
Molecular Basis and Characterization of the Hyperinsulinism/Hyperammonemia Syndrome

Predominance of Mutations in Exons 11 and 12 of the Glutamate Dehydrogenase Gene

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Glutamate dehydrogenase (GDH) is allosterically activated by the amino acid leucine to mediate protein stimulation of insulin secretion. Children with the hyperinsulinism/hyperammonemia (HI/HA) syndrome have symptomatic hypoglycemia plus persistent elevations of plasma ammonium. We have reported that HI/HA may be caused by dominant mutations of GDH that lie in a unique allosteric domain that is encoded within GDH exons 11 and 12. To examine the frequency of mutations in this domain, we screened genomic DNA from 48 unrelated cases with the HI/HA syndrome for exon 11 and 12 mutations in GDH. Twenty-five (52%) had mutations in these exons; 74% of the mutations were sporadic. Clinical manifestations included normal birth weight, late onset of hypoglycemia, diazoxide responsiveness, and protein-sensitive hypoglycemia. Enzymatic studies of lymphoblast GDH in seven of the mutations showed that all had reduced sensitivity to inhibition with GTP, consistent with an increase in enzyme activity. Mutations had little or no effect on enzyme responses to positive allosteric effectors, such as ADP or leucine. Based on the three-dimensional structure of GDH, the mutations may function by impairing the binding of an inhibitory GTP to a domain responsible for the allosteric and cooperativity properties of GDH. *Diabetes* 49:667–673, 2000

Congenital hyperinsulinism is the most frequent cause of persistent hypoglycemia in infancy (1). A severe, neonatal-onset form of the disorder is caused by recessive mutations of the β -cell sulfonylurea receptor or its associated potassium ion pore that

are encoded by the SUR1 and Kir6.2 genes on chromosome 11p (2–5). A milder dominantly inherited form of hyperinsulinism has been reported in one family with a gain of function mutation of glucokinase, the gene that is also involved in one form of maturity-onset diabetes of the young (MODY2) (6). We recently identified a second autosomal dominant form of hyperinsulinism in which the affected children have both hypoglycemia and persistently elevated plasma concentrations of ammonium (7–9). This hyperinsulinism/hyperammonemia (HI/HA) syndrome is associated with mutations of glutamate dehydrogenase (GDH) that produce increased enzyme activity as a consequence of impairing inhibitory control of the enzyme by its allosteric effector, GTP (10,11).

The fact that altered regulation of GDH causes hyperinsulinism indicates the importance of this enzyme in controlling the release of insulin by pancreatic β -cells. Leucine stimulation of insulin secretion is mediated by leucine acting as an allosteric activator of GDH to increase the rate of glutamate oxidation (12,13). In the pancreas of children with the HI/HA syndrome, the increase in GDH activity associated with impaired GTP inhibition leads to inappropriate secretion of insulin. The HI/HA syndrome also demonstrates that GDH is important for regulating ammonium metabolism in the liver. In HI/HA children, excessive GDH enzyme activity may cause hyperammonemia through two mechanisms. The first is a direct increase in the release of ammonium via the oxidative deamination of glutamate to α -ketoglutarate. The second, indirect consequence of excessive GDH activity is to reduce the high intrahepatic concentrations of glutamate that are necessary for synthesis of N-acetylglutamate, a required allosteric activator of urea synthesis (14).

The domain encoded by exons 11 and 12 of human GDH is thought to be important for allosteric regulation, since it is not conserved in prokaryotic forms of GDH that lack allosteric regulation (15). In the first HI/HA patients examined, all had identifiable missense mutations that clustered into a small region within exons 11 and 12 of GDH (10). Thus, the HI/HA syndrome appears to reflect the loss of this important allosteric control function of mammalian GDH. The purposes of the present study were to test the hypothesis that mutations in GDH exons 11 or 12 are a frequent cause of the HI/HA syndrome and to correlate these mutations with their clinical manifestations and abnormalities of enzyme function.

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GDH, glutamate dehydrogenase; HI/HA, hyperinsulinism/hyperammonemia; IC₅₀, half-maximal inhibition; PCR, polymerase chain reaction; SC₅₀, half-maximal stimulation.

