

Expression, purification and characterization of human glutamate dehydrogenase (GDH) allosteric regulatory mutations

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Glutamate dehydrogenase (GDH) catalyses the reversible oxidative deamination of L-glutamate to 2-oxoglutarate in the mitochondrial matrix. In mammals, this enzyme is highly regulated by allosteric effectors. The major allosteric activator and inhibitor are ADP and GTP, respectively; allosteric activation by leucine may play an important role in amino acid-stimulated insulin secretion. The physiological significance of this regulation has been highlighted by the identification of children with an unusual hyperinsulinism/hyperammonaemia syndrome associated with dominant mutations in GDH that cause a loss in GTP inhibition. In order to determine the effects of these mutations on the function of the human GDH homohexamer, we studied the expression, purification and characterization of two of these regulatory mutations (H454Y, which affects the putative GTP-binding site, and S448P, which affects the antenna region) and a mutation designed to alter the putative binding site for ADP (R463A). The sensitivity to GTP inhibition was impaired markedly in the purified H454Y (ED₅₀, 210 μM) and S448P (ED₅₀, 3.1 μM) human GDH mutants compared with the wild-type

human GDH (ED₅₀, 42 nM) or GDH isolated from heterozygous patient cells (ED₅₀, 290 and 280 nM, respectively). Sensitivity to ADP or leucine stimulation was unaffected by these mutations, confirming that they interfere specifically with the inhibitory GTP-binding site. Conversely, the R463A mutation completely eliminated ADP activation of human GDH, but had little effect on either GTP inhibition or leucine activation. The effects of these three mutations on ATP regulation indicated that this nucleotide inhibits human GDH through binding of its triphosphate tail to the GTP site and, at higher concentrations, activates the enzyme through binding of the nucleotide to the ADP site. These data confirm the assignment of the GTP and ADP allosteric regulatory sites on GDH based on X-ray crystallography and provide insight into the structural mechanisms involved in positive and negative allosteric control and in inter-subunit cooperativity of human GDH.

Key words: enzyme inhibitor, gene expression regulation, hyperammonaemia, hyperinsulinism, protein structure.

INTRODUCTION

Glutamate dehydrogenase (GDH; EC 1.4.1.3) is a homohexameric mitochondrial matrix enzyme that catalyses the reversible oxidative deamination of glutamate to α-ketoglutarate plus free ammonia using either NAD or NADP as a co-factor. The enzyme is expressed at high levels in liver, brain, pancreas and kidney, but not in muscle. Allosteric control of mammalian GDH activity by positive effectors (e.g. ADP and leucine) and negative effectors (e.g. GTP) has been studied extensively [1,2]. The physiological significance of this regulation is highlighted by the recent identification of infants and children with an unusual hyperinsulinism/hyperammonaemia (HI/HA) syndrome [3]. This is a novel disorder of intermediary metabolism in which the mutations are expressed dominantly and cause a gain, rather than a loss, of enzyme function due to impairment of GDH sensitivity to inhibition by GTP. Affected children present with recurrent episodes of symptomatic hypoglycaemia, but also have persistent asymptomatic elevations of plasma ammonium ions. The increased GDH activity leads to excessive insulin release in pancreatic β-cells via the pathway of leucine-stimulated insulin secretion, and is manifested by heightened sensitivity of patients to stimulation of insulin release by leucine or protein [4,5]. Simultaneously, in liver, excessive GDH activity results in increased ammonia production and depressed synthesis of N-acetylglutamate, a required allosteric activator of the first step in ureagenesis.

GTP inhibits enzyme turnover over a wide range of conditions by increasing the affinity of GDH for the reaction product, making product release rate limiting under all conditions in the presence of GTP [6]. In contrast, ADP activates GDH by facilitating product release [6,7]. When the enzyme is highly saturated with substrate, an inhibitory abortive complex forms in the active site: NAD(P)H–glutamate in the oxidative deamination reaction at high pH and NAD(P)⁺–α-ketoglutarate in the reductive amination reaction at low pH [8]. Under these conditions, ADP is a potent activator that decreases product affinity and allows the enzyme to reconcile these non-catalytic complexes.

The structures of several bacterial forms of GDH [9–11,13] and bovine GDH complexed with NADH, glutamate and GTP have been determined [14,15]. Whereas mammalian GDH is highly regulated, bacterial GDH is not. The major structural difference between bovine GDH and the bacterial forms is a 48-residue domain in bovine GDH that forms an 'antenna' structure extending from the top of the NAD domain [14]. Therefore, it was suggested that this antenna may be involved in some or all of the allosteric regulation that is unique to mammalian GDH. From the proposed locations of the GTP and ADP sites in the bovine GDH structure, it was suggested that these allosteric regulators exert their effects by changing the energy required to open and close the catalytic cleft during enzymic turnover [14,15].

