 mM. Data points represent mean values of at least two experimental determinations and are expressed as percentage of baseline activity obtained in the absence of GTP. ADP displaces the GTP inhibition curve of the G456A mutant to the right, as is the case for the normal enzyme. However, in comparison to the normal GLUD1-derived GDH, IC₅₀ values for GTP inhibition are 8–15 times higher and the curve is rather hyperbolic instead of sigmoidal.
Regulation of the human recombinant GDHs by GTP was studied essentially as previously described (10) by adding this compound to the reaction mixture at various concentrations (0.025–1000 μM, final concentrations) while keeping the other substrates constant. Also, GTP inhibition was studied in the presence of ADP (0.1 or 1 mM, final concentrations). To study the allosteric activation of recombinant GDHs by ADP, this compound was added to the reaction mixture at various concentrations (varying from 0.01 to 1 mM), while the concentration of the other substrates was kept constant as indicated above.

**Statistical Analyses**

All statistical analyses on the obtained data and plotting were performed by the use of the Origin Program (MicroCal Software, Northampton, MA). $K_m$ and $V_{max}$ values were calculated by application of the weighted hyperbolic fit method in the Hyper program (Dr. J. S. Easterby, Dept. of Biochemistry, University of Liverpool, Liverpool, UK). Differences in kinetic and allosteric behavior were evaluated using Student’s t test. IC$_{50}$ and SC$_{50}$ values were determined graphically. The Hill plot coefficients for GTP inhibition were calculated according to the method discussed by Cornish-Bowden (16). Results were confirmed by the use of the Leonora program (17). Studies of the structural models of bovine GDH were performed with the use of the RasMol© (version 2.7.1.1, R. Sayle), Swiss-PDBViewer© (version 3.7.b2, N. Guex) and Quanta (Accelrys Inc.) programs.

**RESULTS**

**Production of Recombinant Mutant and Wild Type GDHs—** Expression of the G456A, R470H, and N498S GLUD1 mutants and the wild type GLUD1 gene in Sf21 cells produced GDH proteins capable of catalyzing the reversible interconversion of glutamate to α-ketoglutarate in the presence of either NAD(H)

### Table I

| IC$_{50}$ (µM) | Hill coefficient | No activators | 0.1 mM ADP | 1 mM ADP |
|---------------|------------------|---------------|------------|----------|
| G456A         | R470H            | N498S         | GLUD1      |          |
| G456A         | R470H            | N498S         | GLUD1      |          |
| No activators | 2.80 ± 0.15      | 0.17 ± 0.01   | 0.20 ± 0.01| 0.19 ± 0.01| 1.12 ± 0.06 |
| 0.1 mM ADP    | 8.98 ± 0.25      | 0.85 ± 0.09   | 0.93 ± 0.04| 1.02 ± 0.03| 1.30 ± 0.07 |
| 1 mM ADP      | 79.55 ± 3.67     | 10.48 ± 0.32  | 11.81 ± 0.39| 12.23 ± 0.54| 1.07 ± 0.04 |

* Indicates that the IC$_{50}$ and Hill coefficient values for the G456A mutant are significantly different (p < 0.001) as compared to those of the wild type GLUD1-derived GDH, the R470H mutant, and the N498S mutant.

**FIG. 3.** Comparison of GTP inhibition curves of G456A, R470H, and N498S mutant GDHs and the wild type GLUD1-derived GDH (GLUD1). Data points represent mean values of at least two experimental determinations and are expressed as percentage of baseline activity (no GTP added). GDH activity was measured in the direction of reductive amination of α-ketoglutarate in the presence of increasing concentrations of GTP. A, GTP inhibitory curves for the four recombinant enzymes obtained in the absence of ADP, B, GTP inhibitory curves for the four recombinant enzymes obtained in the presence of 0.1 mM ADP. Under both conditions, the G456A mutant is resistant to GTP concentrations that render the wild type GDH essentially inactive. The R470H and the N498S mutant GDHs retain the allosteric properties of the wild type enzyme.

**FIG. 4.** 12% SDS-PAGE analysis of purified human GDHs. HLiv, GDH purified from human liver; G456A, expressed G456A mutant GDH; GL1, expressed wild type GLUD1-derived GDH; MW, molecular weight marker proteins; BLiv, bovine liver GDH.

Regulation of the human recombinant GDHs by GTP was studied essentially as previously described (10) by adding this compound to the reaction mixture at various concentrations (0.025–1000 μM, final concentrations) while keeping the other substrates constant. Also, GTP inhibition was studied in the presence of ADP (0.1 or 1 mM, final concentrations). To study the allosteric activation of recombinant GDHs by ADP, this compound was added to the reaction mixture at various concentrations (varying from 0.01 to 1 mM), while the concentration of the other substrates was kept constant as indicated above.