TABLE 1
Mutations of GDH in HI/HA patients

Exon	Mutation	Amino acid	Restriction enzyme sites	
			Wild-type site	Mutant site
11	C1492A	Phe440Leu	MaeIII	—
11	A1494G	Gln441Arg	BsmAI	—
11	C1506T*	Ser445Leu	EcoRV	—
12	G1508C	Gly446Arg	—	—
12	G1508A*	Gly446Ser	—	TaqI
12	G1509A*	Gly446Asp	—	NsiI
12	G1509T	Gly446Val	—	—
12	G1511A	Ala447Thr	SfaNI	—
12	T1514C*	Ser448Pro	SfaNI	—
12	A1520G	Lys450Glu	—	MnII
12	C1532T*	His454Tyr	Alw44I	RsaI

*Previously identified (10).

RESEARCH DESIGN AND METHODS

Peripheral blood specimens were obtained from 48 probands with the HI/HA syndrome for isolation of DNA and establishment of transformed lymphoblasts. Clinical information on seven of these probands has been reported previously (7-9,16,17). GDH mutations in the first 8 of the 48 families have been reported (10). The diagnosis of hyperinsulinism was based on clinical features, including hypoglycemia with inadequate suppression of plasma insulin concentrations (>2 µU/ml) and evidence of excessive insulin action, such as suppressed levels of plasma β-hydroxybutyrate (<2.0 µmol/l) and free fatty acids (<1.0 mmol/l) and an inappropriately large glycemic response to glucagon stimulation (>30 mg/dl). In all of the patients, plasma ammonium concentrations were elevated (Children's Hospital normal <35 µmol/l). Clinical information on patients and their families was provided by the participating investigators. Studies were reviewed and approved by the Children's Hospital institutional human subject review board, and written informed consent was obtained from subjects or their parents.

Genomic DNA was extracted from lymphoblasts, and GDH exons 11 and 12 were amplified by polymerase chain reaction (PCR) for direct sequencing (10). For exon 11, the forward primer was 5'-TTCTGTTAGCAATATCTTGATCTCTTG-3'. Other primers were as previously reported (10). Mutations were confirmed by repeat PCR amplification and resequencing or restriction enzyme digest analysis. Nucleotides in GDH cDNA were numbered according to the sequence reported by Nakatani et al. (18). Amino acids were numbered according to the sequence of the mature peptide, omitting the 53 amino acid leader sequence.

Lymphoblast GDH enzyme activity and allosteric responsiveness were determined as previously described (10), with the exception of including Triton X-100 at a final concentration of 1% in the homogenization buffer to ensure complete cell disruption. GDH activity was measured spectrophotometrically in the direction of glutamate synthesis from α-ketoglutarate plus ammonium. Concentrations of effectors giving half-maximal stimulation (SC₅₀) or inhibition (IC₅₀) were determined graphically. Results represent means of three to six separate determinations for each cell line. The addition of Triton did not affect the activity or allosteric responses of pure bovine GDH (Sigma) and did not affect basal enzyme activity of lymphoblast GDH from control subjects and patients. Leucine or ADP SC₅₀ values were also not affected. However, IC₅₀ values for GTP were reduced four- to sevenfold in both normal control subjects and patients, possibly due to removal of tightly bound endogenous GTP. Thus, IC₅₀ values for GTP in the present studies are lower compared with our previous report, which did not use Triton (10).

The tertiary structure of bovine GDH reported by Peterson and Smith (15) was used to model the location of GDH mutations with the RasMol program (v2.6, ©1994-1996 Roger Sayle).

RESULTS

HI/HA mutations in GDH exons 11 and 12. Table 1 shows the mutations found by screening GDH exons 11 and 12 in genomic DNA samples from 48 unrelated HI/HA families. Mutations were detected in 25 probands (52%) of the HI/HA patients. All were single nucleotide missense mutations that altered amino acid residues between position 440 and 454 of the mature enzyme. As shown in Fig. 1, 18 of the 25 cases represented de novo mutations, since the mutation was not detected in either parent. Seven of the cases were familial, with