There have been identified 24 different mutations of GDH in patients with the HI/HA syndrome [16–19]. All of these mutations occur at amino acid residues that appear to be involved,

Abbreviations used: GDH, glutamate dehydrogenase; HI/HA, hyperinsulinism/hyperammonaemia; huGDH, human GDH.

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directly or indirectly, in GTP allosteric inhibition, based on the crystal structure of bovine GDH, which is 95% identical with the human enzyme [14]. Evidence that these mutations cause disease has been obtained from pedigrees in which multiple individuals were affected as well as by experiments expressing the HI/HA mutations in COS cells [3]. However, mature GDH exists as a homohexamer, and the effects of HI/HA mutations presumably reflect the activities of GDH heterohexamers comprised of both mutant and wild-type subunits. Since the mutations are dominant, their expression is likely to depend on co-operative interactions among mutant and wild-type subunits within the heterohexamers.

The purpose of the present experiments was to determine the effects of two disease-associated HI/HA mutations on the enzymic properties of human GDH (huGDH) hexamers comprised solely of mutant subunits. These mutations lie at the junction between the GTP-binding site, the pivot helix and the antenna region of GDH. In addition, a third mutation on the pivot helix, not associated with disease, was designed to interfere with the putative site for ADP allosteric activation of the enzyme. These experiments provide a means of confirming that mutations identified in patients are disease-causing and of examining the structural basis for allosteric regulation of GDH enzyme activity.

EXPERIMENTAL

Human GDH cDNA construct

A full-length huGDH cDNA/pcDNA3 construct was modified to delete the mitochondrion-targeting leader peptide sequence using overhanging PCR primers. The forward primer included an *NdeI* restriction site followed by the first 21 bp encoding mature huGDH enzyme. A new ATG start codon, contained within the *NdeI* sequence, was positioned immediately 5' of the mature GDH cDNA sequence. The reverse primer was positioned in the SP6 site of the vector. The resulting 1657 bp PCR product was excised with *NdeI* and *NotI*, ligated into pET-21a(+) vector using T4 DNA ligase, and cloned in *Escherichia coli* DH5 α cells. The huGDH cDNA nucleotides were numbered according to the sequence reported by Nakatani et al. [20] and the amino acid residues numbered according to the mature enzyme sequence, which contains four more residues than bovine GDH at the N-terminus.

Site-directed mutagenesis

Site-directed mutagenesis was used to create two HI/HA mutations, H454Y (cDNA substitution: C¹⁵¹⁹ → T) and S448P (T¹⁵⁰¹ → C), and an ADP-site mutation, R463A (CGT¹⁵⁴⁶⁻¹⁵⁴⁸ → GCC), using overlapping PCR with the leaderless wild-type huGDH cDNA in pET-21a(+) as the template. Two inner primers, containing the desired mutations, and two outer primers were used in three PCRs for creating each mutant. The final products were trimmed with *NheI* and *NotI* and inserted into the GDH/pET-21a(+) vector that had been excised previously with *NheI* and *NotI*. All constructs were confirmed by direct sequencing.

GDH enzymic assays

GDH activity was assayed spectrophotometrically at 340 nm by monitoring oxidation of NADH [21]. Maximum enzyme activities were determined by addition of 200 μ M ADP. Protein concentrations were determined using a Bio-Rad protein assay kit. Purified GDH protein concentrations were determined by absorption at 280 nm, based on an absorption coefficient of 0.93 cm²/mg. The effects on GDH activity of several allosteric effectors were

determined, including two activators, ADP and leucine, and four inhibitors, GTP, palmitoyl-CoA, diethylstilbestrol and ATP. Concentrations of effectors giving half-maximal stimulation or inhibition (ED₅₀) were determined graphically.

GDH co-expression with GroES and GroEL

To enhance expression of correctly assembled active GDH, we employed co-expression with the chaperone proteins GroES and GroEL (gifts from Dr Anthony A. Gatenby of DuPont Experimental Station, Wilmington, DE, U.S.A.), using pGroESL. The dual expression was conducted by first transforming the huGDH/pET-21a(+) cDNA vector into *E. coli* BL21 (DE3), selected with ampicillin. These cells were then transformed with pGroESL, and selected with ampicillin plus chloramphenicol. The transformed bacteria were grown in 15 litres of Luria-Bertani broth at 37 °C to a *D*₆₀₀ value of 0.6, and induced with 0.5 mM isopropyl β -D-thiogalactoside at 23 °C for 20 h. The cells were pelleted at 8000 g, and resuspended in GDH buffer (10 mM NaK₂HPO₄, pH 7.4/1 mM EDTA) with added 5 mM dithiothreitol and Protease Inhibitor Cocktail Tablets (Boehringer Mannheim). Cells were lysed by sonication and solubilized by adding 1% Triton X-100. The supernatant, containing soluble active GDH, was taken for further purification. Expressed huGDH was differentiated from endogenous *E. coli* GDH by comparing total GDH activity with and without 40 μ M GTP. This concentration of GTP completely inhibits huGDH, but does not affect the activity of bacterial GDH.