**Statistical Analyses—** All statistical analyses on the obtained data and plotting were performed by the use of the Origin Program (MicroCal Software, Northampton, MA). $K_m$ and $V_{max}$ values were calculated by application of the weighted hyperbolic fit method in the Hyper program (Dr. J. S. Easterby, Dept. of Biochemistry, University of Liverpool, Liverpool, UK). Differences in kinetic and allosteric behavior were evaluated using Student’s t test. IC$_{50}$ and SC$_{50}$ values were determined graphically. The Hill plot coefficients for GTP inhibition were calculated according to the method discussed by Cornish-Bowden (16). Results were confirmed by the use of the Leonora program (17). Studies of the structural models of bovine GDH were performed with the use of the RasMol© (version 2.7.1.1, R. Sayle), Swiss-PDBViewer© (version 3.7.b2, N. Guex) and Quanta (Accelrys Inc.) programs.

**RESULTS**

**Production of Recombinant Mutant and Wild Type GDHs—** Expression of the G456A, R470H, and N498S GLUD1 mutants and the wild type GLUD1 gene in Sf21 cells produced GDH proteins capable of catalyzing the reversible interconversion of glutamate to α-ketoglutarate in the presence of either NAD(H)
or NADP(H). The expressed proteins were about 5-fold enriched over the endogenous insect cell GDH (data not shown). Baculovirus DNA was isolated from culture media of infected cell cultures and sequenced. Results confirmed that the desired mutation for the corresponding mutant GDHs was present in the virus shed by the infected cells.

Enzyme assays, carried out with the use of either NADH or NADPH as cofactors, revealed that the non-infected host insect cells contained endogenous GDH showing an absolute specificity for NADH in accordance with results of previous studies (7). In contrast, the mammalian-expressed enzymes are capable of using both cofactors. Because non-infected insect cells showed zero GDH activity when assayed with the use of NADPH, all enzyme assays of extracts of Sf21 cells infected with recombinant baculovirus were done in the presence of NADPH. This permitted the study of the recombinant human enzymes in crude extracts without the interference of the endogenous insect GDH.

**Allosteric Regulation of Mutant and Normal GLUD1 GDHs**—Study of the allosteric properties of the produced mutant GDHs (G456A, R470H, and N498S mutants), carried out in crude tissue extracts, revealed that only the G456A mutant exhibited a marked resistance to GTP inhibition (Fig. 2). Under baseline conditions, the IC50 for GTP inhibition was about 15-fold higher for the mutant protein (2.8 μM) as compared with the wild type GLUD1 GDH (0.19 μM) (Table I and Fig. 3A).

Similar results were obtained when GTP inhibition was studied using reaction mixtures of different pH values (7.0–8.0) (data not shown). Also, the differences in GTP sensitivity between the G456A mutant and the normal GLUD1-derived GDH persisted when GTP inhibition was studied in the presence of ADP (0.1 and 1.0 mM, final concentrations) (Table I and Figs. 2 and 3B). In addition, the G456A mutation abolished the cooperative behavior of the enzyme either in the presence or absence of ADP. As shown in Figs. 2 and 3, the GTP inhibition of the G456A mutant lacked the characteristic sigmoidal curve

### Table II

|       | Vmax | Km | Km | Km | Km |
|-------|------|----|----|----|----|
|       | μmol/min/mg | μM | μM | μM | μM |
| G456A | 158.71 | 40.56 ± 5.02 | 1.38 ± 0.48 | 1.49 ± 0.51 | 1.27 ± 0.32 |
| Expressed GLUD1 | 152.95 | 40.36 ± 5.48 | 1.39 ± 0.26 | 1.37 ± 0.44 | 1.44 ± 0.40 |
| Human liver GDH | 137.05 | 32.03 ± 4.70 | 1.26 ± 0.25 | 1.17 ± 0.44 | 1.28 ± 0.15 |

*Vmax* for the purified enzymes was determined in the presence of 250 μM ADP.

*Km* values ±95% confidence intervals are based on experiments carried out with different substrate concentrations as indicated under “Experimental Procedures”. For each substrate, at least six experimental determinations were performed.