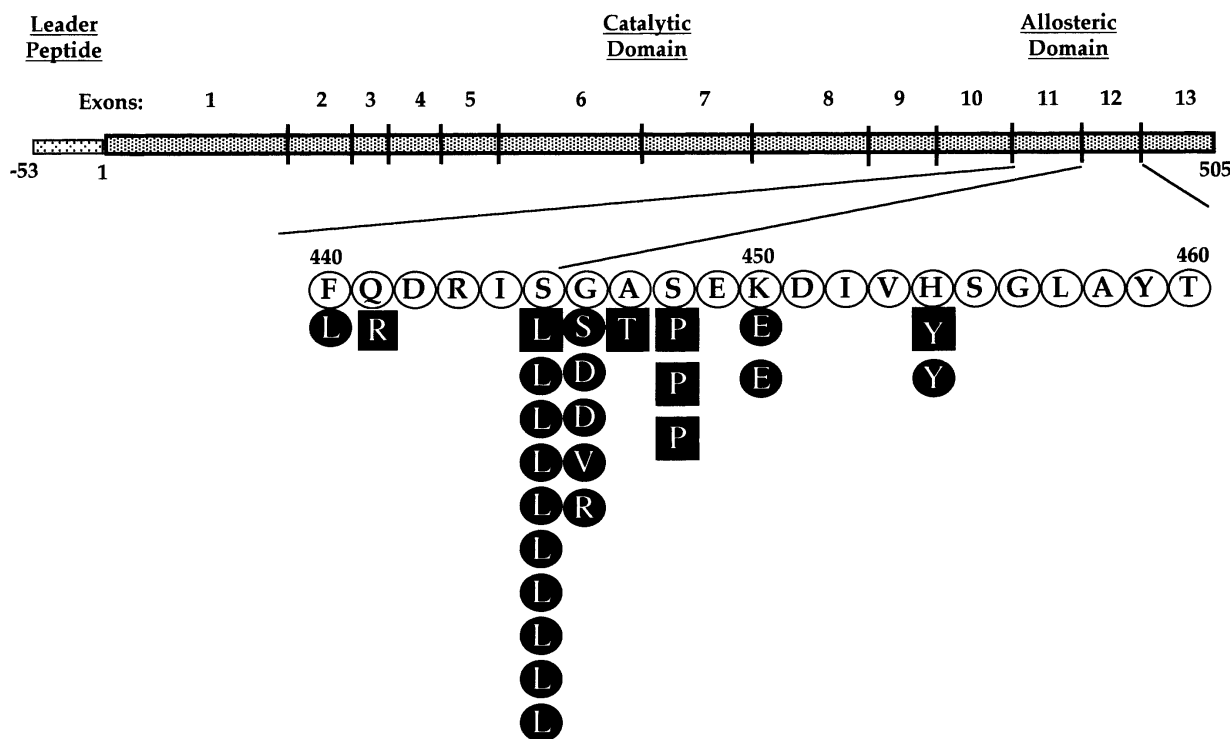


FIG. 1. Loci of HI/HA GDH mutations. Shown is the cDNA diagram of GDH with its mitochondrial targeting leader peptide, catalytic domain, and allosteric domain. The mutations in 25 children with exon 11 or 12 mutations are indicated by circles (sporadic cases) and squares (familial cases). Amino acid residues are numbered according to the sequence of the mature leaderless enzyme.

TABLE 2
Clinical manifestations in HI/HA children with GDH exon 11 or 12 mutations

n	25
Sex (F/M)	10/15
Birth weight (n = 22) (g)	3,500 (2,180–4,310)*
Age of onset (n = 25) (months)	4 (1 h–15 months)
Plasma ammonium (n = 21) ($\mu\text{mol/l}$)	135 (58–360)†
Diazoxide responsive	23/23
Developmental status (n = 22)	
Normal	11
Delayed	11
Transmittance	
Familial	7
Sporadic	18

Data are n or medians (range). *Normal mean = 3,200 g; †normal value = 9–35 $\mu\text{mol/l}$.

affected individuals identified in two or more generations. In one family, 16 of 30 individuals were affected in three generations (17). In two of these seven familial cases, a founder with a de novo mutation was identified. In a third family, two of four siblings were affected, presumably due to gonadal mosaicism, because the mutation was not detected in peripheral blood lymphoblasts from either of the parents. Thus, GDH mutations causing the HI/HA syndrome were either newly or recently acquired in at least 21 of the 25 probands (84%).

All 25 affected HI/HA individuals were heterozygous for their mutation and a normal GDH allele, consistent with dominant expression. There was an unusual clustering of some of the mutations. In particular, a TCG-to-TTG transition at nucleotide 1506 of the GDH cDNA, resulting in a Ser445Leu mutation, occurred in 10 of the 25 families (40%), consistent with this site being a CpG mutational hot spot (19). The adjacent codon for Gly446 was involved in four different mutations, resulting in four separate missense mutations in five families. A Ser448Pro mutation occurred in three of the seven familial cases. These were not due to a common genetic background: the families had different ethnic origins, and a founder with a de novo mutation was identified in two of these families. For two of the other familial mutations (Ser445Leu and His454Tyr), the identical mutation was found in an unrelated sporadic case.