Purification of expressed huGDH

The lysed bacterial supernatant containing huGDH from 15 litres of culture was bound on to a Q-Sepharose Fast Flow anion-exchange column (bed volume, 100 ml, Amersham Bioscience). After washing with 8 litres of GDH buffer until an *A*₂₈₀ value of < 0.1, the bound huGDH was eluted with 1 litre of a linear NaCl gradient (20–250 mM in GDH buffer). Positive fractions were combined and (NH₄)₂SO₄ was added to final concentration of 1.4 M, and then the fractions were loaded on to an ω -aminopentyl hydrophobic-interaction column (bed volume, 100 ml; Sigma), washed with 3 litres of 1.4 M (NH₄)₂SO₄ in GDH buffer until an *A*₂₈₀ value of < 0.1. A linear gradient of (NH₄)₂SO₄ (1.4–0.7 M in GDH buffer; 1.2 litre) was applied to elute the bound GDH. Positive fractions were pooled, concentrated and desalted using an Amicon ultrafilter stirred cell with YM30 membrane. The resulting GDH was bound to 5 ml of GTP-agarose affinity resin (Sigma) and washed with suction over a Nalgene 0.5 mm filter unit with surfactant-free cellulose acetate membrane. The eluate containing GDH was extracted repeatedly on the regenerated GTP affinity resin in batches until 90% of the enzyme activity was recovered. The resin-bound GDH was washed 10 times with 50 ml of GDH buffer until the *A*₂₈₀ value was 0. GDH was eluted finally from GTP-agarose by equilibrating twice with 25 ml of 200 mM NaCl in GDH buffer at 4 °C for 10 min, and collected by filtration. The GTP-agarose matrix was regenerated with GDH buffer. The GDH fractions were pooled, desalted and stored in 60% (NH₄)₂SO₄ at 4 °C.

Data analysis

Data were analysed by Student's *t* test using InStat for MacIntosh 2.0 (GraphPad Software); *P* values of < 0.01 were considered to be significant. GTP inhibition curves for mixtures of purified mutant huGDH with wild-type enzyme were analysed by a two-component mathematical model using the equation: percentage activity = 100 – {M₁([GTP])/(K₁₁ + [GTP])} – {M₂([GTP])/(K₁₂ +

[GTP]], where [GTP] is the concentration of GTP, K_{i1} and K_{i2} are inhibition constants for the two species, and M_1 and M_2 are factors accounting for the maximum extent of GTP inhibition and the fraction of the two species of enzyme in the mixture. Experimental data were fit to either a one- or two-component model using the program Deltagraph (Deltapoint).

RESULTS

Expression and purification of huGDH in *E. coli*

In preliminary experiments, minimal amounts of huGDH activity were produced by *E. coli* transformed with wild-type huGDH/pET-21a(+) alone. Total GDH activity was increased to only 2-fold above that of the endogenous bacterial enzyme. Of this total activity, 30% was inhibitable by 5 μ M GTP, consistent with the presence of trace amounts of huGDH. In contrast, co-expression of huGDH with the chaperonins GroES and GroEL yielded a total GDH activity that was 60-fold greater than that of the endogenous bacterial enzyme. Over 98% of this total GDH activity could be inhibited by GTP, consistent with the allosterically regulated human enzyme. Table 1 illustrates the purification of wild-type huGDH from *E. coli* co-transformed with huGDH/pET-21a(+) and pGroESL. A total of 12 mg of purified wild-type huGDH was obtained with a final yield of 23%. Expression and purification of H454Y and S448P HI/HA mutant forms of huGDH and of the engineered R463A mutant enzyme produced 8.5–12 mg of pure protein with yields of 20–30%. On PAGE, all four forms of expressed huGDH migrated as single zinc-staining protein bands after purification.

As shown in Table 2, the specific maximal activity of wild-type huGDH, in the presence of 200 μ M ADP, was comparable with that of commercial bovine GDH standard (130 compared

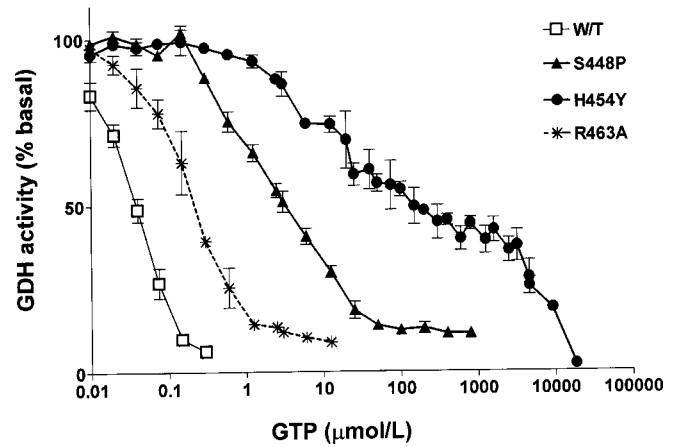


Figure 1 Responses to GTP inhibition of huGDH expressed in *E. coli*

Shown are the responses of the purified expressed wild-type (W/T) huGDH, the H454Y and S448P HI/HA mutant forms, and R463A mutant form (means \pm S.E.M.).