![Molecular model of mammalian GDH](image)

**Fig. 5. Molecular model of mammalian GDH.** A, ribbon diagram of one trimer of the GDH homohexamer based on x-ray crystallographic structure of bovine GDH (11) showing the bound GTP (green) and the two mutations (red). The amino acid numbering of the bovine enzyme (11) is retained. Each of the three subunits is painted in a different color (green, blue, and yellow). B, close up of the G452A (corresponds to G456A of the human GLUD1 GDH) mutation site. The dotted lines indicate possible steric clashes. C, close up of the N494S (corresponds to N498S of the human GLUD1 GDH) mutation site. This analysis was performed by Dr. Michael Karpusas, a Scientist at Biogen and an assistant professor of biochemistry, University of Crete.
the hydroxypatite column revealed that the enzyme was more than 95% pure (Fig. 4). As shown in Fig. 4, the molecular mass of the G456A mutant was identical to that of the recombinant wild type GLUD1-derived GDH and that of the endogenous mature human liver enzyme. Human liver is known to express the GLUD1 gene only (7). This purified human GDHs were slightly larger than the commercially available bovine liver GDH used as a marker (Fig. 4). This is consistent with sequencing data showing the bovine liver enzyme is four amino acids shorter than the human GDHs (18, 19).

The GDH-specific activity (measured at 8.0 mM α-ketoglutarate and in the presence of 1 mM ADP) of the purified G456A mutant (133.97 μmol of NADPH oxidized min⁻¹ mg protein⁻¹) was comparable with that of the recombinant wild type GLUD1-derived GDH (130.50 μmol of NADPH oxidized min⁻¹ mg protein⁻¹). Also, the endogenous human liver GDH gave a similar GDH-specific activity (124.56 μmol of NADPH oxidized min⁻¹ mg protein⁻¹).

Kinetic analyses revealed that the \( K_\text{m} \) values for α-ketoglutarate and for NADPH for the purified G456A mutant were similar to those for the purified recombinant wild type GLUD1 GDH and for the purified endogenous human liver enzyme (Table II). In addition, the maximum velocities \( (V_{\text{max}}) \) of the three purified human GDHs were comparable (Table II).

GTP inhibition studies performed with the use of the three purified enzymes confirmed the above described data on crude extracts by showing that the purified G456A mutant was markedly resistant to GTP inhibition \( (IC_{50} = 121.35 ± 14.89 \mu M \text{ GTP determined in the presence of } 1 \text{ mM ADP}) \) as compared with the wild type recombinant GLUD1 enzyme \( (IC_{50} = 12.95 ± 0.46 \mu M \text{ GTP}) \). The endogenous human liver GDH was as sensitive to GTP inhibition \( (IC_{50} = 15.57 ± 0.57 \mu M) \) as the recombinant normal GLUD1 enzyme. Hill analyses also confirmed that GTP inhibition of the purified G456A mutant lacked the cooperativity \( (HC = 0.99 ± 0.07) \) shown by the purified recombinant wild type GLUD1 GDH \( (HC = 2.15 ± 0.16) \) and the purified human liver enzyme \( (HC = 2.17 ± 0.06) \).

**Structural Models—**Study of the structure of mammalian GDH based on x-ray crystallography of bovine liver GDH (11) suggests that Gly-456 of the human GLUD1 enzyme (corresponds to Gly-452 of the bovine protein) lies in a tightly packed region of the molecule (Figs. 5 and 6). Modeling of the introduced Ala-456 side chain suggests that this chain may be in steric clash with the side chain of Phe-387 of the mutant enzyme (corresponds to Phe-383 of the bovine enzyme) (Fig. 5). The estimated distance between Ala-456 and Phe-387 is \( \sim 2.8 \) Å. This is short enough to indicate a steric clash that may alter the conformation of the Phe-387 side chain, which in turn may affect the nearby Leu-401 (corresponds to Leu-397 of the bovine GDH) side chain from a neighboring subunit. Another steric clash \( (2.3 \) Å) apparent from this model is between the Cβ of Ala-456 and the carbonyl oxygen of residue 452 in the human enzyme (corresponds to residue 448 in the bovine sequence). This clash occurs assuming that the main chain angles do not change after replacement of Gly-456 with Ala. The proximity of these residues to the GTP binding site is shown in Fig. 5.

**DISCUSSION**

To study structure-function relationships in human glutamate dehydrogenase, we performed site-directed mutagenesis in the C-terminal region of the GLUD1 gene. Three residues (Gly-456, Arg-470, and Ser-498) that are different in the GLUD1 as compared with the GLUD2 gene were selected for these studies (Fig. 1). Results showed that replacement of Gly by Ala at site 456 markedly attenuates GTP inhibition and
abolishes the cooperative behavior of the enzyme without affecting its kinetic properties (in the absence of allosteric inhibitors). In contrast, substitution of His for Arg-470 or substitution of Ser for Asn-498 did not alter significantly either the allosteric regulation or the catalytic properties of the enzyme.