Clinical features of GDH exon 11 or 12 mutations. Table 2 shows the clinical features of the children with GDH mutations confined to exons 11 and 12. Most of these HI/HA children had normal birth weights, and only four (16%) weighed more than the 90th percentile. Half were described to have the onset of hypoglycemic symptoms at 4 months of age or later; seven had onset in the first month and only four within the first week after birth. All of the HI/HA probands who were treated with diazoxide to control hypoglycemia were described as being responsive. However, at least five continued to have episodes of symptomatic hypoglycemia while on diazoxide, possibly induced by protein meals. Two children had undergone subtotal pancreatectomy but continued to require treatment for hypoglycemia. Half were considered to have delayed development; this was attributed to episodes of hypoglycemia in all cases. Eleven of 15 (73%) were described as having protein- or leucine-sensitive hypoglycemia. In the remainder, specific tests for protein sensitivity

had not been done. In three of the families with multiple affected individuals (two with Gly446Asp [10] and one with Ala447Thr [17]), subjects with GDH mutations were identified who had not previously been recognized to have symptoms. Plasma ammonium concentrations were elevated in all cases, ranging from 52 to 360 $\mu\text{mol/l}$ (normal <35 $\mu\text{mol/l}$). The hyperammonemia was not affected by protein feeding or protein restriction. In most cases, the hyperammonemia was considered to be asymptomatic. However, attention deficit/hyperactivity disorders were noted in some cases in which a contributing effect of hyperammonemia could not be excluded. The child with the highest recorded plasma ammonia concentration (360 $\mu\text{mol/l}$) was reported as having normal mental development.

GDH enzyme activity in exon 11 or 12 mutations. Table 3 summarizes the results of in vitro studies of lymphoblast GDH activity in HI/HA patients with seven of the eight different mutated residues shown in Table 2. For the Ser448Pro mutation, cells from four different patients were studied. In all cases, enzyme sensitivity to inhibition by GTP was impaired. GTP IC_{50} values ranged from 2 to 10 times normal. Impairment was least for the Gln441Arg mutation. With the exception of the Phe440Leu and Gln441Arg mutations, GDH activity in patient lymphoblasts was not completely suppressed by 3,000 nmol/l GTP. In contrast, enzyme sensitivity to inhibition by diethylstilbestrol or palmitoyl-CoA was not consistently altered.

Most of the mutations did not alter basal enzyme activity (i.e., in the absence of effectors). The sole exception was the Ser448Pro mutation, in which basal activity appeared to be inhibited by 50%, since maximal stimulated activity in the presence of 200 $\mu\text{mol/l}$ ADP or 6 mmol/l leucine was 70–80% of normal. The Ser448Pro mutation also showed modest, but significant decreases in responsiveness to ADP activation and to diethylstilbestrol inhibition. Compared with the Ser448Pro mutation, the seven other mutations had higher basal activity ($22 \pm 3.3 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$, $P = 0.0001$) and lower ADP SC_{50} ($21 \pm 4.8 \mu\text{mol/l}$, $P = 0.0007$), but similar GTP IC_{50} ($265 \pm 121 \text{ nmol/l}$, NS).

The half-maximal effector concentrations for ADP activation and GTP inhibition of lymphoblast GDH shown in Table 3 are two to three orders of magnitude lower than normal tissue concentrations (20). To determine whether the opposing effects of ADP and GTP are competitive in HI/HA patients, we examined the effects of increasing concentrations of GTP in combination with ADP on the activity of lymphoblast GDH from a patient with a mutation of the His454 that directly binds GTP. As shown in Fig. 2, GTP alone suppressed GDH activity less effectively in the patient than in the control subject. Subsequent addition of ADP at 0.4 mmol/l restored GDH activity to the maximum stimulated level seen prior to GTP in both patient and control subject. The effect of ADP could be reversed by further addition of GTP and then restored by additional ADP. However, compared with the control subject, much higher concentrations of GTP were required in the patient to overcome stimulation by ADP. These results indicate that the effects of GTP and ADP on GDH activity are competitive in both the HI/HA patient and the normal control subject.