with 97 μ mol/min per mg of protein). The specific maximal activity of H454Y mutant huGDH was comparable with that of wild-type huGDH. The S448P mutant huGDH was unstable in Tris assay buffer, especially at low protein concentrations or in the absence of allosteric activators. Therefore, activity of the S448P mutation was determined in phosphate buffer. Both basal and maximal specific activities of this mutant were lower than that of the expressed wild-type huGDH (Table 2). The R463A mutation did not alter enzyme specific activity.

Table 1 Purification of wild-type huGDH expressed in *E. coli*

	Total protein (mg)	Total maximum activity (μ mol/min)	Specific maximum activity (μ mol/min per mg)	Yield (%)	Purification ratio
Lysate supernatant from 15 l of culture	6424	6008	0.935	100	1
Q-Sepharose anion-exchange column	482	5318	11.0	89	12
ω -Aminopentyl hydrophobic-interaction column	41.8	3937	94.2	66	101
GTP-agarose affinity resin	12.3	1364	111	23	119

Table 2 Activities of mutant and wild-type huGDH expressed in *E. coli*

Means \pm S.D. are shown, except for lymphoblast basal and maximal enzyme activities, where only mean values are shown. NA, no activation (see Figure 2). Assays were performed in 10 mM $\text{Na}_2\text{HPO}_4/1$ mM EDTA buffer, pH 8.0. The numbers in parentheses indicate the number of determinations.

	Expressed				Lymphoblast		
	S448P	H454Y	R463A	Wild type	S448P	H454Y	Wild type
Basal activity (μ mol/min per mg)	19 \pm 1.6* (5)	74 \pm 11 (9)	73 \pm 9.2 (3)	84 \pm 9.5 (6)	9.8 $\times 10^{-3}$	18 $\times 10^{-3}$	22 $\times 10^{-3}$
Maximum activity (μ mol/min per mg)							
200 μ M ADP	69 \pm 8.9* (4)	140 \pm 45 (7)	NA	130 \pm 22 (5)	36 $\times 10^{-3}$	43 $\times 10^{-3}$	45 $\times 10^{-3}$
6 mM Leucine	55 \pm 6.5* (5)	130 \pm 30 (7)	100 \pm 23 (3)	130 \pm 4.4 (4)	29 $\times 10^{-3}$	42 $\times 10^{-3}$	42 $\times 10^{-3}$
Activator ED ₅₀							
ADP (μ M)	21 \pm 6.8 (5)	9.1 \pm 3.4 (9)	NA	12 \pm 4.5 (7)	34 \pm 2.3† (4)	13 \pm 2.8 (7)	20 \pm 3.4† (7)
Leucine (mM)	0.61 \pm 0.089 (5)	0.47 \pm 0.21 (9)	0.27 \pm 0.06* (3)	0.64 \pm 0.11 (7)	1.0 \pm 0.1† (4)	0.7 \pm 0.15 (7)	0.8 \pm 0.1 (7)
Inhibitor ED ₅₀							
GTP (nM)	3100 \pm 1100* (5)	210000 \pm 150000* (8)	220 \pm 68 (3)	42 \pm 12 (5)	288 \pm 34† (4)	276 \pm 17† (7)	48 \pm 17 (7)
Diethylstilbestrol (μ M)	0.74 \pm 0.09* (5)	0.36 \pm 0.18* (7)	5.1 \pm 0.93 (3)	1.7 \pm 0.24 (5)	1.3 \pm 0.2† (4)	0.65 \pm 0.19 (6)	1.9 \pm 0.2 (7)
Palmitoyl-CoA (nM)	13 \pm 3.0* (4)	15 \pm 5.4† (7)	200 \pm 73 (3)	30 \pm 5.4 (5)	970 \pm 230† (4)	315 \pm 54† (6)	690 \pm 350† (7)

* $P < 0.01$ versus wild type.

† $P < 0.05$ or 0.01 versus purified expressed form.