Our recombinant human GDHs are processed in the cultured insect cells in a manner similar to that of the mammalian GDH, which involves removal of the 53-amino acid-long leader sequence predicted by the GLUD1 cDNA (5, 8). Expressed human GLUD1-derived recombinant proteins purified from our cell extracts, as done here, show the same molecular mass as the mature human protein (Fig. 4) and an N-terminal amino acid sequence (Ser-Glu-Ala-Val-Ala . . . ) identical to that obtained by sequencing of GDH purified from human liver (7, 19).

Cho et al. (20) recently expressed in E. coli bacterial cells a synthetic human GLUD1 gene lacking the sequences that predict the leader peptide. They produced a recombinant GDH protein that had at the N terminus five additional amino acids, which were subsequently removed using the factor Xa. Lee et al. (21) have also used this system to identify Lys-450 as a site for GTP binding. The Gly-456 site studied here lies about a helix turn away from Lys-450, with the side chain of the former amino acid residue protruding out of the helix in the opposite direction than the side chain of the latter according to the proposed structural models (11).

Studies based on chemical modification of bovine brain GDH have indicated that Gly-456 of the human GLUD1 GDH (corresponds to Gly-452 of bovine sequence) lies in the vicinity of the GTP binding domain (22). Structural models of mammalian GDH derived from x-ray crystallographic studies, have suggested that Gly-456 belongs to the α-helix of the GDH protein that comes into direct contact with GTP (11, 23, 24). Specifically, Gly-456 is located at the base ("hinge region") of an "antenna-like" region of GDH, which is thought to be important for the interaction between the subunits of the catalytically active enzyme (11).

In its tertiary form, GDH is a hexameric molecule composed of two trimers. Each of the three polypeptide chains of the trimer includes an α-helix to which GTP binds, as noted above. Gly-456 lies exactly at the point where the GTP-binding α-helix of one subunit comes in direct contact with the antenna-like region of the other (Figs. 5 and 6). As described under "Results," introduction of Ala-456 side chain occurs in a tightly packed region of the molecule, and this may lead to steric clashes with other side chains (Fig. 5). These disturbances may impair the ability of the antenna to facilitate intersubunit communication, a process that may be essential for GTP inhibition and cooperative effects (11).

In contrast to the G456A mutation, substitution of His for Arg-470 or of Ser for Asn-498 did not affect the properties of the GLUD1-derived GDH. Arg-470 is located on the surface of the protein (Fig. 6) where it does not appear to interact with other amino acids. Also, Asn-498 is not located in a tightly packed region of the GDH molecule; its only possible interaction is with Arg-48 (corresponds to Arg-44 in the bovine) (Fig. 5), and as such, the present results suggest that this interaction may not be essential for enzymatic function.

Stanley et al. (9) have described mutations affecting the regulatory domain of the GLUD1-derived GDH in children with the hyperinsulinism-hyperammonemia syndrome. Additional cases have been described by these and other investigators (25–30). GTP inhibition of the mutant proteins, expressed in COS cells or in cultured patient lymphoblasts, was found to be attenuated (the IC_{50} increased 2–6-fold). The Gly-456 residue studied here, lies immediately downstream of the amino acid residues encoded by the 11th and 12th exons of the GLUD1 gene and which are altered by these spontaneous mutations.

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 GLUD2 gene through random mutations and natural selection. Previous functional analyses of the two GDH-specific genes have suggested that the GLUD2 gene might have adapted to particular needs of the nervous system where it is specifically expressed (3). The nerve tissue-specific GDH (GLUD2-derived) that is resistant to GTP inhibition differs at its C-terminal region in three positions (amino acid residues 456, 470, and 498) from the GLUD1 GDH, which is GTP-sensitive (Fig. 1). Our functional analysis showed that only substitution of Ala for Gly-456 is sufficient to confer resistance to GTP. This resistance may permit the nerve tissue-specific GDH to function in the GTP-rich environment that prevails in the nervous tissue.

The present data, however, indicate that the presence of Ala instead of Gly at position 456 in the GLUD2 GDH is not sufficient to explain other functional differences that exist between this and the GLUD1 GDH. These include: a diminished catalytic activity of GLUD2 isoenzyme in the absence of allosteric activators and a marked sensitivity of this isoenzyme to ADP and to l-leucine activation (8, 10). Hence, additional studies are needed to identify the residues responsible for these functional properties unique to the nerve tissue-specific GLUD2 GDH.

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Ioannis Zaganas and Andreas Plaitakis

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