Tertiary structure of GDH exon 11 and 12 mutations. Mature GDH is a homohexamer composed of two sets of trimers bound face to face, with antenna-like projections of

TABLE 3
Activity and allosteric responses of lymphoblast GDH with exon 11 and 12 mutations

	Mutation sites								Control
	Phe440Leu	Gln441Arg	Ser445Leu	Gly446Arg	Gly446Asp	Ser448Pro (P vs. control)	Lys450Glu	His454Tyr	
n	1	1	2	1	1	4	2	1	7
Basal activity (nmol · mg ⁻¹ · min ⁻¹)	22	22	29*, 28*	21	23	10 ± 1.7 (<0.0001)	19, 20	19	22 ± 2.3
Allosteric activators									
ADP SC ₅₀ (μmol/l)	21	20	23*, 19	18	28*	34 ± 2.3 (<0.0001)	24*, 25*	13	19 ± 3.4
Maximum activity (nmol · mg ⁻¹ · min ⁻¹) (with ADP 200 μmol/l)	39*	49	48, 45	46	49	36 ± 5 (0.0077)	55*, 51*	43	46 ± 4.5
Leucine SC ₅₀ (mmol/l)	0.7	0.83	0.90, 0.7	0.75	0.80	1 ± 0.1 (0.013)	1*, 0.98*	0.71	0.8 ± 0.12
Maximum activity (nmol · mg ⁻¹ · min ⁻¹) (with leucine 6 mmol/l)	37	50*	47, 47	36	49*	29 ± 5.3 (0.0066)	35*, 49*	38	42 ± 6.5
Allosteric inhibitors									
GTP IC ₅₀ (nmol/l)	190*	94*	280*, 340*	300*	480*	290 ± 34 (<0.0001)	230*, 190*	280*	48 ± 17
Residual activity (nmol · mg ⁻¹ · min ⁻¹) (% basal with GTP 3 μmol/l)	2.4	0.83	14*, 8*	20*	26*	9.2 ± 3.6 (NS)	22*, 21*	19*	2.4 ± 2.3
Diethylstilbestrol IC ₅₀ (μmol/l)	2.1	2.4*	2.2, 1.4*	3.1*	1.2*	1.3 ± 0.23 (0.0017)	0.85*, 0.73*	0.65*	1.9 ± 0.2
Palmitoyl-CoA IC ₅₀ (μmol/l)	0.64	1.1*	0.73, 0.43	1.2*	0.6	0.97 ± 0.23 (NS)	0.48, 0.74	0.32*	0.69 ± 0.35

Data are n or means ± SD. *Beyond 95% confidence limit of normal control subjects.

three adjacent subunits in each trimer intertwined to mediate cooperative interactions between subunits (15). Based on the published structure of bovine GDH, which is similar in sequence to the human enzyme, the HI/HA exon 11 and 12 mutations lie on one side of the antenna region close to one of two binding sites for GTP (Fig. 3A). The Lys450Glu and His454Tyr mutations lie at the proximal end of the hinge α -helix and directly interfere with hydrogen bonding to oxygen atoms of the inhibitory GTP β - and γ -phosphates (Fig. 3B). The Ser448Pro lies at the beginning of the hinge α -helix, whereas the remaining exon 11 and 12 mutations lie along the antenna loop further than 10 Å from the inhibitory GTP.

DISCUSSION

In this study, slightly more than half of children with the HI/HA syndrome had mutations in a domain of GDH that spans 15 amino acid residues. This domain is encoded within two small exons, 11 and 12. All of these mutations impair the sensitivity of enzyme activity to inhibition by GTP, consistent with the concept that both the hyperinsulinism and the hyperammonemia in these children are caused by an increase in the activity of GDH in pancreatic β -cells and in the liver. In all cases, the affected patients were heterozygous, indicating that the mutations are dominantly expressed. Three-fourths of the cases had de novo, sporadic rather than inherited mutations.

The clinical features of hypoglycemia in the children with the HI/HA syndrome due to exon 11 and 12 mutations are much less severe than in patients with congenital hyperinsulinism due to recessive mutations of SUR1 or Kir6.2 (16). Infants with the latter form of hyperinsulinism present with

large birth weights, have onset of hypoglycemia in the first few days after birth, rarely respond to diazoxide or other medical therapy, and often require near total pancreatectomy to control blood glucose levels (16). In contrast, the patients with HI/HA had normal birth weights and were frequently not recognized to have hypoglycemia until they were several months to >1 year of age. As reported previously (10), some members of their families were not recognized as being affected until adult life; some carriers of disease-causing mutations were described as asymptomatic. This variability in presentation of hypoglycemia may be related to the observation that three-fourths of the HI/HA children were described to have protein-sensitive hypoglycemia. Thus, the loss of sensitivity to GTP inhibition may potentiate the stimulatory effect of leucine on GDH activity. It is possible that infants and children previously described as having leucine-sensitive hypoglycemia actually had the HI/HA syndrome due to mutations of GDH (21–23).