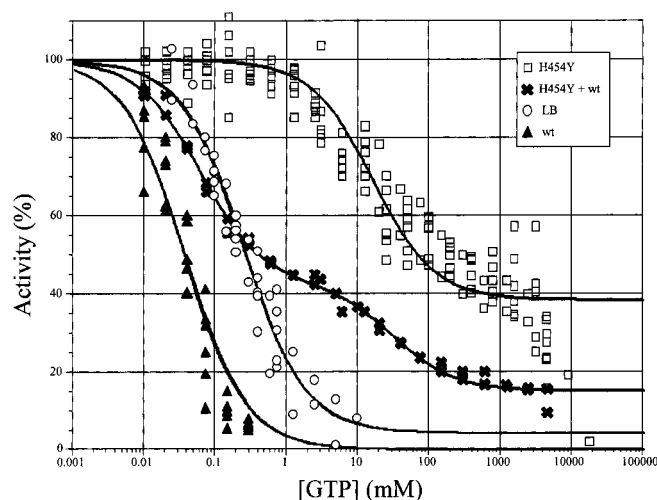


Figure 2 Analysis of GTP inhibition curves for H454Y mutant huGDH heterohexamers compared with H454Y/wild-type enzyme mixture

Shown are data from experiments with enzyme prepared from patient lymphoblasts heterozygous for the H454Y mutation (LB), pure H454Y mutant, pure wild-type (wt) and an equimolar mixture of purified H454Y and wild-type huGDH. Individual data points were obtained from three to eight separate experiments. Curves shown were derived using the equation shown in the Experimental section. Parameters for each curve are shown in Table 3.

Figure 1 shows the responses of the purified expressed forms of huGDH to allosteric inhibition by GTP. The purified wild-type huGDH was potently inhibited by GTP. The purified H454Y mutant form of huGDH had markedly reduced sensitivity to GTP inhibition, whereas the purified S448P mutant huGDH showed an intermediate reduction in sensitivity to GTP inhibition. As shown in Table 2, the ED_{50} for GTP of the purified expressed wild-type huGDH was the same as that found with GDH isolated from normal human lymphoblasts and was similar to the ED_{50} for GTP of commercial bovine GDH (Sigma). The purified S448P mutant huGDH was approx. 10-fold less sensitive to GTP, whereas the purified H454Y mutant was 1000-fold less sensitive compared with the corresponding enzymes isolated from cells of patients with these two mutations [16]. The R463A ADP-site mutation appeared to only slightly reduce sensitivity to GTP inhibition ($P > 0.01$).

The differences in GTP sensitivity between patient lymphoblasts and the expressed mutant forms of GDH may have been due to the fact that the patient cells contained GDH heterohexamers composed of wild-type and mutant subunits, whereas the expressed enzymes consisted of homohexamers of the mutant subunits. This explanation was examined by an experiment mixing equimolar amounts of the purified H454Y mutant and wild-type forms of huGDH. As shown in Figure 2, GDH from heterozygous patient lymphoblasts expressing the H454Y mutant showed a single smooth intermediate inhibition curve that was parallel to the GTP inhibition curve for purified wild-type huGDH. In contrast, the GTP inhibition curve for the mutant/wild-type mixture showed a biphasic response, with a first phase that paralleled the wild-type GDH inhibition curve and a second phase that paralleled the curve of the pure H454Y mutant. In the case of the purified wild-type and mutant enzymes and of enzyme isolated from H454Y heterozygous lymphoblasts, the data could be fit by curves using single inhibition constants (Figure 2 and Table 3). For the mixture of H454Y and wild-type enzyme, the data were best fit by a two-component model with two inhibition

Table 3 Coefficients for the mathematical modelling of GTP inhibition curves in Figure 2

huGDH species	M_1	M_2	K_{11}	K_{12}	R^2
Purified wild type	1.00	—	36 nM	—	0.93
H454Y lymphoblast	0.94	—	250 nM	—	0.94
Purified H454Y	0.61	—	16 μ M	—	0.9
H454Y/wild-type mixture	0.57	0.28	60 nM	30 μ M	0.99

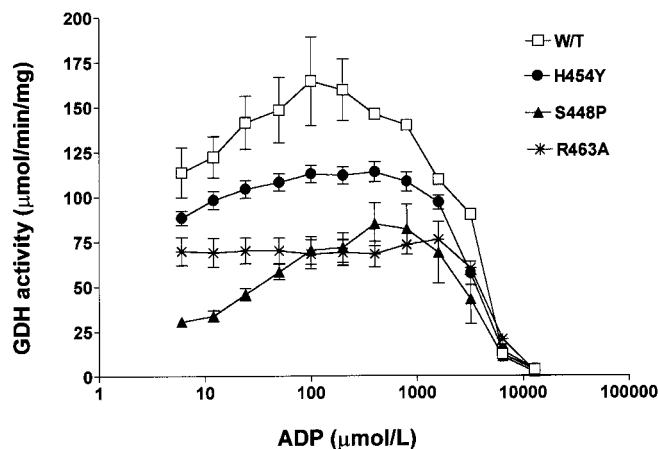


Figure 3 Responses to ADP activation of huGDH expressed in *E. coli*

Shown are the responses of the wild-type (W/T) and the H454Y, S448P and R463A mutant forms of huGDH (means \pm S.E.M.).

constants. In all instances, the inhibition constants for lymphoblast, mutant and wild-type enzymes derived from curve fitting (Table 3) were similar to the experimental ED_{50} values (Table 2). These observations demonstrate the existence of co-operative interactions between mutant and wild-type subunits in the naturally occurring heterohexamers of GDH in patient cells.