We noted a higher frequency of de novo, sporadic, as compared with familial mutations in the HI/HA patients. This might reflect reduced reproduction potential for affected patients in previous times when diazoxide was not available for treatment and permanent brain damage from hypoglycemia was likely to occur. In our earlier report, it appeared that HI/HA patients with familial Ser448Pro mutations had milder disease (10). In the present series, however, as more patients have been identified, there was no clear difference in clinical features among the different mutations. Variability in symptoms of hypoglycemia was particularly apparent in one of the families with a Ala447Thr mutation in which some individuals developed hypoglycemic seizures and died in

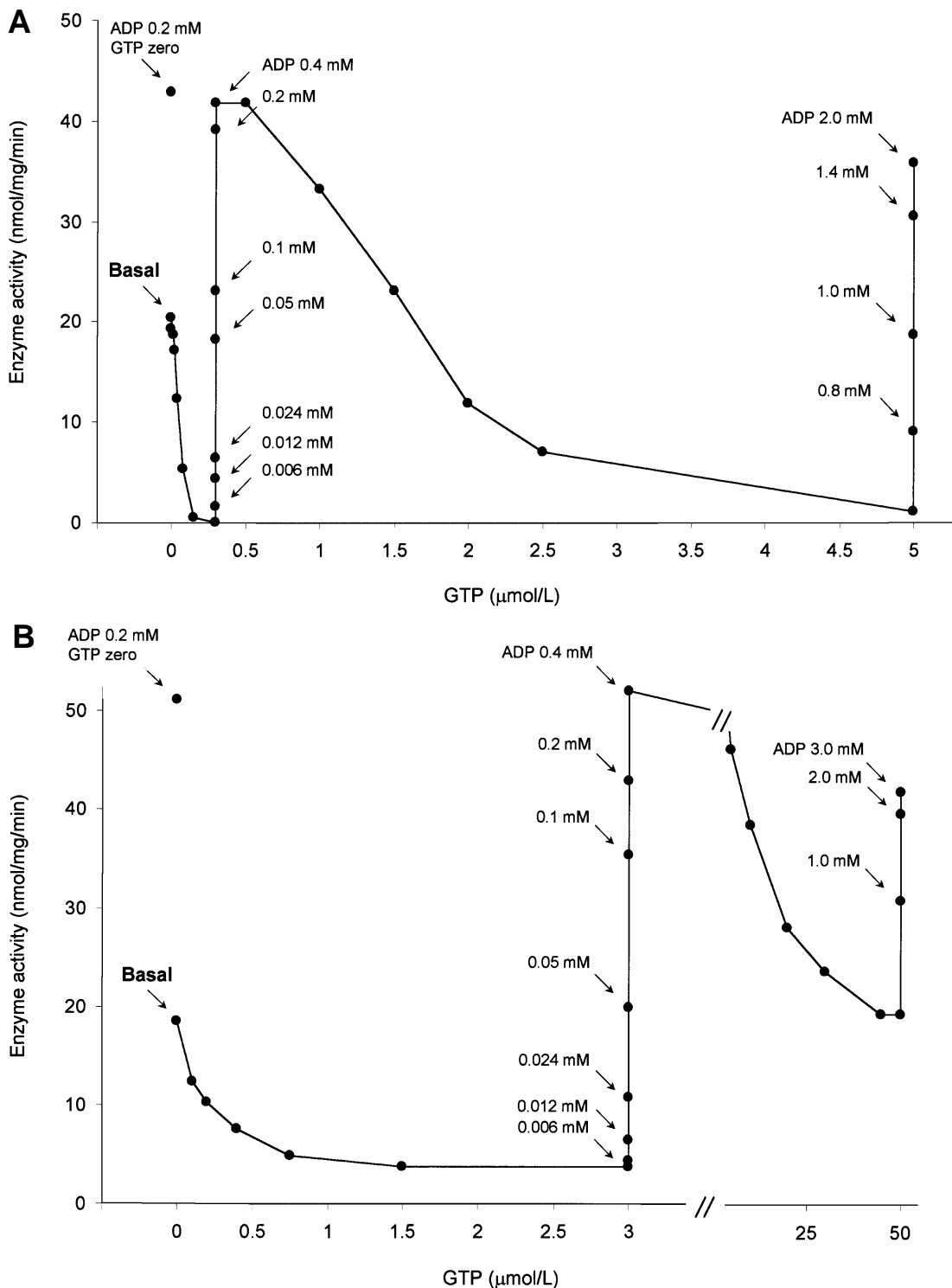


FIG. 2. Counterbalancing effects of GTP inhibition and ADP stimulation on GDH activity. Shown is the activity of GDH from lymphoblast homogenates in which the assay mixtures contained different combinations of GTP and ADP in a control subject (A) and an HI/HA patient with a His454Tyr mutation (B).

early infancy, whereas others survived into adult life without ever showing signs of hypoglycemia (17).

Hyperammonemia appeared to be asymptomatic in HI/HA individuals with mutations in GDH exon 11 and 12 of GDH. Although this does not eliminate concern about possible adverse effects of hyperammonemia in these patients, at present, treatment does not appear to be either effective or

necessary. Unlike patients with urea cycle enzyme disorders, HI/HA children with hyperammonemia do not respond to protein restriction or alternate pathway therapy (7–9). Zamarchi et al. (8) have reported a modest reduction in ammonium concentrations in an HI/HA child using N-carbamoyl-glutamate, an allosteric activator of carbamoyl-phosphate synthetase, the first step in urea synthesis.

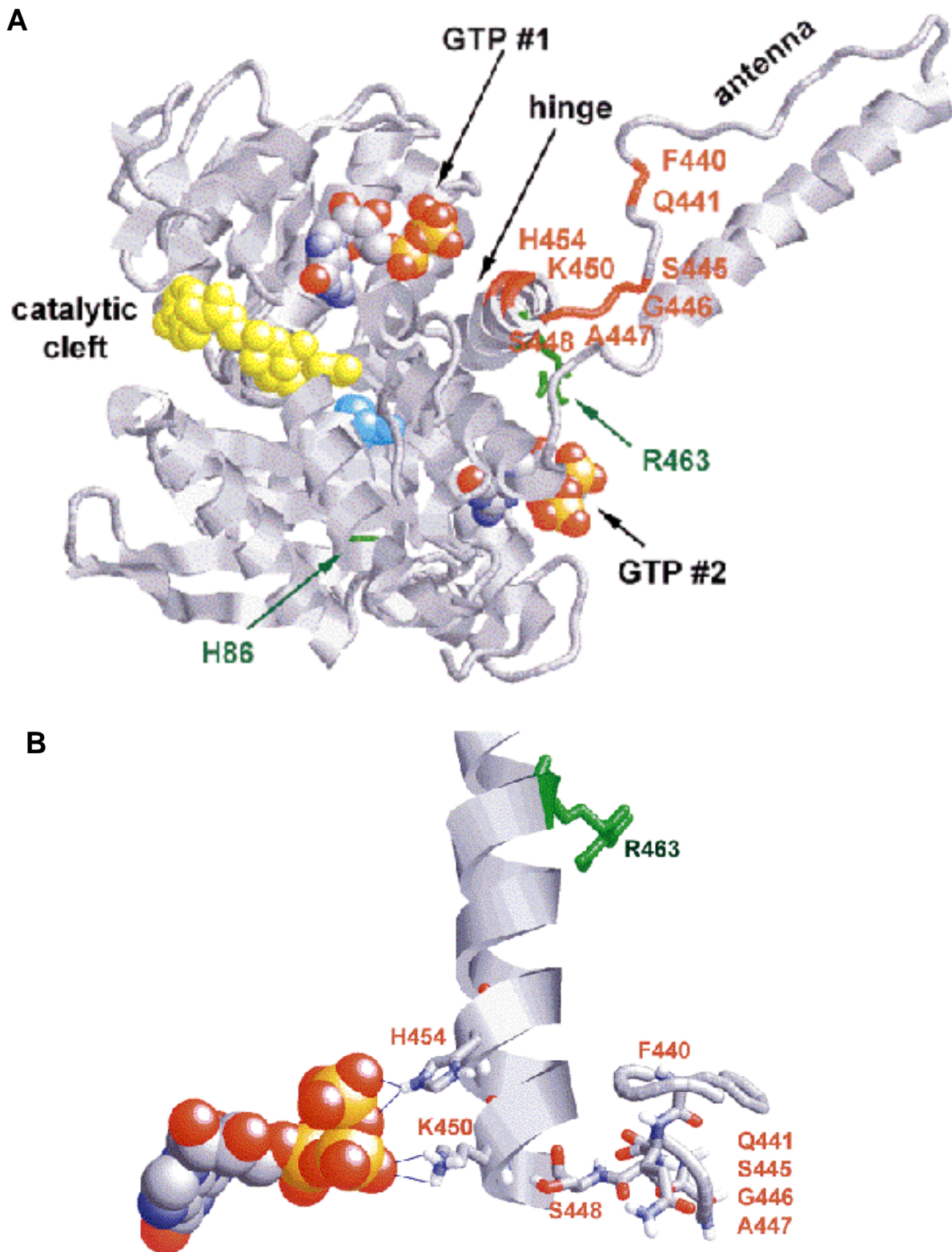


FIG. 3. A: Location of HI/HA exon 11 and 12 mutations in GDH. Shown is a ribbon diagram of one subunit of the GDH homohexamer based on the X-ray crystallographic structure of bovine GDH (15). Facing left is the catalytic cleft binding the enzyme substrates NADH (yellow) and glutamate (light blue). Outside the catalytic cleft, on the front surface of the enzyme, is one of the two bound GTPs (GTP #1). Pointing right is the antenna region containing the eight mutated amino acids found in HI/HA children (red). On the reverse face of the enzyme are two amino acid binding sites (green) for the allosteric activator ADP, which bracket the second bound GTP (GTP #2). B: Close-up of interactions between the inhibitory GTP #1 and sites of GDH mutations in HI/HA syndrome. In the center is the hinge α -helix containing one of the ADP binding residues (green). Mutated amino acid residues are shown as stick diagrams (O, red; N, blue; H, white) projecting from the first two turns of the hinge and the connecting peptide chain of the antenna. H-bond distances from GTP γ - and β -phosphate oxygens (red) to the His454 and Lys450 residues (bovine his450 and lys446) are 4.16, 3.96, 4.42, and 4.22 Å, respectively.

Allosteric control of GDH activity may be mediated by narrowing (GTP) or widening (ADP) of the catalytic cleft, with the α -helix labeled in Fig. 3A acting as a hinge (15). The hinge region contains an Arg463 (boGDH Arg459) residue that is known to interact with the allosteric activator ADP (24,25). The GTP#2 site, shown in Fig. 3A on the other side of the antenna from the inhibitory GTP#1 site, may represent the ADP binding site. Two of the HI/HA mutations (Lys450Glu and His454Tyr) alter amino acids that directly bind GTP in the GTP#1 site. The Ser448Pro mutation inserts a proline