Responses of purified mutant forms of huGDH to ADP and ATP

As shown in Figure 3, the ADP activation curves for the purified H454Y mutant and wild-type forms of huGDH were similar, indicating that this mutation had little effect on either basal enzyme activity or activation by ADP. The ADP activation curve for S448P huGDH paralleled that of the wild-type enzyme, but with lower basal and maximal specific activities. Sensitivity to ADP activation was similar to wild-type for both the S448P and H454Y mutant forms of huGDH (Table 2). Both of the HI/HA mutants and the wild-type forms of huGDH were completely inhibited at very high concentrations of ADP of 5–10 mM.

As shown in Figure 4, the effect of ATP on purified wild-type huGDH activity was triphasic, with inhibition occurring at the low ATP concentration of 100 μ M, followed by an activation peak at 1 mM and, finally, inhibition at 5–10 mM. The H454Y and S448P mutant forms of huGDH both showed complete loss of the first-phase inhibitory response to ATP. The S448P mutant form actually showed a progressive increase in enzyme activity in response to ATP, similar to the response of this mutant form to ADP (Figure 4). The wild-type expressed huGDH and both of the HI/HA mutant forms were suppressed at very high concentrations of ATP.

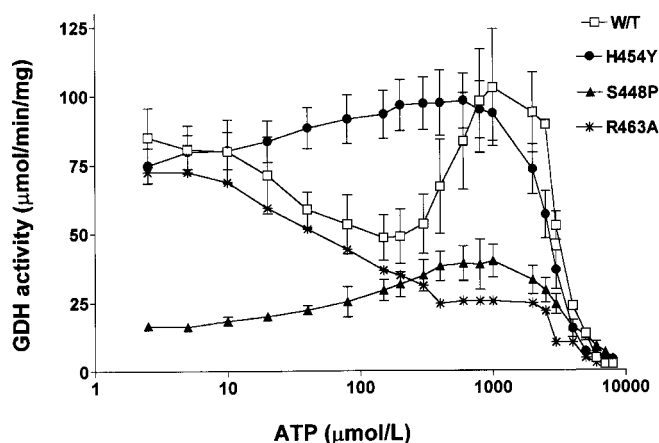


Figure 4 Responses to ATP of huGDH expressed in *E. coli*

Shown are the responses of the wild-type (W/T) and the H454Y, S448P and R463A mutant forms of huGDH (means \pm S.E.M.).

The arginine side chain at position 463 of huGDH is thought to be involved in ADP allosteric activation [15]. The R463A mutation eliminated the stimulatory effect of ADP on enzyme activity (Figure 3). In contrast, the R463A mutation slightly increased the sensitivity to leucine stimulation (Table 2). Compared with wild-type huGDH, the R463A mutation also produced an exaggerated first inhibitory phase and nearly eliminated the second stimulatory phase of responses to ATP (Figure 4).

Responses of purified mutant forms of huGDH to other allosteric effectors

Table 2 compares the sensitivities of the purified expressed forms of huGDH and HI/HA patient lymphoblast enzymes to allosteric activation by leucine or inhibition by the oestrogen analogue diethylstilbestrol and palmitoyl-CoA. Although HI/HA patients have enhanced sensitivity to leucine-stimulated insulin secretion *in vivo* [5], neither the purified HI/HA mutant forms of huGDH nor the mutant forms of the enzyme expressed in lymphoblasts showed altered sensitivity to leucine (Table 2). The expressed forms of both HI/HA mutations were slightly more sensitive to the inhibitory effect of diethylstilbestrol than purified wild-type huGDH, exaggerating the small differences in sensitivity observed with the forms of these mutations expressed in lymphoblasts. The wild-type and HI/HA mutant forms of purified expressed huGDH were considerably more sensitive to inhibition by palmitoyl-CoA than their lymphoblast counterparts; the expressed HI/HA mutant forms were both slightly more sensitive to inhibition by palmitoyl-CoA than wild-type huGDH. The R463A mutant GDH appeared to be slightly less sensitive to all three inhibitors, but with the small number of determinations that were done this only reached significant levels in the case of palmitoyl-CoA.

DISCUSSION

In these experiments, we used *E. coli* to express the mature form of huGDH that is present in the mitochondrial matrix. The enzymic properties of wild-type huGDH expressed with this method were similar to those of endogenous GDH expressed in cultured human lymphoblasts, as well as to those of purified bovine liver GDH. Our method of GDH expression in bacteria appears to be capable of synthesizing larger quantities of enzyme

than was described recently using baculovirus to express a variant form of huGDH [22]. Using co-expression with pGro ESL, we have been able to purify quantities of huGDH sufficient for crystallization experiments [15]. Data obtained with the expressed H454Y and S448P forms of huGDH confirmed that these are disease-causing mutations.