ring, which is likely to cause a severe twist of the peptide chain at the beginning of the hinge α -helix. This may explain why this mutation alters not only responsiveness to GTP inhibition, but also basal enzyme activity and sensitivity to ADP activation. The remaining mutations, which lie further up the antenna, may impair GTP binding indirectly by affecting the alignment of the Lys450 and His454 side chains. The fact that most of the exon 11 and 12 mutations specifically affect GTP inhibition provides evidence that the other positive and negative effectors bind to distinct allosteric domains on the enzyme that are separate from the GTP#1 site.

Remarkably, over half of the patients in this series had mutations in a small region involving only 16 of the 505 amino acids of GDH, and all of these are localized to exons 11 or 12. Thus, screening of these two exons for mutations may provide a convenient molecular test to diagnose patients with the HI/HA form of hyperinsulinism. HI/HA patients without mutations in exons 11 or 12 may have mutations elsewhere in GDH. Mutations outside the exon 11 and 12 domain could occur in other residues thought to be involved in GTP binding, such as the Arg274 site, which has been identified as a potential GTP binding site by cross-linking studies (24). Alternatively, the HI/HA phenotype could be due to mutations in another protein involved in similar metabolic pathways. The present data indicate that the leucine and ADP binding sites for allosteric activation of GDH function independently of the inhibitory GTP binding site. It is possible that mutations at these binding sites could exist in infants with other forms of congenital hyperinsulinism or be associated with diabetes.

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APPENDIX

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