The locations of the mutations used in these experiments are shown in Figure 5. A 48-residue antenna-like projection that extends from the top of each NAD-binding domain and intertwines with the antennae of two adjacent subunits may play an important role in GDH allosteric regulation and negative cooperativity [14]. In the case of clostridial GDH, a large motion in the NAD-binding domain is associated with substrate binding and is required for catalysis [23,24]. In the case of mammalian GDH, it was proposed that allosteric regulation is mediated by the control of this NAD-binding domain motion [14,15].

GTP binds to the junction between the antenna and the NAD-binding domain [14,15]. The H454Y HI/HA mutation disrupts hydrogen bonding between the pivot α -helix and the β -phosphate of the bound GTP molecule. The nearby S448P mutation inserts a proline ring at the junction between the pivot helix and the antenna. The S448 does not contact the bound GTP molecule directly, but the proline mutation affects GTP inhibition by altering the flexibility of the antenna loop, as was made evident by the effects of this mutation on enzyme stability. The R463A ADP-site mutation lies away from the GTP-binding site, further along the pivot helix. From our recent studies on bovine GDH complexes, we proposed that, when the catalytic cleft opens, R463 is rotated down on to the phosphates of ADP [15]. In this way, ADP activates by facilitating the opening of the catalytic cleft. Therefore, the elimination of the R463 positive charge is expected to abrogate ADP activation.

The results of the present experiments confirm the assignment of the GTP inhibitory binding site and the proposed location of the ADP activation site shown in Figure 5 [14,15]. These assignments also agree with previous site-directed chemical-modification studies that identified Y262 and R463 in bovine GDH as important for GTP inhibition and ADP activation, respectively [21,25].

A striking feature of the H454Y GTP-site and the R463A ADP-site mutations was that both eliminated responsiveness to their respective effectors while having essentially no impact on responsiveness to the opposing effector. In the case of the H454Y mutation, there was little or no effect on basal or maximal specific enzyme activity. The R463A mutation also did not affect basal enzyme specific activity, indicating that GDH assumes its basal state configuration in the absence of allosteric effectors, regardless of whether the GTP- and ADP-binding sites are functional. The H454Y mutation had minimal effects on responsiveness to other positive or negative allosteric effectors, implying that these effectors bind at other loci. Most notably, leucine activation of GDH activity remained essentially unchanged by either the GTP-site or ADP-site mutations. This agrees with previous kinetic data suggesting that leucine activation occurs independently of the ADP site by binding elsewhere, perhaps directly within the catalytic cleft [21]. The enhanced responses of HI/HA patients to leucine stimulation of insulin release [5], which result from their impaired sensitivity to GTP inhibition, emphasize the physiological importance of inhibitory control of GDH activity.

ATP has more complex effects on huGDH activity than GTP or ADP; it inhibits at low concentrations, activates at intermediate concentrations and inhibits again at high concentrations. The initial inhibitory effect is mediated through the GTP-binding site since it was eliminated by the H454Y mutation.

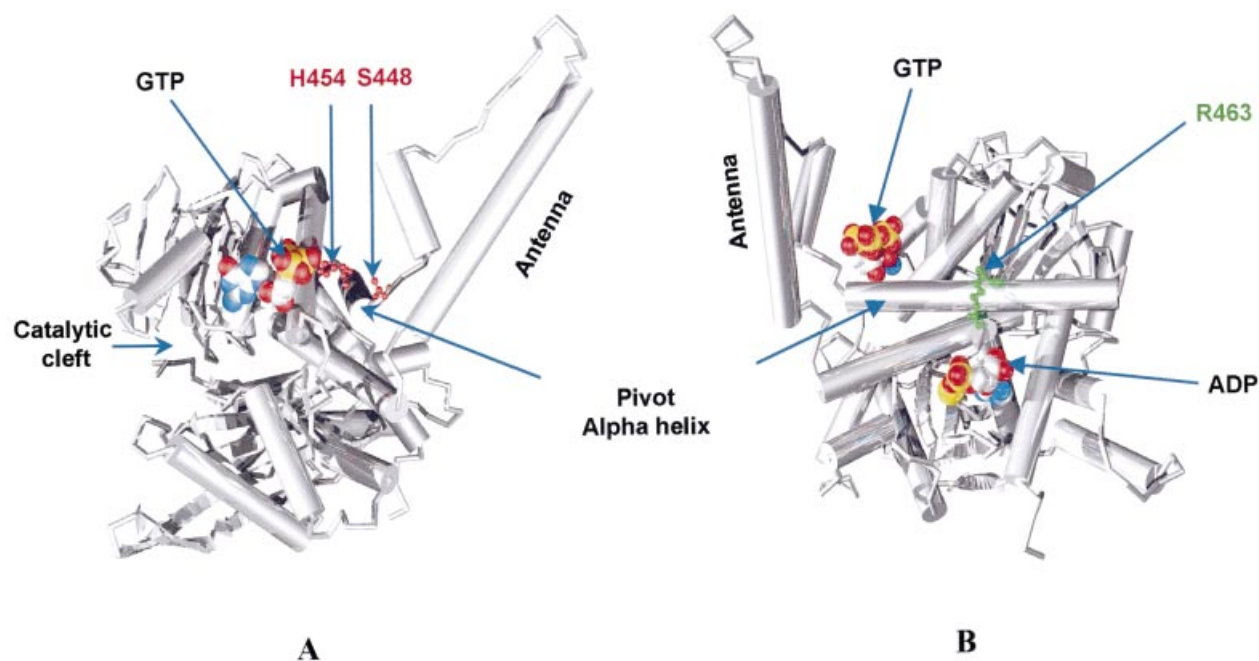


Figure 5 Locations of the H454Y, S448P and R463A mutations and allosteric effector binding sites in GDH

Shown is a single GDH subunit, based on the X-ray crystallographic structure of bovine GDH. (A) A side view of the enzyme showing inhibitory GTP bound to the surface of the upper NAD domain. The S448 residue lies at the junction of the antenna, pivot and GTP β -phosphate. (B) A rear view of the enzyme showing the activating ADP bound in close proximity to the R463 residue projecting from the mid-portion of the pivot α -helix.

Based on the data with the R463A mutation, the affinity of ATP for the GTP site appears to be 1000-fold lower than for GTP. This is consistent with our recent results on bovine GDH demonstrating that, whereas most of the GDH–GTP interactions are via β - and γ -phosphate interactions, there are specific interactions with E292 and K289 (E296 and K293 in huGDH) that favour guanosine over adenosine [15]. The second, stimulatory, phase of ATP regulation is mediated through the ADP effector site, since it was almost completely eliminated in the R463A mutant huGDH. Together, these observations indicate that ATP is able to bind to either regulatory site and that the triphosphate moiety is the major determinant of binding at the GTP site, whereas the nucleotide group is the major determinant of binding to the positive ADP effector site. The inhibition seen at very high concentrations of both ADP and ATP was relatively unaffected by either the H454Y or the R463A mutation, suggesting that this effect is mediated by weak binding at a third site. Binding to this third site is relatively specific for the adenine nucleotide, since the GTP inhibition curve for the H454Y mutation did not suggest the presence of a second inhibitory site apart from the GTP site. Inhibition by high concentrations of ADP has been suggested previously to be due to competition between ADP and the adenosine moiety of the coenzyme at the active site [26].

In contrast with the H454Y mutation, the S448P mutation had pronounced effects on basal enzyme activity. In Tris buffer, the S448P mutant had very low activity in the absence of effectors. Activity of this mutant was stabilized by increasing enzyme concentrations or addition of ADP or phosphate buffer. The effect of phosphate is consistent with reports that bivalent anions stabilize the enzyme [27], and recent structural studies have shown that phosphate molecules bind to the GTP site [15]. The stabilizing effects of enzyme concentration and ADP may be due

to enzyme polymerization, since bovine GDH undergoes a concentration-dependent polymerization [28] that is potentiated by ADP [26]. From the crystal packing observed in the bovine GDH structure, it was suggested that this polymerization is due to the antenna of one hexamer interacting with the antenna and NAD-binding domain of another. The effects of the S448P mutation on enzyme stability and activity indicate that the antenna plays a major catalytic and structural function in the enzyme.

The heterohexamers of S448P huGDH in patient lymphoblasts had reductions in sensitivity to GTP that were similar to the purified mutant homohexamers, whereas homohexamers of the H454Y mutant were much more insensitive to GTP than the heterohexamers expressed in patient lymphoblasts. These differences in the effects of the two HI/HA mutations presumably reflect protein–protein interactions within the GDH hexamers mediated through the antenna projections of adjacent subunits. Direct evidence of co-operative interactions among subunits was provided by comparison of the GTP responsiveness of heterohexamers from patient cells with that of an H454Y/wild-type huGDH equimolar mixture (Figure 2). The fact that the S448P mutation is at the pivot helix/antenna flex point may be an important factor in its more potent dominant effect on antenna-mediated communication. In contrast, the H454Y mutation is at the GTP-binding site and therefore may affect GTP-mediated subunit communication to a lesser extent. It has been reported previously that GTP inhibition of GDH can be abrogated with cross-linking reagents without affecting GTP binding [29]. This observation can now be accounted for by the present observations demonstrating that GTP effects on GDH are not localized solely to the subunit to which it is binding and that the antenna plays an important role in communicating this inhibition to other subunits.

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